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Soil Enzymology in the Recycling of Organic Wastes and Environmental Restoration

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Preface

Soil biological and related biochemical parameters, particularly enzymes, play a fundamental role in many soil processes such as the mineralization of organic matter, the synthesis of humic substances, the degradation of xenobiotics or the mechanisms involved in the biocontrol of plant pathogens. Their direct link with soil microorganisms gives them a key role as biomonitors of the evolution of soil quality, in the determination of their resistance to or their resilience against external environmental impacts, or in the monitoring of the application of organic amendments in the recovery of degraded, eroded or polluted soils. As a consequence of the importance of soil biological and biochemical parameters on soil processes, there is an increasing interest in their study, as well as in the application of new biochemical and molecular techniques, which, when applied to soil, are becoming indispensable diagnostic tools.

It is especially important to assess the evolution of soil quality after the introduction into the soil of exogenous materials which are produced as organic residues in the domestic, industrial or agronomic human activities. This form of waste reutilisation is extremely important in the Mediterranean environment due to the depletion in organic matter content of its soils and the consequent risk of soil erosion and environmental degradation. However, the role of organic amendments in the recovery of soil biodiversity has the counterpart of an increase in the risk of environmental pollution due to an excess or inadequate balance of soil nutrients or the presence of organic and inorganic pollutants. In this book, numerous research papers are presented concerning the effects of organic wastes addition to soil as a consequence of its generalised use in Southern European agroecosystems.

The research on soil enzymology, which has developed in Spain since the 1980s, has achieved a notably high level of relevance. However, its importance has not been recognised in the public and institutional domain and these studies have not been taken into account in terms of environmental monitoring of soil, nor in the design of the management of organic residues, nor environmental restoration. Therefore, at the beginning of December 2008, the Spanish Group of Soil Enzymology organised an international meeting, held in Burgos (Spain), with the intention of disseminating the research in soil enzymology. As a consequence of

this event, at which more than fifty research papers were presented, a collection of the most relevant works have been collected, updated and reviewed in this book presenting interesting topics in the research of applied soil enzymology and related parameters such as microbial biomass quantisation or the use of new molecular tools in soil biochemistry.

The book is divided in three main sections: the first one is dedicated to the discussion of the role of enzymes as indicators in environmental monitoring, the second one deals with the application of soil enzymology and related biochemical parameters to environmental restoration processes, most of them involving the recycling of organic wastes, and finally, the third section tries to introduce new molecular tools or new applications of enzymes to other technological applications of organic residues.

The book has an introductory chapter written by Prof. Nannipieri et al., which is an up to date revision of the history of soil enzymology, the future challenges for it and the emerging tools in molecular ecology. Furthermore, the first chapter of each section contains a deep analysis of its main purpose. The first section addresses the role of enzymes and other biochemical properties in soil and ecosystem monitoring, the second section revises the effect of heavy metal pollution on microbial activity and the changes induced in the microbial community structure, and in the third one, new advances in molecular fingerprinting are applied to the study of microbial communities that play a significant role in organic wastes treatments, such as anaerobic processes.

The book also includes interesting studies about the behaviour of enzymes in contrasting soils, such as those of Mexican *tetapetes*, andosols from the Canary Islands, minimally disturbed Mediterranean soils of Catalonia, grassland soils or eucalyptus plantations from Galicia or fire affected soils. Two chapters are related to the study of humus-enzyme complexes and the information that they give about soil quality or more general aspects related to soil biochemical properties such as the influence of pre treatments of soil samples and their influence in the consequent interpretation of the results.

In section two, after the general introductory chapter, particular aspects are included in several chapters such as the the effect of heavy metals on soil enzymes in soils contaminated after Aznalcollar spill (Seville, Spain) or agricultural soils contaminated with Zn. Other chapters are related to the effect of xenobiotics on soil enzymes such as those dedicated to study the effect of 2,4,5-trichlorophenol, Banvel or mixture of herbicides like oxyfluoren and glyphosate. Finally, the use of organic amendments in restoring degraded soils are treated in three different chapters: one of them is about the recovery of a forest nursery field soil using several organic amendments, another is the recovery of gypsiferous soils with deinking paper sludge, alone or co-amended with other organic residues, and, finally, the middle term effect of the use of compost of sewage sludge to agricultural soils in Catalonia.

The third section concludes the book with chapters dedicated to the study of the extraction and characterisation of humus-enzyme complexes in vermicompost, the characterization of L-glutaminase in compost of urban refuse, the effect of dry

olive residues, after a fungal treatment or not, in the rhizosphere soil of lettuce and finally, the effect of biodisinfection processes on soil microbial populations.

The editors are grateful to Springer and to Ms. Oelschlaeger for the interest shown in publishing the results of this International Conference and to Springer Publishing Group for its help in the production of the book.

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Past, Present and Future in Soil Enzymology

Paolo Nannipieri, Loretta Landi, Laura Giagnoni and Giancarlo Renella

Abstract The bibliography on soil enzymes is extensive as showed by books and many review chapters devoted to the subject. The assays of soil enzymes are generally simple, accurate, sensitive and relatively rapid and for this reasons they have been extensively used to determine the effects of contaminants, changes in management practices and effects of environmental factors and plant cover on soil metabolism. However, the present enzyme assays determine potential rather than real enzyme activities due to the optimal conditions of the assays and they do not discriminate the contribution of extracellular stabilised enzymes from that of intracellular enzyme activities. The determination of the latter is important to evaluate the answer of soil microorganisms to any effect on soil. Methods based on fumigation of soil with chloroform or with the physiological response of soil microorganisms to glucose addition to soil present drawbacks.

Presently, enzyme activities are still used to evaluate the response of soil metabolism to any effect not only in arable soils but also in forest soils. However, not always the past bibliography and the limits of the present enzyme assays are considered. A few innovative studies have been carried out. Measurements of enzyme activities have been combined with those on microbial diversity evaluated by molecular techniques. Both synthesis and persistence of phosphomonoesterases have been quantified in studies based on the stimulation of microbial growth by adding easily degradable organic compounds to soil. Metcalfe et al. (Appl Environ Microbiol 68:5042–5050, 2002) covered all events from gene presence, through gene expression and up to the detection of target enzyme in soil. The addition of sludge to a pasture soil increased chitinase activity and the number of actinobacteria but selected actinobacterium-like chitinase sequences.

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Enzyme assays distinguishing the contribution of extracellular stabilised enzymes from that of intracellular enzyme activities are needed. Future research should increase the number of enzyme activities which can be determined in soil. For example, an accurate assay for determining nuclease activity in soil is not available. It is important to set up accurate methods for extracting intracellular and stabilised extracellular proteins, which are largely prevailing, so as to be able to carry out the proteomic approach in soil. The understanding of microbial synthesis of proteins (functional proteomic) as affected by different environmental conditions can increase our knowledge on the synthesis of enzymes in soil whereas the characterization of proteins protected against microbial degradation by their interactions with surface-reactive particles or their inclusion within humic component (structural proteomic) can give insights on the stabilization of organic N, including enzymes, in soil. The set up of suitable techniques is needed to visualise the location of stabilised enzymes in soil sections by both scanning electron microscopy and transmission electron microscopy. Acid phosphatase activity has been detected in small (7×20 nm) fragments of microbial membranes, roots, mycorrhizae, etc. of soil but not in naturally-electron dense soil components (minerals) and in soil components reacting with OsO_4 (humus) and this does not permit to localize extracellular enzymes or proteins stabilized by clay minerals or humic materials (Ladd and Butler, Aust J Soil Res 4:41–54, 1966).

1 Introduction

The bibliography on soil enzymes is extensive and several reviews and some books (Burns 1978a; Kiss et al. 1998; Burns and Dick 2002) have been published on the topic. The widespread interest on soil enzymes is mainly due to the fact that the relative activities are involved in the degradation and release of inorganic nutrients from organic matter and the enzyme assays are simple, rapid, accurate and generally tested in soils with a broad spectrum of properties. In addition enzyme activities of soil are thought to be sensitive indicators of changes in soil metabolism and thus soil quality.

The aim of this review is to discuss: 1) the history of soil enzymology by mentioning the main scientists who have contributed to its advance; 2) the present gaps in our knowledge and some drawbacks of the current approaches to the study of soil enzymes. Finally we shall propose future researches to better understand the origin and the state of enzymes in soil.

2 History of Soil Enzymes

As shown by Skujins (1978) the first report on the presence of enzymes in soil was that by Woods, who in 1899 hypothesised the presence of peroxidase in the decay of plant residues. He was followed by Conn (1901, in Skujins 1978) who

hypothesised the importance of enzymes from bacteria and yeast in the degradation of plant residues. Due to its easy detection catalase was the most studied enzyme activity of soil in the 1920s and 1930s in Soviet Union (Kurtyakov), Rumenia (Radu), Italy (Rotini and Galletti), Germany (Scharrer), Belgium (Baeyensand Livens), Hungary (Valy), Czechoslovakia (Smolik) and Japan (Matsuno and Ichikava). In the 1930s Rotini reported the presence of pyrophosphatase and urease activities in soil (Skujins 1978). The presence of the latter enzyme activity in soil was also confirmed and studied in more details by Conrad in the 1940s (Conrad 1940, 1942, 1944). In the same years Rogers and co-workers studied the hydrolysis of organic P to inorganic P in soil (Rogers et al. 1941, 1942). However, the development of soil enzymology reached its maximum after 1950 with the set up of accurate methods to measure enzyme activities in soil and the use of theoretical approaches to understand the origin and state of enzymes in soil. In Soviet Union Kuprevisch and co-workers (Kuprevich and Shcherbakova 1971) and Galstyan (Skujins 1978) carried out an extensive research measuring several enzyme activities under different vegetative covers, different soils and different climates. In Rumania, Kiss and co-workers monitored several enzyme activities in polluted and unpolluted soils and they discussed the state of several enzyme locations whose activities contributed to the measured enzyme activity (Kiss et al. 1975). In the Western Europe, Hofman and collaborators improved methods to determine soil enzyme activity such as acid and alkaline phosphomonoesterase (commonly called phosphatase) activities (Hofmann and Hoffmann 1966).

In USA Arthur Douglas McLaren emphasised the role of extracellular enzymes adsorbed by clays or entrapped by the humic matter (McLaren 1975). He studied the peculiarity of enzyme kinetics in soil due to the heterogeneity of soil (McLaren and Packer 1970). Cervelli et al. (1973) quantified the effect of substrate (*p*-nitrophenyl phosphate) adsorption by soil particles on the Km value of phosphatase activity of four soils and the subject of enzyme kinetics in soil was reviewed by Nannipieri and Gianfreda (1998). John Skujins, who collaborated with McLaren, reviewed the history of soil enzyme research by underling the conceptual mistake of considering a single enzyme activity of soil as indicator of soil microbial activity or soil fertility, since both include a multitude of enzyme reactions and depend on many soil properties (Skujins 1978). Despite this problem has been also discussed by Nannipieri et al. (1990), nowadays there are still reports which take a single enzyme activity as an index of microbial activity, soil fertility and even soil quality. What stated by Skujins (1978) "It is evident enzymes are substrate specific and individual enzymatic measurements cannot reflect the total nutrient status of the soil" should be considered prior to any study involving measurements of enzyme activities in soil.

In the same years Jack Bremner and collaborators studied factors affecting urease activity in soil and the production and the stabilization of these enzymes in soil (Bremner and Mulvaney 1978). Ali Tabatabai, who collaborated with Bremner at the beginning of his scientific carrier, set up several enzyme assays (Tabatabai 1994). His protocol for setting up these methods involved the determination of either the disappearance of the substrate or the formation of the reaction product,

and the determination of the optimal substrate concentration, temperature and buffer for the enzyme activity; the new assay was always tested on several soils with a broad range of properties. This approach should be considered in setting up new enzyme assays. Researchers such as Dick R., Dick W.A., Eivazi F., Frankemberger W.T. Jr and Juma N.G. have collaborated with Tabatabai on studying effects of agricultural and forest practices, pollutants, environmental conditions and soil properties on the activity of several soil enzymes.

In Australia, Jeff Ladd set up set up methods to determine soil proteases active against high and low molecular substrates (Ladd and Butler 1972) and also suggested the presence of humus-enzyme complexes in soil (Ladd and Butler 1975). In New Zealand Speir and Ross carried out extensive research on enzyme activities of grassland soils (Speir and Ross 1978). In Japan, Hayano (1993) suggested the bacterial origin of protease in soil and this has been confirmed by molecular techniques (Mrkonjic Fuka et al. 2008a, 2008b). Hayano (1977) also extracted phosphodiesterase from soil.

Burns et al. (1972a, 1972b) hypothesised that extracellular hydrolases acting on low molecular weight substrates were stabilized in soil because surrounded by a network of humic molecules with pores large enough to allow the passage of the substrates and products of reactions but not that of the high molecular weight proteases. This hypothesis was supposed to be supported by the fact that humus-urease (Nannipieri et al. 1978) and humus-phosphatase complexes (Nannipieri et al. 1988) of higher molecular weight extracted from soil were more resistant to thermal and proteolytic degradation than those with low molecular weight. Humus-enzyme complexes of higher molecular weight were supposed to possess molecular arrangements more likely to that proposed by Burns et al. (1972a, 1972b; Nannipieri et al. 1996a). However the model proposed by Burns et al. (1972a, 1972b) was supposed to be not valid for enzymes acting on high molecular weight substrates because they would also be accessible to proteolytic enzymes with the consequent their degradation (Ladd and Butler 1975). The resistance of humus-enzyme complexes to thermal denaturation and proetolysis may also be due to the presence of glycoproteins which are more resistant than non-glycosilated proteins; pyrolysis-gas chromatography of humus-enzyme complexes extracted from soil showed the presence of glycoproteins (Bonmatí et al. 1998). Humus-enzyme complexes studied by Nannipieri et al. (1996a) were extracted by neutral sodium pyrophosphate, normally used to solubilise organic matter from soil under mild conditions; the fractionation procedure involves ultrafiltration of the soil extract against pyrophosphate separating the retained material into fractions of molecular weight higher (A_I) and lower (A_{II}) than 10^5, followed by gel chromatography of the two fractions (Ceccanti et al. 1978). By considering all his research on enzymes (laccase, polyphenol oxidase, phosphomonoesterase, phosphodiesterase, arylsulfatase, cellulose, xylanase, β-glucosidase, invertase and protease) extracted from soil by phosphate–EDTA at pH 7–8 for 1 h. Mayaudon (1986), suggested that these enzymes were fungal glycoproteins entrapped by negatively charged bacterial lypopolysaccharides, which were linked to negatively charged humic molecules by Ca ions.

An alternative approach to study the state of the humus-enzyme complexes was based on the preparation of artificial humus-enzyme complexes by oxidative coupling or condensation reactions of phenolic compounds with immobilization of the selected enzyme (Burns 1986; Nannipieri et al. 1996a). Rowell (1974) immobilised trypsin, pronase, subtilisn, papain, carboxypeptidase A, urease and acid phosphatase by oxidative coupling of *p*-benzoquinone modifying the procedure by Ladd and Butler (1966). Usually the synthetic humus-enzyme complexes have properties similar to those of the naturally occurring complexes and the entrapped enzyme is usually active and resistant to thermal and proteolytic degradation (Rowell et al. 1973; Burns 1986; Nannipieri et al. 1996a).

The bibliography on clay-enzyme complexes is extensive. The clay-enzyme interaction is a very complex process depending on several factors such as type and surface area of clay, clay moisture, nature of exchangeable cation, pH of the bulk phase and pH of the clay-water interface (Theng 1979; Burns 1986; Stotzky 1986; Boyd and Mortland 1990; Quiquampoix 2000). Characteristics of the enzyme molecule such as solubility, molecular weight, isoelectric point, polar and ionisable functional groups, are also important (Nannipieri et al. 1996a)

The meaning of measurements of enzyme activities was also discussed. Measurements of enzyme activities give potential rather than real enzyme activities because soil is submerged and incubated with buffer at optimal substrate concentrations, optimal pH and temperature values in the present assays whereas *in situ* rarely soil is submerged and substrate concentration and pH are rarely at optimal values whereas temperature and moisture can fluctuate widely (Burns 1978b). The site of enzymes contributing to the measured enzyme activities was also discussed; in addition to the intracellular location, enzymes could be active as extracellular proteins associated to the microbial plasmic membrane, contained and attached to the walls of the periplasmic space or released into the soil aqueous phase; the latter, the free extracellular enzymes, are short-lived unless they are adsorbed by surface-reactive particles or entrapped in the humic matter (Kiss et al. 1975; Burns 1982; Nannipieri et al. 2002). Some periplasmic extracellular enzymes are glycosylated before being released in the extracellular environment and this make these glycosylated extracellular enzymes more resistant to thermal and proteolytic degradation (Feldman et al. 2005).

Extracellular enzymes stabilised by adsorption on surface-reactive particles or by entrapment by humic matter are rather stable (Nannipieri et al. 1996a) and they may represent an important enzyme activity when microbial activity is low due to unfavourable conditions (Nannipieri et al. 2002).

Burns (1982) proposed an ecological role for the extracellular stabilised enzymes in soil; the activity of these enzymes can release reaction products triggering the synthesis of extracellular enzymes by soil microorganisms; it is unreasonable the continuous release of extracellular enzymes by microbial active cells inhabiting soil because it can not be energetically supported if the reaction substrate is not available in the microenvironment surrounding the microbial cell. Unfortunately it is difficult to verify this hypothesis due to the problems in setting up experiments simulating the soil microenvironment.

3 From the Nineties to the Present Time

Nowadays enzyme activities of soil are increasingly determined by assays based on the use of conjugates of the fluorescent compound methylumbelliferone (MUB), which upon enzymatic release can be determined at low concentration being highly fluorescent and this makes these assays very sensitive (Marx et al. 2001). The MUP assay gave lower Km values than the *p*-nitrophenyl phosphate assay (Marx et al. 2001) probably because the former substrate better simulated the hydrolysis of naturally occurring soil organic phosphate esters (Freeman et al. 1995). However, further research involving several soils with a broad spectrum of properties and the relative comparison with the conventional short-term assays is required.

Despite the conceptual problems in using a single enzyme activity as an indicator of soil quality, microbial activity or activity of microbial processes, such as N mineralization, have been discussed several times (Skujins 1978; Nannipieri et al. 1990; Nannipieri 1994), single enzyme activities are still assumed to represent them. For example, β-glucosidase is assumed to represent C cycling and urease activity or single protease or peptidase activities are frequently used as indicators of N mineralization. β-glucosidase activity is involved in the hydrolysis of glucosidic bonds with release of glucose whereas C cycling involves a multitude of enzyme reactions concerning substrates such as cellulose, lignin, starch, lipids, etc. Urease activity is involved in the hydrolysis of urea and this just one of the many N organic substrates which are mineralised to NH_4^+ in soil. Glycine aminopeptidase activity has been taken as an indicator of N mineralisation (Allison and Vitousek 2005); even assuming protein N mineralisation as the main metabolic process involved in N mineralization, glycine aminopeptidase being one of the several exopeptidases can not represent the whole processes which involves proteases, exopeptidases, endopeptidases and several enzymes releasing NH_4^+ (Ladd and Jackson 1982).

The problem of distinguishing the stabilised extracellular enzyme activity from the enzyme activity associated to active microbial cells in soil was addressed by using bacteriostatic such as toluene. This has not solved the problem (Nannipieri 1994) and in the case of enzymes like urease, toluene can increase the permeability of microbial membranes to urea thus conducting to an overestimation of the urease activity (Nannipieri et al. 2002). When soil irradiation was used to eliminate enzyme activity of viable cells, differences in stabilised extracellular β-glucosidase but not in the stabilised arylsulfatase activities were related to changes in soil management (Knight and Dick 2004). However, further research is needed before using this physical treatment to distinguish intracellular from stabilised extracellular enzyme activities because we do not know the effect of soil irradiation on the release of active enzymes from killed cells and on the efficient elimination of active microbial cells protected by their association with soil particles. Another approach (the physiological approach-see Nannipieri et al. 2002) was used to calculate the stabilised extracellular enzyme activity of soil. Mc Laren and Pukite (1973) correlated the urease activity versus number of ureolytic microorganisms

determined by Paulson and Kurtz (1969) at various times after adding glucose and nitrate to soil. The correlation was positive and the extrapolation of ureolytic microorganisms to zero gave a positive intercept, which was assumed to represents the extracellular urease activity of soil. Nannipieri et al. (1996b) discussed that ureolytic bacteria determined by plate counts only represent a minor proportion of soil microorganisms (Torsvik et al. 1996) and thus a more accurate determination of microbial numbers or biomass was required. Nannipieri et al. (1996b) stimulated microbial growth of several soils by adding glucose and a N source and correlated the phosphomonoesterase activity measured at pH 6.5 with microbial biomass determined by the ATP content; in some soils the extrapolation of the ATP content at zero gave a positive intercept, which was assumed to represent the stabilised extracellular phosphomoesterase activity of soil. Since it was argued that the ATP content may not represent the microbial biomass when soil samples are analysed immediately after sampling (Nannipieri et al. 2002), Renella et al. (2006) monitored the changes in the content of double stranded DNA, an indicator of microbial biomass, with the changes in acid and alkaline phosphomonoesterase activities when soils were treated with ryegrass residues; both enzyme activities were positively correlated with microbial biomass and the intercept at zero of microbial biomass gave positive intercepts of both enzyme activities (Renella, Landi and Nannipieri, unpublished data). The physiological approach can only be valid with constitutive enzymes whose activity increases with microbial growth but not with enzymes, whose synthesis is not related to microbial growth; for example, the microbial synthesis of phosphatases is repressed by the presence of phosphates even if the microbial growth is stimulated in soil (Nannipieri et al. 2002). Another approach was set up by Klose and Tabatabai (1999a, 1999b), the so called chloroform fumigation method. By assuming that the present short term enzyme assays measure the extracellular stabilised enzyme activity, it was suggested that the enzyme activity of chloroform fumigated soils was due to the extracellular plus the intracellular enzyme activity since the lysis of microbial cells by chloroform fumigation allows the interaction between the reaction substrate and the intracellular enzymes. By subtracting the enzyme activity of the unfumigated soil from the enzyme activity of the fumigated soil was thus possible to calculate the intracellular enzyme activity. Renella et al. (2002) showed that during chloroform fumigation proteases partially degraded intracellular hydrolases and thus there was an underestimation of intracellular hydrolase activities. Another critical problem of this method is the assumption that the present shot-term enzyme assays measures the stabilised extracellular enzyme activity since this assumption has never been proved. The fact that hydrolase activities, such as urease and phosphomonoesterase activities can increase with the increase in microbial biomass, after the addition of C and N sources to soil, seems to indicate that these short-term enzyme assays also determine the intracellular enzyme activity (Nannipieri et al. 2002).

Production (Pr) and persistence (Pe) of acid and alkaline phosphomonoesterase, phosphodiesterase, urease, protease and β-glucosidase activities have been calculated in soils treated with glucose and N source by the following

relationships: Pr = H/t_H and Pe = $(r/H)/\Delta t$, where H is the peak of enzyme activity at time t_H, r is the residual activity at time t_r and Δt is the time interval $t_r - t_H$ (Renella et al. 2007).

Since it is unrealistic to assume that a single enzyme activity can be an indicator of microbial activity or microbial functional diversity in soil a more reasonable approach is to integrate a range of enzyme measurements in a single index or to perform the statistical analysis of several enzyme measurements so as to calculate microbial activity or microbial functional diversity in soil (Nannipieri et al. 2002). The first attempts to integrate different enzyme activities in a single index were carried by Stefanic et al. (1984), who proposed the Biological Index integrating dehydrogenase and catalase activity, and by Beck (1984), who proposed the Enzyme Activity Number (EAN) integrating dehydrogenase, catalase, phosphatase, protease and amylase activities. The EAN index gave more realistic indications than the BIF index (Perucci 1992). Nannipieri underlined that indices proposed by Stefanic et al. (1984) and by Beck (1984) were based con enzyme assays less accurate and sensitive than those currently used. In addition, the rationale of the choice of enzyme activities was not clear, since, for example, the cellulase activity should be preferred over amylase activity since cellulose is a more abundant than starch in plant residues reaching soil. Trasar-Cepeda et al. (1998) found the empirical relationship: total N = (0.38×10^{-3}) microbial biomass C + (1.4×10^{-3}) mineralized N + (13.6×10^{-3}) phosphatase activity + (8.9×10^{-3}) β-glucosidase activity + (1.6×10^{-3}) urease activity. However, also in this case the measurement of protease and deaminase activities would be more appropriate than the measurement of urease activity as indexes of soil organic N mineralization (Nannipieri et al. 2002). The integrated lignocellulase activity index proposed by Sinsabaugh et al. (1992) was calculated by integrating β-glucosidase, β-xylosidase, endocellulase, exocellulase and phenol oxidase activities by principal component analysis and this index was significantly correlated with the mass loss of birch ice-cream sticks. The choice of the measured enzyme activities was conceptually reasonable since all enzymes are involved in the degradation of the lignocellulose complex, the most important quantitative component of plant residues (Nannipieri et al. 2002). Pugliesi et al. (2006) calculated three indexes by canonical discriminant analysis of seven enzyme (arylsulphatase, β-glucosidase, phosphatase, urease, invertase, dehydrogenase and phenol oxidase) activities of three different agricultural sites. The three indexes discriminate between altered and control soils but the third index (AI3 = 7.87 β-glucosidase—8.22 phosphatase—0.49 urease) was able to discriminate contaminated from control soils not only for the investigated soils but also for those of published papers. Multivariate statistics can be an alternative approach to the use of single indexes (Nannipieri et al. 2002). By using this approach Kandeler et al. (1996) showed that dehydrogenase and arylsulfatase activities were the most important variables explaining more than 94% of total variance of the data set. According to Dilly and Blume (1998) a sun ray plot displaying the measured enzyme activities can give an overall idea showing differences among these enzyme activities whereas these differences are lost by their integration in a single index.

The mean ratio for β-1, 4-glucosidase (it was assumed to represent C acquisition by microflora and it catalyses the hydrolysis of cellobiose with release), β-1, 4-N acetyl glucosaminidase and leucine aminopeptidase (they were assumed to represent N acquisition by microflora and catalyse the hydrolysis of glucosamine from chitibiose and amino acid from the N terminus of proteins and peptides) and acid or alkaline phosphomonoesterase (they were assumed to represent P acquisition by microflora and catalyse the hydrolysis of organic ester P to phosphate) activities was near to 1:1:1 in some soils and freshwater sediments and this was suggested to reflect the equilibrium between the elemental composition of microbial biomass and organic residues and represent the efficiencies of microbial nutrient assimilation and growth (Sinsabaugh et al. 2009). This interesting and innovative approach suffers of the drawback of considering single enzyme activities representative of multienzyme processes.

The ecological dose 50 (ED_{50}), which is the concentration of the toxicant inhibiting the enzyme activity by 50%, allows quantifying the effect of the pollutant on the measured enzyme activity; the ED_{50} value can been calculated by sigmoidal dose-response model (Haastra et al. 1985; Doelman and Haastra 1989) or by the Michaelis–Menten kinetic with two different models (Speir et al. 1995, 1999). The model 1 is based on the relationship $v = c/(1 + bi)$, where v is the enzyme activity, i the concentration of the toxicant and c and b are constants; it was the most successful in calculating the ED_{50} (given by 1/b) of the ATP content, urease and dehydrogenase activities of two Cd polluted soils (Moreno et al. 2001). The model 2, which describes the partial inhibition according to the relationship $v = c\ (1 + ai)/(1 + bi)$, where v, b, c and i have the same meaning of what stated for model 1 and where a is another constant, was used to calculate the ED_{50}, here given by $(1-ab)/(b-a)$, values of acid and alkaline phosphomonoesterase activities of soils polluted with few heavy metals (Renella et al. 2003).

Models of soil organic matter decomposition are based on first order kinetic whereas the enzyme kinetic has been neglected in these models despite the decomposition is a multienzyme process. A conceptual model based on exoenzyme catalysing decomposition has been proposed by Schimel and Weintraub (2003) but it needs to be tested in different soils and compared with the other models.

Stemmer et al. (1998) developed a method to separate soil particle-size fractions with low-energy sonication so as to evaluate enzyme activities of the moist soil fractions. Invertase activity was higher in the fine than coarse fractions as it was the organic C content and microbial biomass whereas xylanase, a mainly extracellular enzyme, activity showed an opposite behaviour. Alkaline phosphomonoesterase activity was mainly located in silt and clay fractions whereas protease activity showed high values in coarse sand and in the clay fraction (Kandeler et al. 1999a). The location of xylanase activity in coarser fractions was also found during maize straw composition (Stemmer et al. 1999). An imaginative and interesting approach was set up by Kandeler et al. (1999b) to sample the thin soil layers in the detritusphere so as to monitor changes in enzyme activities at the microscale; xylanase, invertase and casein-hydrolysing activities of the maize

straw were higher than the corresponding values of soil samples taken from 1 to 2 mm from the straw interface.

The effect of raising atmospheric temperature on enzyme activities is another hot topic of the present time. According to Trasar-Cepeda et al. (2007) only β-glucosidase activity among nine tested enzyme activities of three soils had the Q_{10} at 20°C value higher than 2 in one of the soils whereas most of the other enzyme activities had a value closer to 1.5. However, temperature sensitivity of soil enzyme activity changes with season (Trasar-Cepeda et al. 2007; Wallenstein et al. 2009), probably because different enzyme catalysing the same reaction contribute to the measured enzyme activity. Indeed temporal changes in both laccase and peroxidase isoenzymes involved in litter decomposition were observed by Di Nardo et al. (2004). However sensitivity of enzyme activity to increasing temperature in situ may differ from that measured under laboratory conditions due to the marked differences between enzyme assays and in situ environmental conditions (Wallenstein et al. 2009). Caution is required in relating measurements in enzyme temperature sensitivity to organic matter temperature sensitivity because the latter is still poorly known and the measured enzyme activity represents a potential rather than a real enzyme activity as mentioned above (Von Lutzow and Kögel-Knabner 2009).

4 Future Research

A challenge in soil enzymology is to identify the functional location of extracellular enzyme in soil and possibly to quantify its contribution to the measured enzyme activity (Wallestein and Weintraub 2008). This approach would also permit detecting the soil components responsible for the enzyme stabilization (Nannipieri 2006) Visualization of both intracellular and extracellular enzyme in the soil matrix was approached by Foster using cytochemical and histochemical techniques and electron microscopy (Ladd et al. 1996). Acid phosphatase activity was detected in small (7×20 nm) fragments of microbial membranes, roots, mycorrhizae, etc. of soil but not in naturally-electron dense soil components (minerals) and in soil components reacting with OsO_4 (humus) and this did not permit to localize extracellular enzymes or proteins stabilized by clay minerals or humic materials (Ladd et al. 1996). Potential advances in the visualization of enzymes may be obtained by using Quantum Dots, nano-scale crystals emitting in the near-infrared wavelength; the Quantum Dots are quenched unless they bind the target enzyme. This technique has been used to detect protease activity in biomedical research and it may be adapted to soil (Whiteside et al. 2009).

Molecular techniques for detecting DNA sequences have been extensively used in soil and with metagenomic technologies and their use has improved the determination of composition of complex microbial communities such as those inhabiting soil (Urich et al. 2008). Changes in the acid and alkaline phosphomonoesterase, phosphodiesterase and protease activities have been related to

changes in bacterial and fungal diversity determined by Denaturing Gradient Gel Electrophoresis (DGGE) to evaluate the in situ stabilization of an arsenic-contaminated soil (Ascher et al. 2009). However these DNA-based studies do not allow determining gene expression in soil and thus are not indicative of soil functioning. Monitoring the expression of gene sequences at both transcription and translational levels is needed in soil science in analogy to post genomic studies of cultivated organisms. The analysis of the target mRNA is supposed to be an indicator of the transcription activity in soil. Despite extraction procedures of soil RNA and DNA are similar in principle, successful extraction and characterization of mRNA from soil has lagged behind those of DNA due to problems such as activity of nucleases and fast turnover rate of prokaryotic mRNA (Costa et al. 2004; Bakken and Frostegård 2006). However, several methods are now available to characterise mRNA and thus to measure gene expression in soil (Metcalfe et al. 2002; Krsek et al. 2006). An RNA meta-transcriptome approach involving extraction of both mRNA and rRNA from a sandy soil, reverse transcription to cDNA and direct pyrosequencing, produced both cDNArRNA-tags and m-RNA-tags and this allowed the quantification of abundant microorganisms and information of the activity of enzymes involved in ammonia oxidation and CO_2 fixation (Urich et al. 2008).

The first study linking the presence and expression of genes to the measured enzyme activity (chitinase activity) in soil was carried out by Metcalfe et al. (2002). Primers targeted to a gene fragment from family 18 (one of the two main groups in which chitinases have been classified according to amino acid similarities within the catalytic domain of the enzyme molecule) were used to determine the diversity and origin of chitinases after analysis of DNA extracted from a pasture soil. The addition of sludge to the pasture soil increased the chitinase activity and the number of actinobacteria but decreased the diversity of chitinase enzymes. Extraction of transcripts was unsuccessful, probably due to the adsorption of mRNA by soil colloids, and the target enzyme proteins were not monitored. As already mentioned, genes codifying protease and protease activity in soil has been already compared (Mrkonjic Fuka et al. 2008a, 2008b). The interrelation of DNA, RNA, proteins and substrate activity has been called the meta- approach.

Multiple protein isoforms can be synthesised by a single gene because mRNA molecules can be subjected to post-transcriptional control such as alternative splicing, polyadenylation and mRNA editing (Graves and Haystead 2002). The analysis of expressed proteins in pure culture is rapid and sensitive and involves extraction of proteins, their separation by 2-dimensional gel, the solubilisation of excised band and successive trypsin digestion followed by analysis of tryptic peptides by ionization mass spectrometers (Pandey and Mann 2000). The use of bioinformatics for processing a huge mass of data is also required.

Studies about soil proteomic should consider that on average microbial N accounts for 4% of organic N in soil whereas most of the total organic N is present as extracellular protein N or peptides N stabilised by soil colloids (Nannipieri 2006). Indeed information on microbial gene expression should be based on the characterization of intracellular microbial proteins unless information on microbial

processes involving extracellular enzymes is required. The characterization of extracellular proteins, protected against proteolysis by their association with soil colloids, should give insights on mechanisms responsible of such stabilization. The two approaches have been termed soil functional proteomics and soil structural proteomics, respectively (Nannipieri 2006). The study of functional proteomics can improve our understanding of degradation of organic pollutants and organic debris, nutrient cycling, blockage of inorganic pollutants, molecular colloquia between microorganisms, between plant roots and microorganisms and between plant roots (Nannipieri 2006). A successful extraction of intracellular proteins from soil should lyse microbial cells, inhibit proteases, avoid the adsorption of proteins with soil colloids once released after cell lysis and be representative of the status of microorganisms inhabiting soil. The manipulation of the sample prior to extraction should not alter microbial physiology.

Indeed most of the reports on soil proteomics are based on the direct extraction method (Nannipieri 2006). However, caution is required in using both colorimetric (Bradford method) or immunological (ELISA) methods to determine extracted proteins since these techniques can also determine phenolic compounds and litter and humic components (Rosier et al. 2006; Whiffen et al. 2007; Roberts and Jones 2008).

The phylogenetic origin of proteins, from dissolved organic matter of forest soil, was evaluated and bacterial proteins were classified in ribosomal, transcription, membrane and enzyme proteins according to their function (Schulze 2004). The procedure involved, after extraction, purification of proteins by gel filtration (with removal of humic acids, phenolic compounds and small molecules) concentration by ethanol before SDS–PAGE, silver staining, cutting of each protein band and digestion by trypsin; mixtures of tryptic peptides were separated by nanoflow liquid chromatography prior to analysis by mass spectrometry. The power of analytical MS tool was also shown by detecting chloro catechol dioxygenase, enzymes involved in the degradation of 2,4-D (2,4-dichlorophenoxy acetic acid); proteins were extracted by 0.1 M NaOH from 2,4-D treated soils, successively purified and separated by SDS–PAGE; the excised bands of gels were digested with trypsin prior to mass spectrometry analysis of tryptic peptides (Benndorf et al. 2007).

An alternative approach to extract microbial proteins from soil is the indirect extraction method, that is, the separation of microbial cells from soil particles and successive cell lysis with release of proteins. The microbial extraction prior to cell lysis should give purer samples but with lower yields than the direct extraction method. However, artefacts due to possible changes in the physiology of microorganisms can occur during the extraction of microbial cells from soil (Nannipieri 2006) making it difficult to relate the microbial proteins to the effects of the studied factors (i.e. stresses, agriculture management, etc.). Maron et al. (2008) used the indirect extraction method to show that copper or mercury pollution of soil stimulated the synthesis of protein with molecular weight ranging from 20 to 50 kDa; some of these proteins, such as those of heavy metal efflux pumps, were involved in heavy metal resistance mechanisms.

Phosphate is more efficient than other buffers in extracting proteins from soil (our unpublished data). However each procedure for extracting proteins from soil should be tested with a microbial strain with a known proteome so as to evaluate any effect of the used procedure on the microbial proteome. We are testing our extraction procedure on *Cupriavidus metallidurans* CH34 (unpublished data).

In conclusion advances in soil enzymology require: (1) to better understand the meaning of measurements of enzyme activity in soil by discriminating activities due to enzymes associated to active microbial cells from activities due to the stabilized extracellular enzymes. The determination of the former is important to evaluate the answer of soil microorganisms to any effect to soil; (2) setting up accurate assays for determining enzyme activities such as nuclease activity, whose determination is not possible nowadays; (3) standardization of methods with comparisons of potential enzyme activities determined with the current assays with the relative enzyme activities occurring *in situ*; (4) visualization of functional enzymes in the soil matrix. This may improve our understanding of the stabilization of extracellular proteins in soil; (5) determining genes codifying enzymes, and their expression at both transcription and translational level and relating these measurements to the measured enzyme activity in different soils. This approach can improve our understanding of the origin of enzyme sin soil.

The last statement is an excuse to many researchers who have contributed to the development of soil enzymology and who have not been mentioned due to the limits imposed to the length of this contribution.

References

Allison SD, Vitousek PM (2005) Responses of extracellular enzymes to simple and complex nutrient inputs. Soil Biol Biochem 37:937–944

Ascher J, Ceccherini MT, Landi L, Mench M, Pietramellara G, Nannipieri P, Renella G (2009) Composition, biomass and activity of microflora, and leaf yields and foliar elemental concentrations of lettuce, after in situ stabilization o fan arsenic-contaminated soil. Soil Biol Biochem 41:351–359

Bakken LR, Frostegård A (2006) Nucleic acid extraction from soil. In: Nannipieri P, Smalla K (eds) Nucleic acids and proteins in soil. Springer, Berlin, pp 49–73

Beck T (1984) Methods and application of soil microbial analyses at the Landnsanstalt fur Bodenkultur und Pflanzenbau (LBB) in Munich for the determination of some aspects of soil fertility. In: Nemes MP, Kiss S, Papacostea P, Stefanic C, Rusan M (Eds) Fifth symposium on soil biology, Roman National Society of Soil Science, Bucharest, Rumania, pp 13–20

Benndorf D, Balcke GU, Harms H, Von Bergen M (2007) Functional metaproteome analysis of protein extracts from contaminated soil and groundwater. The ISME J 1:224–234

Bonmatí M, Ceccanti B, Nannipieri P (1998) Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biol Biochem 30:2113–2125

Boyd AS, Mortland MM (1990) Enzyme interactions with clays and clay-organic matter complexes. In: Bollag J-M, Stotzky G (eds) Soil Biochemistry, vol 6. Marcel Dekker, New York, pp 1–28

Bremner JM, Mulvaney RL (1978) Urease activity in soils. In: Burns RG (ed) Soil Enzymes. Academic Press, London, pp 149–196

Burns RG (1978a) Soil Enzymes. Academic Press, London
Burns RG (1978b) Enzyme activity in soil: some theoretical and practical considerations. In: Burns RG (ed) Soil Enzymes. Academic Press, London, pp 295–340
Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423–427
Burns RG (1986) Interactions of enzymes with soil mineral and organic colloids. In: Huang PM, Schnitzer M (eds) Interactions of soil minerals with natural organics and microbes. Soil Science Society of America, Madison, pp 429–431
Burns RG, Dick RP (2002) Enzymes in the environment. Activity, ecology and applications. Marcel Dekker, New York
Burns RG, El-Sayed MH, McLaren AD (1972a) Extraction of an urease-active organo-complex from soil. Soil Biol Biochem 4:107–108
Burns RG, Pukite AH, McLaren AD (1972b) Concerning the location and persistence of soil urease. Soil Sci Soc Am Proc 36:308–311
Ceccanti B, Nannipieri P, Cervelli S, Sequi P (1978) Fractionation of humus-urease complexes. Soil Biol Biochem 10:39–45
Cervelli S, Nannipieri P, Ceccanti B, Nannipieri P (1973) Michaelis constant of soil acid phosphatase. Soil Biol Biochem 5:841–845
Conrad JP (1940) Hydrolysis of urea in soils by the termolabile catalysis. Soil Sci 49:253–263
Conrad JP (1942) The occurrence and origin of urease-like activities in soils. Soil Sci 54:367–380
Conrad JP (1944) Some effects of developing alkalinities and other factors upon urease-like activities in soils. Soil Sci Soc Am Proc 8:171–174
Costa R, Gomes NCM, Milling A, Smalla K (2004) An optimized protocol for simulteneous extraction of DNA and RNA from soils. Braz J Microbiol 35:230–234
Di Nardo C, Cinquegrana A, Papa S, Fuggi A, Fioretto A (2004) Laccase and peroxidase isoenzymes during leaf litter decomposition of *Quercus ilex* in a Mediterranean ecosystem. Soil Biol Biochem 36:1539–1544
Dilly O, Blume HP (1998) Indicators to assess sustainable land use with reference to soil microbiology. Adv Geoecol 31:29–39
Doelman P, Haastra L (1989) Short- and long-term effects of heavy metals on phosphatase activity: an ecological dose-response model approach. Biol Fertil Soils 8:235–241
Feldman MF, Wacker M, Hernández M, Hitchen PG, Marolda CL, Kowarik M, Morris HR, Dell A, Valvano MA, Aebi M (2005) Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. Proc Natl Acad Sci USA 102:3016–3021
Freeman C, Liska G, Ostle NJ, Jones SE, Lock MA (1995) The sue of fluorogenic substrates for measuring enzyme activity in peatlands. Plant Soil 175:147–152
Graves PR, Haystead TAJ (2002) Molecular biologist's guide to proteomics. Microbiol Mol Biol Rev 66:39–63
Haastra L, Doelman P, Oude Voshaar JH (1985) The use of sigmoidal dose response curve in soil ecotoxicological research. Plant Soil 84:293–297
Hayano K (1977) Extraction and properties of phosphodiesterase from a forest soil. Soil Biol Biochem 9:221–223
Hayano K (1993) Protease activity in a paddy field soil: origin and some properties. Soil Sci Plant Nutr 39:53–546
Hofmann E, Hoffmann G (1966) Determination of soil biological activity with enzymatic methods. Adv Enzymol 28:365–390
Kandeler E, Kampicher C, Horak O (1996) Influence of heavy metals on the functional diversity of soil microbial communities. Biol Fertil Soils 23:299–306
Kandeler E, Palli S, Stemmer M, Gerzabeck MH (1999a) Tillage changes microbial biomass and enzyme activities in particle-size fractions of a Haplic Chernozem. Soil Biol Biochem 31:1253–1264
Kandeler E, Luxhøi, Tscherko D, Magid J (1999b) Xylanase, invertase and protease at the soil-litter interface of a loamy sand. Soil Biol Biochem 31:1171–1179

Kiss S, Drăgan-Bularda M, Rădulescu D (1975) Biological significance of enzymes accumulated in soil. Adv Agron 27:25–87
Kiss S, Pasca D, Drăgan-Bularda M (1998) Enzymology of disturbed soils. Development in soil science 26. Elsevier, Amsterdam
Klose S, Tabatabai MA (1999a) Arylsulphatase activity of microbial biomass in soils. Soil Sci Soc Am J 63:569–574
Klose S, Tabatabai MA (1999b) Urease activity of microbial biomass in soils. Soil Biol Biochem 31:205–211
Knight TR, Dick RP (2004) Differentiating microbial and stabilized β-glucosidase activity relative to soil quality. Soil Biol Biochem 36:2089–2096
Krsek M, Gaze WH, Morris NZ, Wellington EMH (2006) Gene detection, expression and related enzyme activity in soil. In: Nannipieri P, Smalla K (eds) Nucleic acids and proteins in soil. Springer, Berlin, pp 217–255
Kuprevich VF, Shcherbakova TA (1971) Comparative enzymatic activity in diverse types of soil. In: McLaren AD, Skujins JJ (eds) Soil biochemistry, vol 2. Marcel Dekker, New York, pp 167–201
Ladd JN, Butler JHA (1966) Comparison of some properties of soil humic acids and synthetic phenolic polymers incorporating amino derivatives. Aust J Soil Res 4:41–54
Ladd JN, Butler JHA (1972) Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates. Soil Biol Biochem 4:19–30
Ladd JN, Butler JHA (1975) Humus-enzyme systems and synthetic organic polymer-enzyme analogs. In: Paul EA, McLaren AD (eds) Soil biochemistry, vol 4. Marcel Dekker, New York, pp 143–194
Ladd JN, Jackson RB (1982) Biochemistry of ammonification. In: Stevenson FJ (ed) Nitrogen in agricultural soils. American Society of Agronomy, Madison, pp 173–227
Ladd JN, Forster RC, Nannipieri P, Oades JM (1996) Soil structure and biological activity. In: Stotzky G, Bollag J-M (eds) Soil biochemistry, vol 9. Marcel Dekker, New York, pp 23–78
Maron P-A, Maitre M, Mercier A, Lejon DPH, Nowak V, Ranjard L (2008) P and DNA fingerprinting of a soil bacterial community inoculated into three different sterile soils. Res Microbiol 159:231–236
Marx MC, Wood M, Jarvis SC (2001) A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biol Biochem 33:1633–1640
Mayaudon J (1986) The role of carbohydrate in the free enzymes in soil. In: Fuchsman CH (ed) Peat and Water. Elsevier, New York, pp 263–309
McLaren AD (1975) Soil as a system of humus and clay immobilized enzymes. Chem Scr 8:97–99
McLaren AD, Packer L (1970) Some aspects of enzyme reactions in heterogenous systems. Adv Enzymol 33:245–308
McLaren AD, Pukite A (1973) Ubiquity of some soil enzymes and isolation of soil organic matter with urease activity. In: Povoledo D, Gloltermaan ML (eds) Humic substances and function in the biosphere. Pudoc, Wageningen, pp 187–193
Metcalfe AC, Krsek M, Gooday GW, Prosseer JI, Wellington EM (2002) Molecular analysis of a bacterial chitinolytic community in an upland pasture. Appl Environ Microbiol 68:5042–5050
Moreno JL, García C, Landi L, Falchini L, Pietramellara G, Nannipieri P (2001) The ecological dose value (ED_{50}) for assessing Cd toxicity on ATP content and dehydrogenase and urease activities of soil. Soil Biol Biochem 33:483–489
Mrkonjic Fuka M, Engel M, Gattinger A, Bausenwein U, Sommer M, Munch JC, Schloter M (2008a) Factors influencing variability of proteolytic genes and activities in arable soils. Soil Biol Biochem 40:1646–1653
Mrkonjic Fuka M, Engel M, Haesler F, Welzi G, Munch JC, Schloter M (2008b) Diversity of proteolytic community encoding for subtilisin in an arable field: spatial and temporal variability. Biol Fertil Soils 45:185–191
Nannipieri P (1994) The potential use of soil enzymes as indicators of productivity, sustainability and pollution. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota: management in sustainable farming systems. CSIRO, Melbourne, pp 238–244

Nannipieri P (2006) Role of stabilized enzymes in microbial ecology and enzyme extraction from soil with potential applications in soil proteomics. In: Nannipieri P, Smalla K (eds) Soil biology nucleic acids and proteins in soil, vol 8. Springer, Heidelberg, pp 75–94

Nannipieri P, Gianfreda L (1998) Kinetics of enzyme reactions in soil environments. In: Huang PM, Senesi N, Buflle J (eds) Structure and surface reactions of soil particles. Wiley and Sons, New York, pp 450–479

Nannipieri P, Ceccanti B, Cervelli S, Sequi P (1978) Stability and kinetic properties of humus-urease complexes. Soil Biol Biochem 10:143–147

Nannipieri P, Ceccanti B, Bianchi D (1988) Characterization of humus-phosphatase complexes extracted from soil. Soil Biol Biochem 20:683–691

Nannipieri P, Grego S, Ceccanti B (1990) Ecological significance of the biological activity in soil. In: Bollag J-M, Stotzky G (eds) Soil biochemistry, vol 6. Marcel Dekker, New York, pp 293–355

Nannipieri P, Sequi P, Fusi P (1996a) Humus and enzyme activity. In: Piccolo A (ed) Humic substances in terrestrial ecosystems. Elsevier, Amsterdam, pp 293–328

Nannipieri P, Sastre J, Landi L, Lobo MC, Pietramellara G (1996b) Determination of extracellular neutral phosphomonoeterase activity in soil. Soil Biol Biochem 28:107–112

Nannipieri P, Kandeler E, Ruggiero P (2002) Enzyme activities and microbiological and biochemical processes in soil. In: Burns RG, Dick RP (eds) Enzymes in the environment: activity ecology and applications. Marcel Dekker, New York, pp 1–33

Pandey A, Mann M (2000) Proteomic to study genes and genomes. Nature 405:837–846

Paulson KN, Kurtz LT (1969) Locus of urease activity. Soil Sci Soc Am Pro 33:897–901

Perucci P (1992) Enzyme activity and microbial biomass in a field soil amended with a municipal refuse. Biol Fertil Soils 14:54–60

Pugliesi E, Del Re AAM, Rao MA, Gianfreda L (2006) Development and validation of numerical indexes integratine enzyme activities of soils. Soil Biol Biochem 38:1673–1681

Quiquampoix H (2000) Mechanisms of protein adsorption on surface and consequences for extracellular enzyme activity in soil. In: Bollag J-M, Stotzky G (eds) Soil biochemistry, vol 10. Marcel Dekker, New York, pp 171–206

Renella G, Landi L, Nannipieri P (2002) Hydrolase activities during and after the chloroform fumigation of soil as affected by protease activity. Soil Biol Biochem 34:51–60

Renella G, Ortigoza ALR, Landi L, Nannipieri P (2003) Additive effects of copper and zinc on cadmium toxicity on phosphatase activities and ATP content of soil as estimated by the ecological dose (ED_{50}). Soil Biol Biochem 35:1203–1210

Renella G, Landi L, Ascher J, Ceccherini MT, Pietramellara G, Nannipieri P (2006) Phospho-monoesterase production and persistence and composition of bacterial communities during plant material decomposition in soils with different pH values. Soil Biol Biochem 38:795–802

Renella G, Szukics U, Landi L, Nannipieri P (2007) Quantitative assessment of hydrolase production and persistence in soil. Soil Biol Biochem 44:321–329

Roberts P, Jones DL (2008) Critical evaluation of methods for determining total protein in soil solution. Soil Biol Biochem 40:1485–1495

Rogers HT, Pearson RW, Pierre WH (1941) Absorption of organic phosphorus by corn and tomato plants and the mineralizing action of exoenzyme systems of growing roots. Soil Sci Soc Am Proc 5:285–291

Rogers HT, Pearson RW, Pierre WH (1942) The source of phosphatase activity of enzymes hydrolysing sucrose and starch. Soil Sci 54:353–366

Rosier CL, Hoye AT, Rilling MC (2006) Glomalin-related protein: assessment of current detection and quantification tools. Soil Biol Biochem 38:2205–2211

Rowell M (1974) Production and characterization of enzymes stabilised as humic acid analogues. Ph.D dissertation, University of Saskatchewan, Saskatoon

Rowell M, Ladd JN, Paul EA (1973) Enzymatically active complexes of proteases and humic acid analogues. Soil Biol Biochem 5:699–703

Schimel JP, Weintraub MN (2003) The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. Soil Biol Biochem 35:549–563

Schulze W (2004) Protein analysis in dissolved organic matter: what free proteins from soil leachate and surface water can tell us-a perspective. Biogeosciences Discuss 1:825–853
Sinsabaugh RL, Antibus RK, Linkins AE, McClaugherty CA, Rayburn L, Repert D, Weiland T (1992) Wood decomposition over a first-order watershed: mass loss as a function of lignocellulase activity. Soil Biol Biochem 34:743–749
Sinsabaugh RL, Hill BH, Follstad Shah JJ (2009) Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature 462:795–799
Skujins J (1978) History of abiontic soil enzyme research. In: Burns RG (ed) Soil enzymes. Academic Press, London, pp 1–49
Speir TW, Ross DJ (1978) Soil phosphatase and sulphatase. In: Burns RG (ed) Soil enzymes. Academic Press, London, pp 197–250
Speir TW, Kattles HA, Parshotam A, Searle PL, Vlaar LNC (1995) Simple kinetic approach to derive the ecological dose values, ED_{50}, for the assessment of Cr(VI) toxicity to soil biological properties. Soil Biol Biochem 27:801–810
Speir TW, Kattles HA, Parshotam A, Searle PL, Vlaar LNC (1999) Simple kinetic approach to determine the toxicity of As(V) to soil biological properties. Soil Biol Biochem 31:705–713
Stefanic C, Eliade G, Chirnageanu J (1984) Researches concerning a biological index of soil fertility. In: Nemes MP, Kiss S, Papacostea P, Stefanic C, Rusan M (Eds) Fifth symposium on soil biology, Roman National Society of Soil Science, Bucharest, Rumania, pp 35–45
Stemmer M, Gerzabeck MH, Kandeler E (1998) Organic matter and enzyme activity in particle-size fractions of soils obtained after low-energy sonication. Soil Biol Biochem 30:9–17
Stemmer M, Gerzabeck MH, Kandeler E (1999) Invertase and xylanse activity of bulk soil and particle-size fractions during maize straw decomposition. Soil Biol Biochem 31:9–18
Stotzky G (1986) Influence of soil mineral colloids on metabolic processes, growth, adhesion and ecology of microbes and viruses. In: Huang PM, Schnitzer M (eds) Interactions of soil minerals with natural organics and microbes. Soil Science Society of America, Madison, pp 305–428
Tabatabai MA (1994) Soil enzymes. In: Weaver RW, Angle S, Bezdicek D, Smith S, Tabatabai MA, Wollum A (eds) Methods of soil analysis. Part 2 microbiological and biochemical properties. Soil Science Society of America, Madison, pp 775–833
Theng BKG (1979) Formation and properties of clay-polymer complexes. Elsevier, New York
Torsvik V, Sørheim R, Gorksøyr J (1996) Total bacterial diversity in soil and sediment communities-a review. J Ind Microbiol 17:170–178
Trasar-Cepeda C, Leirós MC, Gil-Sotres F, Seoane S (1998) Towards a biochemical quality index for soils: an expression relating several biological and biochemical properties. Biol Fertil Soils 26:100–106
Trasar-Cepeda C, Gil-Sotres F, Leirós MC (2007) Thermodynamic parameters of enzymes in grassland soils from Galicia, NW Spain. Soil Biol Biochem 39:311–319
Urich T, Lanzen A, Qi J, Huson DH, Schleper C, Scuster SC (2008) Simultaneous assessment of soil microbial community structure and function through analysis of the metatranscriptome. PLoS ONE 3:1–13
Von Lutzow M, Kögel-Knabner I (2009) Temperature sensitivity of soil organic matter decomposition-what do we know? Biol Fertil Soils 46:1–16
Wallestein MD, Weintraub MN (2008) Emerging tool for measuring and modelling the in situ activity of extracellular enzymes. Soil Biol Biochem 40:2098–2106
Wallestein MD, McMahon SK, Schimel JP (2009) Seasonal variation in enzyme activity and temperature sensitivity in Arctic tundra soils. Glob Change Biol 15:1631–1639
Whiffen LK, Midgley D, McGee PA (2007) Polyphenolic compounds interfere with quantification of protein in soil extracts using the Bradford method. Soil Biol Biochem 39:691–694
Whiteside MD, Treseder KK, Atsatt PR (2009) The brighter side of soils: quantum dots track organic nitrogen through fungi and plants. Ecology 90:100–108

Part I
Enzymes as Indicators of Environmental Soil Quality

Role of Humo-Enzyme Complexes in Restoring of Soil Ecosystems

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Abstract Conventional chemical (total organic carbon, total nitrogen, C/N ratio), biochemical (total and extracellular β-glucosidase, ATP, dehydrogenase) and unconventional IEF parameters (active humic carbon, humic-bound β-glucosidase activity), were used in the monitoring of the changes in biochemical properties caused by organic amendment practices. Two soil ecosystems characterized by a gradient of different grass covers, i.e. (1) a natural Catena (control) and (2) a managed Catena altered by amending practices were selected in a semi arid zone of the Mediterranean (Murcia region, Spain). Both natural and managed Catenas showed activation of carbon cycle which gradually shifted vegetal carbon toward the humic substance formation and accumulation of active humic-β-glucosidase complexes. The model of study and the correlation among the selected parameters, has permitted to discriminate even little differences in soil biochemical properties and, on the basis of these properties, to rank the soil ecosystems in a decreasing order of quality: forest > shrub > bare. There was a narrow correlation between amount of humic carbon forms and humic-associated enzyme-activity, demonstrating that a sort of humification in situ was steadily taking place even after years and likely sustained by plant root exudates. The combination of UF and IEF resulted very efficient in the characterization of humic-enzyme complexes and biochemical processes which drive the humic substances formation, storage and activity in soils.

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1 Introduction

Native soil systems are characterised by an equilibrium between the main physical, chemical, and biochemical processes taking place in function of environmental conditions. The alteration of this equilibrium by natural or anthropogenic activity, may cause instability and stress (Doran and Parkin 1994). Usually microbial biomass and activity can be considered good indicators (bioindicators) of soil biological status, however, microorganisms are increasingly criticized because they are vulnerable to temporal and spatial variation (Chen et al. 2003; Gil-Sotres et al. 2005). For instance, dehydrogenase activity and ATP, and enzymes activities belonging to the microbial cells are likely changeable to transitory fluctuations in response to recent management and climatic effects, thus they are not reliable marker to assess of soil degradation (Gil-Sotres et al. 2005).

The extracellular biochemical activities stabilized out of the microbial cells, result suitable in assessing significant changes caused on soil ecosystems by external pressures. The extracellular enzymes, complexed with or entrapped within soil clays and humic matter (humo-enzyme complexes), are relatively stable and can persist for extended periods, thereby providing a long-term perspectives in indicating the history of the soil and not just a snapshot of the time of soil sampling (Lähdesmäki and Piispanen 1992; Ceccanti et al. 2008). Their importance arises from the fact that they can represent a reservoir of biochemical energy and nutrients capable to reactivate the ecosystem functionality even in heavy stressed situations, thus representing the necessary conditions for soil resilience (Ceccanti and Masciandaro 2003; Benítez et al. 2004). Since they have been found in a great variety of natural and managed soils humic-enzyme complexes they are supposed to constitute structural components of soil organic matter that significantly contribute to the empowering the biological barrier at the protection of the final and irreversible soil degradation (Klein et al. 1985; Ceccanti and Masciandaro 2003). The biochemical techniques to purify and characterize active humic-enzyme complexes were initially proposed by Ceccanti et al. (1978) and Ceccanti and Masciandaro (2003), revised by Ceccanti et al. (2008) and they are based on three steps: (1) sodium pyrophosphate extraction (pH 7.1) of humic matter, (2) purification by membrane cut off (mol wt $> 10^4$ Da) ultrafiltration (UF) of the organic extracts previously dialysed on Visking tube (1,000 Da), followed by (3) fractionation and characterization through analytical isoelectric focussing technique (IEF) (Fig. 1). IEF is an electrophoretic technique, which has been used with the purpose of the in-depth investigation of humic matter and humic-bound enzymes extracted from soils (Ciavatta and Govi 1993; Ceccanti et al. 1989) or other organic materials (Canali et al. 1998; Benítez et al. 2000). IEF is based on the separation of different humic substances on the basis of their isoelectric point (*pI*), that is, according to their net surface electric charges. Since the IEF is a non-denaturing technique, the humic-enzyme activity on the focussed bands can easily be detected.

The objective of this work was to compare the evolution of chemical and biochemical markers of soil quality in two soil ecosystems characterized by

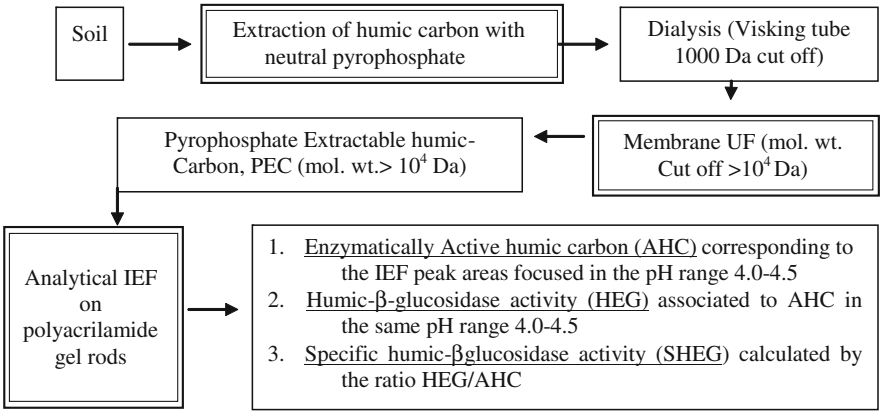

Fig. 1 Schematic flow-sheet of humic-enzyme complexes preparation and purification

(1) naturally altered soil caused by different plant cover (natural Catena) and (2) soil under a gradual practice of amelioration through organic amendment (managed Catena). For this purpose traditional parameters related to soil agro-chemical fertility and unconventional parameters related to the amount of the enzymatically active humic carbon have been proposed.

2 Materials and Methods

2.1 Case Studies

The sites selected for the study are located in the province of Murcia, Spain. The climate of the region is semi-arid Mediterranean with a mean annual rainfall of 300 mm, about 75% of which falls in April and October. One characteristic of the rainfall is its irregularity; it is infrequent but usually it is intense and gives rise to serious episodes of soil erosion. The mean annual temperature is 17°C and mean potential evapotranspiration reaches 800 mm $year^{-1}$.

2.2 Managed Catena

An experimental field (sandy clay loam soil, USDA classification) was split into plots in which 16 years ago was added a single dose of fresh easily degradable municipal organic waste (MOW) in such dose as to increase the soil organic matter by 0.5, 1.0, 1.5 and 2.0%; MOW fraction (dry matter 55%; water 45%, ashes 22.4%, organic matter 32.6; pH 6.5; electric conductivity 4.2 dS m^{-1}) was

incorporated into the top 15 cm of soil using a rotovator. The aim was to restore biochemical and microbial properties and to contrast the erosion through a stimulation of spontaneous establishment of grass cover. One plot was used as control (C). The plots were checked for three years: following organic amendments a 60–70% plant coverage developed and persisted throughout the experiment until today, while 20–30% plant coverage was found in the control soil. After 16 years from the treatment the same vegetated plots have been sampled in order to assess and investigate the changes occurred in consequence of the vegetal cover establishment.

2.3 Natural Catena

A site characterized by three different (sandy loam, USDA classification) gradual degradation states, related to different natural plant cover establishment (natural Catena) has been sampled in Santomera area (1) a natural soil with a vegetation of *Pinus halepensis* (50–60% vegetation cover) (forest); (2) a partially degraded soil with a 20–30% of vegetation cover (autochthonous xerophytic shrub) (shrub); and (3) a bare soil with only a 5–10% of vegetation cover (bare).

2.4 Soil Sampling

Three samples were taken from each sites: each sample consisted of eight subsamples taken from the top 15 cm of soil. The subsamples were mixed, homogenised, sieved (2 mm) and stored dry at room temperature until laboratory analysis.

2.5 Chemical and Biochemical Parameters

Electrical conductivity (EC) and pH were measured in 1:10 (w:v) aqueous solution. Total organic carbon (TOC) and total nitrogen (TN) were determined by dry combustion with a RC-412 multiphase carbon and a FP-528 protein/nitrogen determinator, respectively (LECO Corporation). Pyrophosphate-extractable carbon $> 10^4$ Da (PEC $> 10^4$) was extracted at 37°C for 24 h under shaking at 200 oscillation min^{-1}, using $Na_2P_4O_7$ (0.1 M, pH 7.1) as extractant in a 1:10 w:v ratio following the Ceccanti et al. (2008) method. Then, pyrophosphate extract was filtered on a 0.22 μm Millipore membrane and passed through an ultrafiltration AMICON PM10 cut-off membrane to obtain fractions >10,000 and <10,000 Da. The C content of PEC $> 10^4$ Da was determined by dichromate oxidation (Yeomans and Bremner 1988). ATP was extracted from soil using the Webster et al. (1984) procedure and determined by the firefly luciferin–luciferase enzyme assay as described by Ciardi and Nannipieri (1990).

Dehydrogenase activity (DH-ase) was measured using 0.4% 2-*p*-iodophenyl-3 *p*-nitrophenyl-5-tetrazolium chloride (INT) as substrate; iodonitrotetrazolium formazan (INTF) produced in the reduction of INT was measured with a spectrophotometer at 490 nm (Masciandaro et al. 2000).

The *β*-glucosidase activity was determined on 1 g of air-dried soil (total *β*-glucosidase activity, TG) or 1 ml of soil pyrophosphate extract fraction $> 10^4$ Da (extracellular *β*-glucosidase activity, EG), using 0.05 M *p*-nitrophenyl-*β*-D-glucanopyranoside (PNG) as substrate. The *p*-nitrophenol (PNP) produced by both hydrolases was extracted and determined spectrophotometrically at 398 nm (Masciandaro and Ceccanti 1999).

2.6 Humic Matter Extraction

Pyrophosphate-extractable carbon (PEC) was extracted at 37°C for 24 h under shaking, using $Na_2P_4O_7$ (0.1 M, pH 7.1) as extractant in a 1:10 w:v ratio (Ceccanti et al. 1978). Then, the suspension was centrifuged and filtered through a 0.22 μm Millipore membrane to remove bacterial cells.

Dialysis: PEC was dialysed against distilled water using 1,000 MW Visking tube (1,000 Da), until reaching electrical conductivity values of less than 0.5 dS $m^{-1.}$

Membrane ultrafiltration (UF): The dialyses extract was recovered and concentrated by an Amicon PM-10 diaflomembrane (molecular cut-off 10.000 Da) under N_2 atmosphere (1.5 bar) (Ceccanti et al. 1989). The concentration was carried out at 4°C, maintaining the sample constantly under stirring, until a final volume of about 10 ml. The fraction with molecular weigh $> 10^4$ Da (PEC $> 10^4$) was recovered and used for further analysis.

2.7 Isoelectric Focussing (LEF)

IEF was carried out in cylindrical gel rods (0.5 × 8 cm) containing polyacrylamide gel (5% w:v) and carrier ampholines in the pH range 4–6 (Bio-Rad Laboratories, Richmond, California, USA) at a final concentration of 2% (Ceccanti et al. 1989). TEMED (N,N,N′,N′-Tetramethyl-1,2-diaminomethane) and ammoniumperoxy-disulfat were also added in gel solution at 0.03%. An aliquot of 100 μl of organic material (PEC $> 10^4$ Da) at 4.4% of glycerine was applied at the top of the gel rod (cathode). A little amount of glycerine at 2.2% was put on the sample to avoid interference and mixing with the cathodic solution (NaOH 0.02 N); 0.01 M H_3PO_4 was used for the anodic cell. A pre-run of one hour at the same current intensity and voltage used for the samples run was carried out for each gel tube (1.5 mA for each tube, 100–800 Volt); subsequently the samples run was carried out for 2 h or more until a stable IEF banding was reached.

The electrophoretic bands were scanned by a Bio-Rad GS 800 densitometer, obtaining a typical IEF densitogram for each soil investigated. Gel pH was measured at 0.5 cm intervals with an Orion microprocessor (model 901, Orion research) connected to a microelectrode gel-pHiler (Bio-Rad Laboratories, Richmond, California, USA).

2.8 Specific Selected Parameters

2.8.1 Active Humic Carbon (AHC)

The IEF peaks area was determined for each soil IEF densitogram, assuming as 100% the area under the entire IEF profiles (representative of the total loaded carbon). The peaks area focused in the pH range 4.0–4.5 was calculated and expressed as mg C kg_{ds}^{-1}; this was labelled as active humic carbon (AHC).

2.8.2 *β*-glucosidase Activity in Humic Bands (HEG)

To analyse *β*-glucosidase activity of the humic bands obtained by IEF, the gel was gently removed from the inside of the glass tubes. The bands were cut, pre-washed for 1 h with 2 ml 0.1 M phosphate buffer, pH 6.4, at 37°C. Pre-washing removes the carrier ampholytes, salts and other impurities from the gel, without freeing the gel-trapped humic matter (Ceccanti et al. 1989). After removal of buffer, 2 ml of fresh 0.1 M phosphate buffer, pH 6.4 and 0.5 ml 0.05 M *p*-nitrophenyl-*β*-D-glucopyranoside (PNG) were added in order to assay the enzyme activity. Incubation was carried out at 37°C under shaking for 17 h.

2.8.3 Specific Humic-*β*-glucosidase Activity (SHEG)

The activity was calculated by the ratio HEG/AHC and was expressed as mg PNP g C^{-1} h^{-1}.

2.9 Statistical Analysis

All results are the means of three replicates. Differences among the soils were tested by analysis of variance (ANOVA). The means were compared by using least significant differences calculated at $P < 0.05$ (Fisher's Test) (STATISTICA 6.0 software). A correlation matrix ($P < 0.01$, $P < 0.05$) of the data, for each site, was also calculated in order to determine the relationship between the parameters. Stepwise linear forward regression analysis was carried out. The stepwise

Table 1 Chemical parameters of soil

Soil samples (%)	pH	EC	TOC	TN	C/N
0.0	7.82 ± 0.04^{a}	642 ± 46^{d}	13.9 ± 1.6^{e}	0.95 ± 0.11^{e}	14.6
0.5	7.67 ± 0.06^{b}	927 ± 35^{c}	23.4 ± 0.9^{d}	1.92 ± 0.06^{c}	12.2
1.0	7.39 ± 0.12^{c}	$2{,}051 \pm 60^{b}$	27.2 ± 1.4^{c}	1.60 ± 0.18^{d}	17.0
1.5	7.45 ± 0.08^{c}	$2{,}666 \pm 115^{a}$	33.7 ± 1.2^{b}	2.47 ± 0.13^{a}	13.6
2.0	7.50 ± 0.10^{c}	$2{,}713 \pm 88^{a}$	38.8 ± 1.3^{a}	2.21 ± 0.10^{b}	17.6

Different letters indicate statistically different values ($P < 0.05$). *EC* electrical conductivity (Ds m^{-1}), *TOC* total organic carbon (mg C g^{-1}), *TN* total nitrogen (mg N g^{-1})

regression analysis was used to quantify the relation between two and more co-variables and an outcome variable (Feinstein 1996). Stepwise selection indicates covariables with a statistically significant effect, simultaneously adjusting for the other covariables in the regression model.

3 Results

3.1 Managed Catena

The content of total organic carbon (TOC) increased significantly with organic matter addition (Tables 1 and 2), as expected. Not expected was, instead, the persistence of such an increase during almost two decades as it has been found in our soils after 16 years. This demonstrated that the effect of a single application of organic matter, stimulated the growth and maintenance of a vegetation cover which still persisted until now. Similarly the pyrophosphate extractable carbon (PEC $> 10^4$) and the β-glucosidase activity detected in the whole soil (TG) showed increasing values going from 0 to 2% treatments, these parameters resulted, in fact, significantly correlated at $P < 0.01$ (TOC-PEC $> 10^4$ r = 0.99; TOC-TG r = 0.99; PEC $> 10^4$-TG r = 0.98) (Tables 2 and 4). This result suggested that carbon cycle persisted active and capable to transform most of added organic carbon into a more stable form (PEC $> 10^4$); this pathways was probably sustained by plant root exudates released by the persistent plant coverage.

The DH-ase activity and ATP were correlated (r = 0.99, $P < 0.01$) and generally increased with the increase of organic amendment (Table 2). In general, the biochemical parameters showed a peak at 1.5% of MOW, meaning that some inhibition can be occurred at the highest (2.0%) MOW concentration (Table 2). The 1.5% treatment showed a maximum value of total nitrogen and, as a consequence, a minimum of C/N ratio (Table 1).

The relation between soil humic substances and associated extracellular enzyme activities was evidenced also by IEF results. Figure 2 and Table 3 showed an increase of humic carbon (AHC) and associated β-glucosidase activity (HEG)

Table 2 Biochemical parameters of soil

Soil samples (%)	PEC $> 10^4$	TG	EG	DH-ase	ATP
0.0	522 ± 10^{d}	70 ± 6^{e}	3.6 ± 0.2^{c}	1.76 ± 0.09^{d}	25.0 ± 4^{c}
0.5	977 ± 22^{c}	206 ± 1^{d}	6.9 ± 0.5^{c}	1.72 ± 0.1^{d}	27.8 ± 3^{c}
1.0	1,070 ± 38^{b}	346 ± 13^{c}	20.4 ± 1.6^{b}	3.60 ± 0.22^{c}	62.2 ± 8^{b}
1.5	1,485 ± 75^{a}	407 ± 25^{b}	30.7 ± 2.4^{a}	5.07 ± 0.41^{a}	110 ± 7^{a}
2.0	1,501 ± 103^{a}	544 ± 43^{a}	21.8 ± 1.3^{b}	4.81 ± 0.33^{a}	106 ± 9^{a}

Different letters indicate statistically different values ($P < 0.05$). *PEC* $> 10^4$ pyrophosphate extractable carbon fraction $> 10^4$ Da (mg C kg^{-1}), *TG* total β-glucosidase activity (mg PNP kg^{-1} h^{-1}), *EG* extracellular β-glucosidase activity (mg PNP kg^{-1} h^{-1}) *DH-ase* dehydrogenase activity (mg INTF kg^{-1} h^{-1}), *ATP* (ng g^{-1})

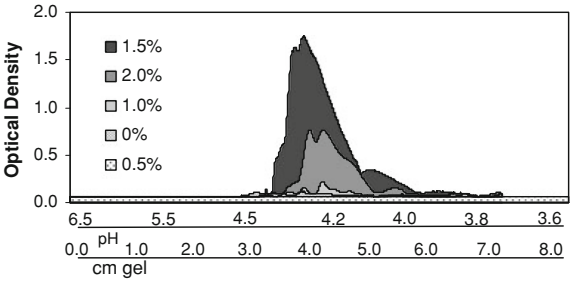

Fig. 2 IEF densitograms of humic matter (PEC $> 10^4$ Da) extracted from each soil

with the increase of amendments with the exception that the values in 1.5% treatment were higher than in 2.0%. The low humic-bound β-glucosidase activity (HEG) in 2.0% amendment was also evident in the specific humic-β-glucosidase activity (SHEG) found in 2.0% treatment.

3.2 Natural Catena

Total organic carbon (TOC) and pyrophosphate extractable carbon (PEC $> 10^4$), as expected, resulted higher in the forest site (Table 5), while not statistically significant differences were found in bare and shrub soils indicating that the vegetal cover in shrub site is not sufficient to preserve soil organic matter content (Table 7). All biochemical parameters resulted markedly higher in the forest site, generally showing values 2–3 fold those of the shrub and bare soils or even much higher (15-fold) as found for EG (Table 5).

Among these biochemical parameters, only dehydrogenase (DH-ase) and the extracellular β-glucosidase (EG) resulted able to discriminate the soils in the following order: forest > shrub > bare (Table 5). In the Fig. 3 are reported the densitograms tracing the profiles of the humic carbon PEC $> 10^4$ after IEF; in terms of PEC $> 10^4$, the forest site showed two broad peaks corresponding to averagely 5 times the height of peaks in shrub and bare soils. Similarly the

Table 3 Amount of enzymatically active humic carbon (AHC) and associated extracellular β-glucosidase activity (HEG) focussed in the pH range 4.0–4.5

Soil samples organic matter (%)	AHC (mg C kg^{-1})	HEG (mg PNP kg^{-1} h^{-1})	SHEG (mg PNP g C^{-1} h^{-1})
0.0	101 ± 6^{e}	1.01 ± 0.09^{e}	9.96
0.5	183 ± 11^{d}	1.20 ± 0.06^{c}	6.56
1.0	256 ± 15^{c}	2.11 ± 0.12^{b}	8.25
1.5	596 ± 45^{a}	7.27 ± 0.22^{a}	12.20
2.0	384 ± 26^{b}	1.31 ± 0.05^{d}	3.41

Different letters indicate statistically different values ($P < 0.05$). *AHC* active humic carbon calculated from the IEF peak areas focused in the pH range 4.0–4.5, *HEG* humic-bound β-glucosidase activity pH 4.0–4.5, *SHEG* specific humic-β-glucosidase activity: HEG/AHC

associated β-glucosidase activity (HEG) focussed in the 4–4.5 pH range (Fig. 3, Table 6) resulted particularly higher in the forest soil suggesting a higher metabolic efficiency in this ecosystem. The same trend was observed for the specific humic-β-glucosidase activity (SHEG) (Table 6). These parameters ranked the soil in the following order of degradation: bare > shrub > forest, confirming their contribute and reliability to explain the dynamics of the chemical and biological processes taking place in soil.

4 Discussion

The addition of organic matter in the restoration of arid soils provoked an increase of total organic carbon, demonstrating that the effect of a single application of organic matter 16 years ago, is still manifested in the accumulation and persistence of β-glucosidase humic complexes. This was probably due to the growth and maintenance of grass cover with time that notoriously has a double effect: (1) provide a source of slow degradable ligno-cellulosic material in the surface and (2) a release of an easily bioavailable carbon source constituted by root exudates (Manns et al. 2007; Fuentes et al. 2009). Both surface and underground carbon sources might have induced microbial synthesis of active β-glucosidase enzyme (García et al. 1997; Ros et al. 2003) which act in the last step of cellulose degradation, promoting a flow of easily metabolizable carbon from the plants to the soil, where part of which was transformed into stable humic substance capable to bind the extracellular enzymes. In our soils this mechanisms was generally confirmed by the evolution of the analytical results and especially by the narrow correlation found among the chemical carbon forms and biochemical parameters (Table 4).

The DH-ase and ATP are here confirmed as good indicator of soil microbial activity that although they are notably sensible and changeable in relation to minor change of soil conditions, they allow us to follow the activation of carbon cycle and turnover in arid soil environments. A high significant correlation was

Table 4 Correlation matrix between chemical and biochemical parameters of soil samples of Natural Catena

	pH	EC	TOC	TN	C/N	PEC	TG	EG	DH-ase	ATP	AHC	HEG	SHEG
pH	+1.00	−0.99*	−0.93	−0.99**	−0.99**	−0.87	−0.90	−0.99*	−0.99	−0.88	−0.91+	−0.99	−0.92
EC		+1.00	+0.96	+0.99*	+0.99*	+0.91	+0.93	+0.99**	+0.99**	+0.91	+0.94	+1.00	+0.89
TOC			+1.00	+0.93	+0.93	+0.99	+0.99	+0.95	+0.96	+0.99	+0.99*	+0.98	+0.72
TN				+1.00	+0.99**	+0.87	+0.90	+0.99*	+0.99	+0.88	+0.91	+0.99	+0.92
C/N					+1.00	+0.87	+0.90	+0.99*	+0.99	+0.88	+0.90	+0.99	+0.92
PEC						+1.00	0.99*	+0.89	+0.91	+0.99**	+0.99*	+0.94	+0.61
TG							+1.00	+0.92	+0.93	+0.99*	+0.99*	+0.96	+0.65
EG								+1.00	+0.99*	+0.90	+0.92	+0.99	+0.90
DH									+1.00	+0.91	+0.94	+0.99	+0.89
ATP										+1.00	+0.99*	+0.94	+0.62
AHC											+1.00	+0.96	+0.67
HEG												+1.00	+0.84

Coefficient correlation values significantly different at $*P < 0.05$ and $** P < 0.01$

EC electrical conductivity, *TOC* total organic carbon, *TN* total nitrogen, *PEC* $> 10^4$ pyrophosphate extractable carbon fraction $> 10^4$ Da, *TG* total β-glucosidase activity, *EG* extracellular β-glucosidase activity, *DH-ase* dehydrgenase activity; *AHC* active humic carbon calculated from the IEF peak areas focused in the pH range 4.0–4.5, *HEG* humic-bound β-glucosidase activity pH 4.0–4.1; *SHEG* Specific humic-β-glucosidase activity: HEG/AHC

Table 5 Chemical and biochemical parameters of soil samples

Soil samples	pH	EC	TOC	TN	C/N	PEC $> 10^4$	TG	EG	DH-ase	ATP
Bare	7.90 ± 0.05^a	248 ± 8^c	10.6 ± 0.9^c	0.64 ± 0.04^c	14.6	$1{,}100 \pm 70^b$	552 ± 44^b	7.02 ± 0.61^c	6.21 ± 0.5^c	444 ± 16^b
Shrub	7.74 ± 0.08^b	312 ± 19^b	13.8 ± 1.4^b	1.55 ± 0.09^b	15.6	$1{,}150 \pm 120^b$	608 ± 36^b	25.7 ± 2.1^b	9.48 ± 0.9^b	469 ± 22^b
Forest	7.59 ± 0.04^c	415 ± 22^a	46.0 ± 2.5^a	3.62 ± 0.21^a	16.6	$5{,}050 \pm 240^a$	$1{,}603 \pm 111^a$	107 ± 5^a	16.2 ± 0.6^a	$1{,}590 \pm 43^a$

Legend: see Tables 1 and 2

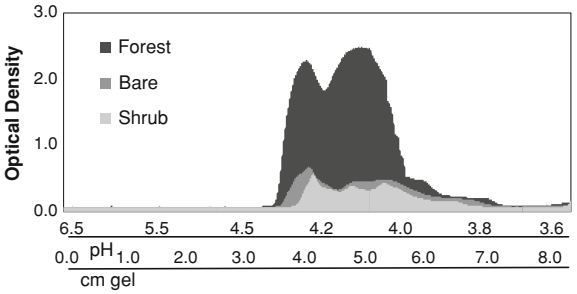

Fig. 3 IEF densitograms of humic matter (PEC $> 10^4$ Da) extracted from each soil

Table 6 Amount of enzymatically active humic carbon (AHC) and associated extracellular β-glucosidase activity (HEG) focussed in the pH range 4.0–4.5

Soil samples	AHC (mg C kg^{-1})	HEG (mg PNP kg^{-1} h^{-1})	SHEG (mg PNP gC^{-1} h^{-1})
Bare	273 ± 16^c	1.22 ± 0.10^c	4.48
Shrub	331 ± 20^b	3.21 ± 0.06^b	9.68
Forest	$1{,}574 \pm 47^a$	16.9 ± 1.8^a	10.7

Legend: see Table 3

also found between PEC $> 10^4$ and the activity of the crude pyrophosphate extract (EG) (Table 4), confirming the capability of the humic substance to protect and accumulate active free enzymes. This finding is very important because allows us to recover the biochemical fertility and soil functionality through the application of a source of degradable organic matter which promote a sort of humification in situ (Ceccanti and Masciandaro 2003; Doni et al. 2009) capable of permanently accumulate humic carbon and restore biological properties which are fundamental factors to resist soil degradation and desertification. The evolution of these biochemical processes have generally been found in various managed Catenas, whose dynamics were investigated through conventional and biochemical parameters (Gil-Sotres et al. 1992; Ceccanti et al. 1994; Ros et al. 2003), and was also confirmed in a natural Catena. Moreover, the managed Catena showed negative not significant correlations between the specific activity measured in the active humic-enzyme complex after IEF (SHEG) and almost all parameters considered, while in the natural Catena SHEG showed a weak positive correlation with the other parameters that could change into significant correlation with the time, as usually found. If confirmed in other similar managed and natural Catenas, this result could be considered a reference threshold for assessing the re-naturalization of a stressed soil ecosystem. Both in the time (Gil-Sotres et al. 1992) and in the space (Ceccanti et al. 1994), we are capable to reconstruct and control a Catena in order to drive a degraded soil ecosystem to an acceptable soil functionality. The analytical UF and IEF techniques have resulted very promising in discriminating between positive and inhibitory effects of organic matter addition such as it occurred at 1.5 and 2%,

Table 7 Correlation matrix between chemical and biochemical parameters of soil samples of managed Catena

	pH	EC	TOC	TN	C/N	PEC	TG	EG	DH-ase	ATP	AHC	HEG	SHEG
pH	1.00	0.92*	−0.83	−0.70	−0.46	−0.83	−0.89*	−0.95*	−0.85	−0.83	−0.82	−0.64	+0.10
EC		1.00	0.94*	0.79	0.50	0.92*	0.95*	0.99**	0.97**	0.98**	0.94*	0.64	−0.26
TOC			1.00	0.92*	0.34	0.99**	0.99**	0.92*	0.86	0.90*	0.92*	0.51	−0.45
TN				1.00	−0.05	0.96**	0.89*	0.81	0.69	0.75	0.90*	0.59	−0.29
C/N					1.00	0.22	0.39	0.40	0.55	0.50	0.18	−0.13	−0.46
PEC						1.00	0.98**	0.92*	0.84	0.88*	0.95*	0.59	−0.36
TG							1.00	0.94*	0.86	0.88*	0.90 *	0.50	−0.43
EG								1.00	0.95*	0.95*	0.95*	0.73	−0.13
DH									1.00	0.99**	0.9*	0.67	−0.17
ATP										1.00	0.94*	0.67	−0.22
AHC											1.00	0.79	−0.10
HEG												1.00	+0.53

Coefficient correlation values significantly different at $*P < 0.05$ and $**P < 0.01$

Legend: see Table 4

respectively, or in ranking the soil subsystems of a natural catena in a decreasing order of quality: forest > shrub > bare.

In order to explain more clearly the factors controlling carbon metabolism and humification process in the two Catenas, stepwise linear regression analysis was performed.

Active humic carbon (AHC) was selected as the more suitable dependent variable. The study of the stable organic C fraction (AHC) is relevant since it determines the potential for soil resilience and resistance of stressed soils, particularly in extreme environments (Ceccanti and Masciandaro 2003).

$$\mathrm{AHC} = +0.330\ \mathrm{HEG} + 1.07\ \mathrm{TG} + 0.431\ \mathrm{PEC} - 0.25\ \mathrm{C/N}$$
$$R^2 = 0.998; \quad p = 0.0393$$

The active humic carbon (AHC), isolated through UF and IEF, clearly represents a significant fraction of the whole soil β-glucosidase activity (TG) and, in addition, supports the finding of previous studies (Doni et al. 2009; Masciandaro and Ceccanti 1999) that most of hydrolases (including β-glucosidase) in soil are associated with stable humic complexes. The C/N ratio is confirmed here as a factor controlling microbial metabolism shifting most of metabolized organic carbon and biochemical energy of the soil ecosystem towards the formation and accumulation of active humic-enzyme complexes.

5 Conclusions

- Stressed soil can be recovered through induced or spontaneous vegetal cover able to promote a carbon flow from the plants into the soil where part of which was transformed into stable humic substance capable to bind the extracellular enzymes.
- The use of UF and IEF in combination with common methodologies and other biological parameters, resulted very efficient in the characterization of humic-enzyme complexes aimed to better understand those biochemical processes which drive the humic substances formation, storage and activity in soils.
- The empowering of separative-purification methodologies and the use of IEF at preparative scale could provide in the future sufficient amount of humic-enzyme complexes to be further investigated through molecular structural analysis such as pyrolysis and the more recent powerful biomolecular technique.

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References

Benítez E, Nogales R, Masciandaro G, Ceccanti B (2000) Isolation by isoelectric focusing of humic urease complexes from earthworm (*Eisenia foetida*)-processed sewage sludges. Biol Fertil Soils 31:489–493

Benítez E, Melgar R, Nogales R (2004) Estimating soil resilience to a toxic organic waste by measuring enzyme activities. Soil Biol Biochem 36:1615–1623

Canali S, Trinchera A, Pinzari F, Benedetti A (1998) Study of compost maturity by means of humification parameters and isoelectrofocusing technique. In: Proceedings of the 16th world congress of soil science. Montpellier, France, 20–26 August, Paper no. 778 (CD-ROM)

Ceccanti B, Masciandaro G (2003) Stable humus-enzyme nucleus: the last barrier against soil desertification. In: Lobo MC, Ibañez JJ (eds) Preserving soil quality and soil biodiversity: the role of surrogate indicators CSIC-IMIA. Madrid, Spain, pp 77–82

Ceccanti B, Nannipieri P, Cervelli S, Sequi P (1978) Fractionation of humus-urease complex. Soil Biol Biochem 10:39–45

Ceccanti B, Bonmatí-Pont M, Nannipieri P (1989) Microdetermination of protease activity in humic bands of different sizes after analytical isoelectic focusing. Biol Fertil Soils 7:202–206

Ceccanti B, Pezzarossa B, Gallardo-Lancho FJ, Masciandaro G (1994) Bio-tests as markers of soil utilization and fertility. Geomicrobiol J 11:309–316

Ceccanti B, Doni S, Macci C, Cercignani G, Masciandaro G (2008) Characterization of stable humic-enzyme complexes of different soil ecosystems through analytical isoelectric focussing technique (IEF). Soil Biol Biochem 40:2174–2177

Chen CR, Condron LM, Davis MR, Sherlock RR (2003) Seasonal changes in soil phosphorus and associated microbial properties under adjacent grassland and forest in New Zealand. For Ecol Manag 177:539–557

Ciardi C, Nannipieri P (1990) A comparison of methods for measuring ATP in soil. Soil Biol Biochem 22:725–727

Ciavatta C, Govi M (1993) Use of insoluble polyvinylpyrrolidone and isoelectric focusing in the study of humic substances in soils and organic wastes. J Chromatogr 643:261–270

Doni S, Macci C, Peruzzi E, Masciandaro G, Ceccanti B (2009) Factors controlling carbon metabolism and humification process in different soil agro-ecosystems. In: Dilly O, Pfeiffer E-M (eds) Book of abstracts XIXth International symposium on environmental biogeochemistry environmental changes and sustainability of biogeochemical cycling. University of Hamburg-Klima Campus, Hamburg, Germany, p 86

Doran JW, Parkin TB (1994) Defining and assessing soil quality. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (eds) Defining soil quality for a sustainable environment. Special Publication 35. Soil Science Society of America, Madison, pp 3–21

Feinstein AR (1996) Multivariable analysis. Yale University Press, New Haven

Fuentes M, Govaerts B, de León F, Hidalgo C, Dendooven L, Sayre KD, Etchevers J (2009) Fourteen years of applying zero and conventional tillage, crop rotation and residue management systems and its effect on physical and chemical soil quality. Eur J Agron 30:228–237

García C, Roldán A, Hernández T (1997) Changes in microbial activity after abandonment of cultivation in a semiarid Mediterranean environment. J Environ Qual 26:285–291

Gil-Sotres F, Trasar-Cepeda MC, Ciardi C, Ceccanti B, Leirós MC (1992) Biochemical characterization of biological activity in very young mine soils. Biol Fertil Soils 13:25–30

Gil-Sotres F, Trasar-Cepeda C, Leirós MC, Seoane S (2005) Different approaches to evaluating soil quality using biochemical properties. Soil Biol Biochem 37:877–887

Klein DA, Sorensen DL, Redente EF (1985) Soil enzyme: a predictor of reclamation potential and progress. In: Tate RL, Klein DL (eds) Soil reclamation processes: microbiological analysis and applications. Marcel Dekker, New York, pp 141–171

Lähdesmäki P, Piispanen R (1992) Soil enzymology: role of protective colloid systems in the preservation of exoenzyme activities in soil. Soil Biol Biochem 24:1173–1177

Manns HR, Maxwell CD, Emery RJN (2007) The effect of ground cover or initial organic carbon on soil fungi, aggregation, moisture and organic carbon in one season with oat (*Avena sativa*) plots. Soil Till Res 96:83–94

Masciandaro G, Ceccanti B (1999) Assessing soil quality in different agro-ecosystems through biochemical and chemical structural properties of humic substances. Soil Till Res 51:129–137

Masciandaro G, Ceccanti B, Ronchi V, Bauer C (2000) Kinetic parameter of dehydrogenase in the assessment of the response of soil to vermicompost and inorganic fertilisers. Biol Fertil Soils 32:479–483

Ros M, Hernández MT, García C (2003) Soil microbial activity after restoration of a semiarid soil by organic amendments. Soil Biol Biochem 35:463–469

Webster JJ, Hampton GJ, Leach FR (1984) ATP in soil: a new extractant and extraction procedure. Soil Biol Biochem 4:335–342

Yeomans JC, Bremner JM (1988) A rapid and precise method for routine determination of organic carbon in soil. Commun Soil Sci Plan 19:1467–1476

IEF Technique to Study the β-Glucosidase-Humic Complexes in Organic and Mineral Amended Soils

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Abstract In this work, fields with different slopes (0, 2 and 6%) located in an erodible area due to topographic and climatic conditions and improper management, were submitted to rehabilitation practices that included almond tree cultivation under organic or mineral fertilization. Conventional parameters usually related to soil fertility and quality (total organic carbon, total nitrogen, water soluble carbon, humic carbon, etc.) along with unconventional parameters (β-glucosidase-humic complexes, soil enzyme activity) were measured with the aim of evaluating the capability of the rehabilitation practices to improve soil quality and/or prevent soil degradation. The application of both organic and mineral fertilizers and the presence of almond trees resulted effective in increasing general soil chemical properties and in particular biochemical properties related to the humic-bound β-glucosidase enzyme (HEG). The analytical isoelectric focussing technique (IEF), was able to isolate and characterise extracellular humic-enzyme complexes discriminating among different slopes and treatments. The organic and mineral fertilisers and plants were more efficient in increasing amount and activity of humic-enzyme complexes in 0 and 2% slopes, while 6% slope showed a significative increase only with organic amendment. The active humic-β-glucosidase enzyme complexes isolated and purified through analytical IEF, even though they represent only a small part of soil humic substances are relevant indicators for assessing amount and role of humic carbon stored in degraded soils, following regeneration practices.

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1 Introduction

In many European regions inappropriate agricultural practices, together with the adverse environmental and climatic factors, make the soil ecosystems very susceptible to erosion processes. The main effects of soil erosion are the decline of productivity and quality by increased compaction and runoff and removal of plant nutrients and organic matter (Smith et al. 2001). For this reason, there is growing recognition for the need to select management practices that improve the productivity and increase humic carbon storage in soil. In particular, it is widely recognised that the conservation of the humic carbon fraction in soil may provide a nutrient resource, physical aggregates stability and protection of enzymes and microorganisms; these are fundamental properties to maintain soil quality in ecosystems affected by agricultural practices or adverse climate conditions (Ceccanti et al. 2008). It has been found that the humic substances, despite of their relatively low amount in soils, are efficient for binding extracellular enzymes (humic-enzyme complexes) and preserve them by proteolysis and chemical degradation (Nannipieri et al. 1982; Ruggiero and Radogna 1988; Lähdesmäki and Piispanen 1992). The extracellular stabilized soil enzymes may support microorganisms in carrying out biochemical processes under unfavourable microbial life limiting conditions. The humic-enzyme complexes have also been considered the last barrier against irreversible soil desertification, thus representing a necessary condition for soil resilience (Ceccanti and Masciandaro 2003). In particular, the persistence of humic-bound β-glucosidase activity has been considered relevant for assessing the organic carbon evolution and the biochemical functionality of natural and managed soil ecosystems (Ceccanti et al. 2008). This enzyme has been one of the most widely used in the evaluation of soil quality in soils subjected to different management procedures (Bandick and Dick 1999; Saviozzi et al. 2001). Extraction and purification are necessary steps to study the origin, location and persistence of the extracellular β-glucosidase enzymes in soil. The biochemical techniques to fractionate humus-enzyme complexes are available (Ceccanti and Masciandaro 2003) and based on three steps: (1) sodium pyrophosphate extraction (pH 7.1) of humic matter, (2) ultrafiltration (UF) of the organic extracts on molecular weight cut-off membranes (mol wt $> 10^4$), followed by (3) analytical isoelectric focussing technique (IEF). IEF is an electrophoretic technique, which has been used with the purpose of the in-depth investigation of humic matter and humic-bound enzymes extracted from soils (Ciavatta and Govi 1993; Ceccanti et al. 1989) or other organic materials (Canali et al. 1998; Benítez et al. 2000). IEF is based on the separation of different humic substances on the basis of their isoelectric point (*pI*), that is, according to their net surface charges. The importance of this technique is due to its capacity to isolate humic-enzyme complexes that retain their properties, such as enzyme activity and molecular structure.

In this work, fields with different slopes (0, 2 and 6%) located in an area with a moderate risk of soil erosion due to topographic and climatic conditions and the lack of proper environmental management, were submitted to rehabilitation practices

that included almond tree cultivation under organic or mineral management systems. Conventional parameters usually related to soil fertility and quality (total organic carbon, total nitrogen, water soluble carbon, humic carbon, etc.) along with the measure of a persistent β-glucosidase-humic complex were studied with the aim to evaluate the capability to improve soil quality and/or prevent soil degradation.

2 Materials and Methods

2.1 Experimental Layout

The field experiment was located in the Metapontino area (province of Matera, Basilicata region) in the South of Italy (latitude 40° 23′ N and longitude 16° 46′ E). The climate in this area is predominantly Mediterranean with dry hot summers and cold winters, an average annual temperature of 16.6°C and an average rainfall of 46.2 mm. The soil was a sandy clay loam (sand 67%, silt 12%, clay 21%, USDA texture classification) with low content of organic matter (1.37%). Three different fields of about 3000 m^2 (85 × 35 m) each were set up on the land of the Pantanello farm. The different fields were characterized by different slope gradients (0, 2 and 6%). Each field was split up into two parts, one assigned to organic management and the other assigned to mineral management. For each slope a plot without amendment was also used as a control (control soil). The almond variety *tuono* in the *franco* rootstock was chosen for its high pedological and climatic adaptiveness. The planting was carried out in March 2006 with a distance of 4 × 5 m. In the middle of April 2006 organic fields were fertilized using 1.5 t ha^{-1} of commercial manure (in pellets). This organic amendment contained 25% organic carbon, 3% organic nitrogen and 3% phosphorus oxide. In the conventional field a fertilisation was carried out containing 15% ammonium nitrate, 7.5% phosphorus oxide and 20% potassium oxide spread over three phases during the spring-summer period and with a total quantity of 0.3 t ha^{-1}. A drip irrigation system with about 2000 m^3 ha^{-1} of water per year was established. The soils sampling were carried out in November 2006 (t1) and November 2007 (t2). Each sample consisted of five subsamples taken from the soil's top layer (15 cm) (150 cm^3 soil cores), randomly collected at the rhizosphere zone (plant) and in the plant inter-rows (inter-row). Analytical characteristics were determined on soil samples which were air-dried and passed through a 2 mm sieve. Because of soil biochemical and microbiological properties are susceptible to change under sample storage even at low temperatures, air-dried soil can facilitate routine soil testing procedures and a better comparison of soil properties evolution with time especially in field scale investigation (Zornoza et al. 2009). In addition air-dried soils allow long term storage and attenuate or eliminate any casual, uncontrolled variables occurring in soil samples.

2.2 Chemical Parameters

The particle size analysis was calculated by a pipette procedure (Indorante et al. 1990). The textural class of the soil was defined by the USDA textural triangle. Water-soluble carbon (WSC) was extracted from the soil with distilled water in a 1:10 solid:liquid ratio by mechanical shaking at 60°C for 1 h. WSC was analysed in the supernatant after centrifugation. Pyrophosphate-extractable carbon (PEC) was extracted at 37°C for 24 h under shaking, using $Na_2P_4O_7$ (0.1 M, pH 7.1) as extractant in a 1:10 (w/v) solid:liquid ratio (Ceccanti et al. 1978). Then, the suspension was centrifuged and filtered through a 0.22 μm Millipore membrane. The PEC-derived fraction $>10^4$ Da was obtained through ultrafiltration on AMICON PM10 membrane of the PEC extract. The C content of WSC, and PEC $> 10^4$ Da were determined by acid digestion with $K_2Cr_2O_7$ and H_2SO_4 at 140°C for 2 h. A spectrophotometric method was used to quantify the Cr^{3+} produced by the reduction of Cr^{6+} (λ 590 nm) (Yeomans and Bremner 1988). Total soil C and N contents were determined by dry combustion with a RC-412 multiphase carbon and a FP-528 protein/nitrogen determinator respectively (LECO Corporation).

2.3 Isoelectric Focussing (IEF)

IEF was carried out in cylindrical gel rods (0.5 × 8 cm) containing polyacrylamide gel (5% w/v) and carrier ampholines in the pH range 4–6 (Bio-Rad Laboratories, Richmond, California, U.S.A.) at a final concentration of 2% (Ceccanti et al. 1986, 1989). TEMED (N,N,N′,N′-Tetramethyl-1,2-diaminomethane) and ammonium peroxydisulfate were also added in gel solution at 0.03%. A volume of 100 μl of pyrophosphate extract derived fraction $>10^4$ Da and glycerine at 4.4% was applied at the top of the gel rod (cathode). A little amount of glycerine at 2.2% was put on the sample to avoid interference and mixing with the cathodic solution (NaOH 0.02 M); 0.01 M H_3PO_4 was used for the anodic cell. A pre-run of 1 h at the same current intensity and voltage used for the samples run was carried out for each gel tube (1.5 mA for each tube, 100–800 Volt); subsequently the samples run was carried out for 2 h or more until a stable IEF banding was reached. The electrophoretic bands were scanned by a Bio-Rad GS 800 densitometer, obtaining a typical IEF profile for each soil investigated. The IEF peaks area was determined for each soil IEF profile, assuming as 100% the area under the entire IEF profiles (representative of the total loaded carbon). The peaks area focused in the pH (or *pI*) range 4.5–4.0 was calculated and expressed as mg C kg^{-1} dry soil; this was labelled as active humic carbon. Gel pH was measured at 0.5 cm intervals with an Orion microprocessor (model 901, Orion research) connected to a microelectrode gel-pHiler (Bio-Rad Laboratories, Richmond, California, USA).

2.4 β-Glucosidase Activity

To determine β-glucosidase activity, 0.05 M *p*-nitrophenyl-β-D-glucopyranoside (PNG) was used as substrate (Masciandaro and Ceccanti 1999). The *p*-nitrophenol (PNP) released as a product by β-glucosidase activity was extracted and determined spectrophotometrically at 398 nm (Tabatabai and Bremner 1969). One g of air-dried soil (total β-glucosidase activity) or 1 mL of soil pyrophosphate extract fraction $> 10^4$ Da (extracellular β-glucosidase activity) was used.

2.5 β-Glucosidase Activity in Humic Bands

To analyse β-glucosidase activity of the humic bands obtained by IEF, the gel was gently removed from the inside of the glass tubes. The bands were cut, pre-washed for 1 h with 2 mL 0.1 M phosphate buffer, pH 6.4, at 37°C. Pre-washing removes the carrier ampholytes, salts and other impurities from the gel, without freeing the gel-trapped humic matter (Ceccanti et al. 1989). After removal of buffer, 2 mL of fresh 0.1 M phosphate buffer, pH 6.4 and 0.5 ml 0.05 M *p*-nitrophenyl-β-D-glucopyranoside (PNG) were added in order to assay the enzyme activity. Incubation was carried out at 37°C under shaking for 17 h. The β-glucosidase activity was expressed as mg PNP kg^{-1} dry soil h^{-1}.

2.6 Statistical Analysis

We tested for significance using one-way analysis of variance (ANOVA) within STATISTICA 6.0 software. Post-hoc mean separations were done using the Fisher LSD test with the threshold for significance adjusted to $P < 0.05$.

Stepwise linear forward regression analysis was carried out. The stepwise regression analysis was used to quantify the relation between two or more co-variables and an outcome variable (Feinstein 1996). Stepwise selection indicates covariables with a statistically significant effect, simultaneously adjusting for the other covariables in the regression model.

3 Results

In general, total organic carbon (TOC) and total nitrogen (TN) content of organic and mineral management systems resulted, at both sampling times, significantly higher than those of the control soils (Table 1). Higher values of these parameters were also observed in the presence of plant with respect to inter-row. At t1 sampling, water soluble carbon (WSC) resulted quite unchanged after both organic

Table 1 Chemical parameters at t1 (November 2006) and t2 (November 2007) sampling

Slope		pH		TN		TOC		WSC		PEC $> 10^4$ Da	
		t1	t2	t1	t2	t1	t2	t1	t2	t1	t2
0%	C	8.79	8.31	817 ± 72	745 ± 134	7339 ± 146	5180 ± 130	213 ± 8	220 ± 19	909 ± 17	793 ± 12
	OMp	7.79	8.57	1163 ± 29	1450 ± 118	8173 ± 223	8335 ± 91	265 ± 28	120 ± 23	1142 ± 49	119 ± 21
	OMi	8.28	8.45	1160 ± 26	1095 ± 77	7550 ± 315	6635 ± 132	199 ± 25	110 ± 15	1123 ± 19	1022 ± 19
	CMp	8.61	8.47	1037 ± 11	925 ± 25	8260 ± 256	8065 ± 127	228 ± 28	150 ± 4	808 ± 15	896 ± 17
	CMi	9.26	8.92	1023 ± 15	800 ± 14	7307 ± 208	6140 ± 72	194 ± 13	124 ± 21	888 ± 22	838 ± 11
2%	C	8.32	8.48	827 ± 46	810 ± 99	8263 ± 310	5605 ± 170	178 ± 15	131 ± 23	969 ± 22	1058 ± 10
	OMp	8.32	8.09	1027 ± 55	1290 ± 42	9403 ± 293	9180 ± 135	249 ± 21	193 ± 19	1262 ± 6	1386 ± 14
	OMi	8.55	8.76	1040 ± 22	1275 ± 35	9420 ± 228	8275 ± 271	233 ± 34	177 ± 27	1225 ± 16	1457 ± 17
	CMp	9.17	8.31	833 ± 87	1050 ± 84	8740 ± 311	6870 ± 101	190 ± 16	177 ± 11	1127 ± 12	809 ± 20
	CMi	9.27	8.45	760 ± 68	1110 ± 71	8030 ± 335	6740 ± 94	172 ± 22	217 ± 8	1200 ± 14	821 ± 17
6%	C	8.97	8.65	727 ± 148	695 ± 7	7853 ± 114	6345 ± 176	204 ± 22	190 ± 13	834 ± 11	649 ± 10
	OMp	8.52	8.61	1217 ± 67	1195 ± 40	8207 ± 157	7695 ± 188	261 ± 15	177 ± 19	925 ± 31	839 ± 15
	OMi	9.40	8.36	1280 ± 26	1093 ± 31	7953 ± 388	6765 ± 121	242 ± 28	169 ± 8	988 ± 64	776 ± 18
	CMp	9.28	8.77	1230 ± 79	1180 ± 69	7667 ± 381	8190 ± 145	220 ± 22	179 ± 23	817 ± 19	723 ± 19
	CMi	8.37	8.34	1037 ± 36	1010 ± 83	7825 ± 100	7160 ± 198	190 ± 5	190 ± 8	793 ± 61	570 ± 10

C control, *OMp* organic management and plant, *OMi* organic management and inter-row, *CMp* mineral management and plant, *CMi* mineral management and inter-row. t1 November 2006, t2 November 2007. *TN* total nitrogen in mg N kg^{-1} , *TOC* total organic carbon, *WSC* water soluble carbon and *PEC* $> 10^4$ Da pyrophosphate extractable carbon fraction $>10^4$ Da in mg C kg^{-1}

Table 2 Biochemical parameters at t1 (November 2006) and t2 (November 2007) sampling

Slope	Soil samples	TG		STG		EG		SEG	
		t1	t2	t1	t2	t1	t2	t1	t2
0%	C	35.7 ± 3.7	29.9 ± 5.1	4.86	5.77	2.39 ± 0.21	1.76 ± 0.21	2.63	2.22
	OM plant	84.1 ± 3.0	175 ± 5	10.39	21.1	3.67 ± 0.32	4.19 ± 0.28	2.95	3.51
	OM inter-row	80.9 ± 2.5	151 ± 10	10.61	22.7	3.29 ± 0.42	3.18 ± 0.15	2.93	3.11
	CM plant	66.0 ± 4.2	102 ± 11	7.99	12.6	2.17 ± 0.08	2.13 ± 0.10	2.69	2.38
	CM inter-row	51.1 ± 8.3	69.1 ± 11.0	6.99	11.25	1.24 ± 0.11	1.38 ± 0.12	1.40	1.65
2%	C	35.3 ± 7.1	27.2 ± 5.2	4.28	4.85	1.19 ± 0.21	1.21 ± 0.09	1.23	1.14
	OM plant	84.3 ± 11.9	120 ± 12	8.97	13.0	2.69 ± 0.21	4.38 ± 0.11	2.13	3.16
	OM inter-row	74.5 ± 14.3	80.8 ± 11.0	7.91	9.8	2.47 ± 0.11	3.48 ± 0.10	2.01	2.39
	CM plant	32.3 ± 4.5	69.4 ± 2.3	3.67	10.1	2.17 ± 0.06	3.00 ± 0.18	1.93	3.71
	CM inter-row	33.5 ± 4.2	42.4 ± 11.0	4.09	6.29	1.01 ± 0.03	1.42 ± 0.14	0.84	1.73
6%	C	29.8 ± 1.6	37.7 ± 3.8	3.80	5.94	1.42 ± 0.10	1.57 ± 0.18	1.71	2.42
	OM plant	45.3 ± 2.2	103 ± 3	5.42	13.4	2.38 ± 0.09	1.68 ± 0.16	2.57	2.00
	OM inter-row	39.5 ± 1.5	64.2 ± 1.9	5.43	9.49	2.21 ± 0.04	1.12 ± 0.15	2.24	1.44
	CM plant	44.3 ± 1.3	59.7 ± 7.2	5.78	7.29	1.61 ± 0.18	1.31 ± 0.11	1.97	1.81
	CM inter-row	36.2 ± 7.8	54.4 ± 10.8	4.62	7.60	1.24 ± 0.07	0.97 ± 0.12	1.57	1.70

C control, *OM* organic management, *CM* mineral management, *TG* total β-glucosidase activity, *EG* extracellular β-glucosidase activity in mg PNP kg^{-1} h^{-1}, *STG* TG/TOC in mg PNP g C^{-1} h^{-1}, *SEG* EG/PEC $> 10^4$ Da in mg PNP g C^{-1} h^{-1}

and mineral treatments even if a higher amount was often found in the presence of plant. This parameter decreased during the time, showing at t2 values often lower than the respective control soil (Table 1). In each slope, the pyrophosphate extractable high-molecular-weight humic fraction (PEC $> 10^4$ Da) resulted, at both sampling times, significantly higher in organic than in mineral management systems.

Total β-glucosidase activity (TG) reached maximum values in the organic with respect to mineral management system, and a positive effect of plants was also generally observed (Table 2). By comparing t1 and t2 sampling times, TG activity significantly increased in the organically amended soils, both in the presence and absence of plants, while, in mineral amended soil, higher values were observed only in presence of plants. By comparing the slopes at t1 sampling, it was found a lowest stimulation of TG in 6% slope which, in addition, showed no significant differences between organic and mineral treatments; at t2 sampling time TG activity increased particularly in the presence of plant. A similar pattern was observed for the specific

total β-glucosidase activity (STG: TG/TOC) but generally no differences between plant and inter-row samples were showed (Table 2). Both absolute (EG) and specific extracellular β-glucosidase activity (SEG: EG/PEC $> 10^4$ Da) resulted generally lower in the mineral with respect to the organic treatment. At t1 sampling significant differences between plant and inter-row samples were found only in the mineral management systems (Table 2), while, at t2 this difference resulted significative also in organic management systems.

The fraction PEC $> 10^4$ Da was used to characterize the different soil managements on the basis of the different IEF patterns. In the Fig. 1 are reported the IEF profiles of humic matter extracted from control, plant and inter-row soils under organic and mineral management systems in each slope at t1 and t2 sampling. The more intense IEF peaks focussed in the pH range 4.5–4.0 (Fig. 1). The sum of the peaks area focussed in this range was almost 40–60% of the total area in all soil samples. These peaks were higher in organic than in mineral management, as expected, and a positive effect of plants was observed in both management systems, in particular at t2 sampling. In plant and inter-row soils under the mineral farming system a decrease of optical density was also observed along the slope gradient. After IEF the largest portion of β-glucosidase activity was located in the two broad organic matter peaks in the pH range 4.5–4.0 (Table 3, HEG). At both sampling times, this activity (HEG) represents only 18–24% of EG in all the controls, while in organically treated soils, percentages higher than 30% were generally observed. Both absolute (HEG) and specific (SHEG) activities in the pH range 4.5–4.0 resulted higher in the organic with respect to the mineral treatment and they were also generally higher in the presence of plants than in the inter-row soils (Table 3); this pattern was particularly evident in 0 and 2% slopes at t2 sampling.

4 Discussion

The chemical and biochemical parameters considered in this work gave information about differences in soil quality between organic and mineral soil managements. In both farming systems the biochemical soil metabolism seems to be stimulated by the increase in soil nutrient and energy pools (higher TOC and TN). The great increase of the chemical and biochemical parameters, generally occurring since t1 sampling time in 0 and 2% slopes with respect to control soils following organic amendment and in 6% slope at t2, may be both due to the stimulation of microbial biomass and activity (Benítez et al. 2005) and by a direct addition of enzymes with organic amendment (Perucci 1992; Pascual et al. 1998). Plants may also contribute to microbial stimulation through organic exudates at the root-soil interface (Newman 1985). The enzymatic activities increased also between t1 and t2, in particular in presence of plant confirming the plant actions.

Although all soil plots investigated may reasonably be considered comparable on the basis of their nutrient and organic substrate contents (TOC, TN and WSC),

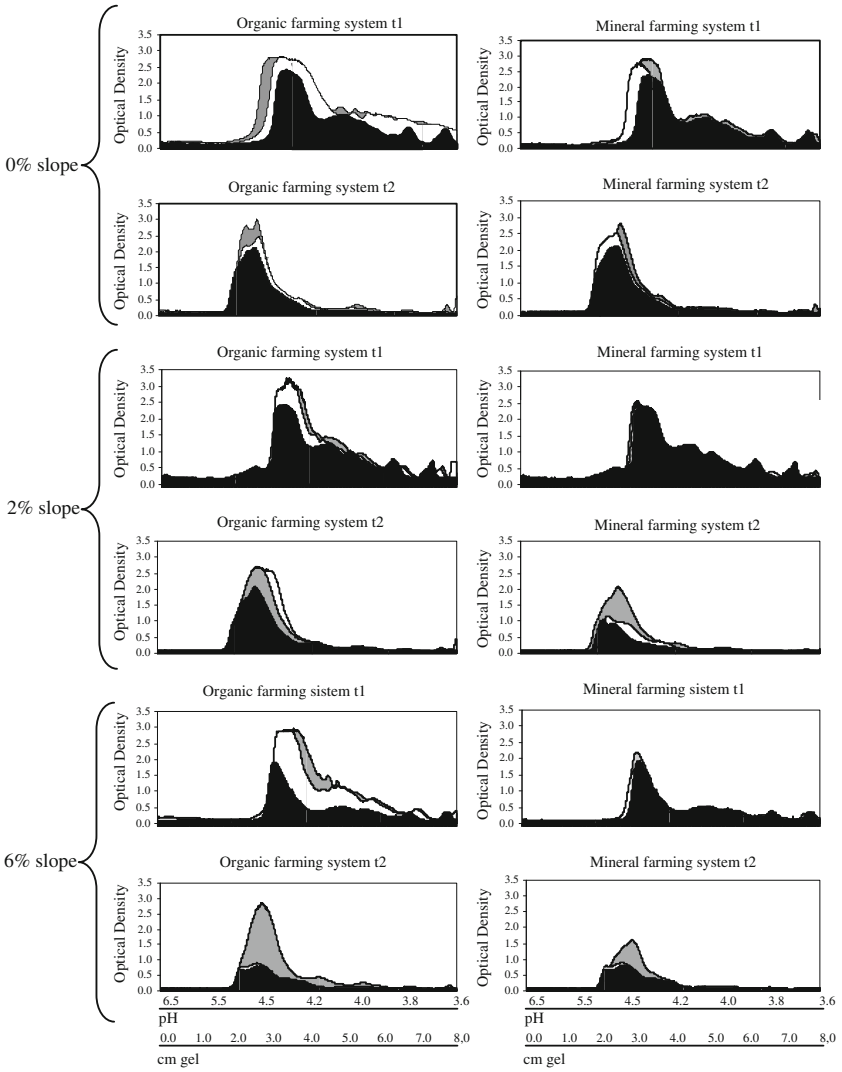

Fig. 1 IEF profiles of humic matter extracted from control (black), plant (grey) and inter-row soils (white) under organic and mineral management systems in 0, 2 and 6% slopes at t1 (November 2006) and t2 (November 2007) sampling

the 6% slope resulted less capable to storage humic carbon and to preserve extracellular enzyme activity; in fact, a decrease of PEC $> 10^4$ Da and extracellular β-glucosidase activity (EG) were evident between t1 and t2. Knight and Dick (2004) have highlighted that EG, even representing a small part of TG, may result in an ecological advantage for some soil microorganisms which can benefit by not having to synthesize this enzyme and/or degrade substrates that are too large or insoluble to be taken up by microbial cells. Masciandaro and Ceccanti (1999),

Table 3 Enzymatically active humic carbon (HC) and extracellular β-glucosidase activity, both absolute (HEG) and specific (SHEG), in soil pyrophosphate extract fraction $> 10^4$ Da in the pH range 4.5–4.0 (HEG) after isoelectric focussing at t1 (November 2006) and t2 (November 2007) sampling

Slope	Soil samples	HC t1	HC t2	HEG t1	HEG t2	SHEG t1	SHEG t2
0%	C	501 ± 11	517 ± 15	0.538 ± 0.016	0.605 ± 0.031	1.074	1.170
	OM plant	675 ± 9	656 ± 23	1.273 ± 0.044	1.914 ± 0.015	1.886	2.918
	OM inter-row	579 ± 12	484 ± 12	1.067 ± 0.030	0.977 ± 0.018	1.843	2.019
	CM plant	384 ± 6	340 ± 19	0.642 ± 0.026	1.011 ± 0.006	1.672	2.974
	CM inter-row	414 ± 4	365 ± 21	0.366 ± 0.020	0.465 ± 0.012	0.884	1.274
2%	C	413 ± 10	518 ± 14	0.291 ± 0.008	0.375 ± 0.012	0.705	0.724
	OM plant	669 ± 14	707 ± 17	0.825 ± 0.025	1.290 ± 0.009	1.233	1.837
	OM inter-row	481 ± 12	787 ± 16	0.504 ± 0.017	0.615 ± 0.022	1.048	0.781
	CM plant	582 ± 12	307 ± 11	0.570 ± 0.007	0.654 ± 0.005	0.979	2.130
	CM inter-row	441 ± 9	351 ± 10	0.228 ± 0.010	0.282 ± 0.003	0.517	0.803
6%	C	360 ± 8	318 ± 21	0.280 ± 0.004	0.231 ± 0.003	0.778	0.726
	OM plant	387 ± 7	364 ± 16	0.741 ± 0.008	0.717 ± 0.015	1.915	1.970
	OM inter-row	416 ± 6	357 ± 19	0.458 ± 0.004	0.536 ± 0.011	1.101	1.501
	CM plant	413 ± 12	383 ± 8	0.396 ± 0.015	0.317 ± 0.007	0.959	0.828
	CM inter-row	337 ± 6	240 ± 15	0.303 ± 0.024	0.262 ± 0.009	0.899	1.092

C control, *OM* organic management, *CM* mineral management, *HC* humic carbon calculated from the IEF peak areas focused in the pH range 4.5–4.0 in mg C kg^{-1}, *HEG* humic-bound β-glucosidase activity pH 4.5–4.0 in mg PNP kg^{-1} h^{-1}, *SHEG* HEG/HC in mg PNP g C^{-1} h^{-1}

studying different soil management systems, found a relation between extracellular enzyme activities and chemico-structural properties of associated humic compounds. Major information on humic-enzyme complexes could be obtained adopting the powerful analytical IEF analysis. The IEF profiles have defined the level of stability of humic substances isolated from each soil ecosystem on the basis of its isoelectric focussing pattern. It is well known the more humified the organic matter, the higher is its isoelectric point, so that the organic molecules focus at higher pH values (Ceccanti and Nannipieri 1979; Govi et al. 1994). The organically managed soils, showing more humified organic compounds that focus at higher pH values (4.5–4.0) in all slopes, may reasonably be considered at a higher degree of humification. A positive effect of plants on soil organic matter humification has been observed in both management systems, due to the organic input by plant roots exudates and litter to humus formation (Horner et al. 1988; Palm and Rowland 1997).

A higher content of humic carbon (PEC $> 10^4$ Da and HC, Tables 2 and 3, respectively) was observed in 0 and 2% slopes under organic management with

respect to the control soil. A moderate increase of humic carbon content was instead observed in the 6% slope indicating that relatively high slope gradient negatively affected carbon storage as reported by Cheng et al. (2008). As regards the activity of humic-bound β-glucosidase, the higher values observed in the organic almond tree plantation suggested an increase in metabolic efficiency, which corresponded to a better ability to store and accumulate energy.

The maintenance of the active humus (pH 4.5–4) in all soil samples indicated the presence of a stable nucleus of humic substances able to protect the extracellular enzyme in an active form (Ceccanti and Masciandaro 2003). Moreover, the percentage of the stable β-glucosidase-humic complexes activity (HEG) with respect to extracellular β-glucosidase activity (EG) resulted generally higher in the plant systems, both under organic and mineral management, confirming the better capacity of these ecosystems to preserve metabolic energy.

In order to explain more clearly the factors controlling β-glucosidase-humic complexes accumulation in the different ecosystems, stepwise linear regression analysis was performed.

Specific humic-bound β-glucosidase activity (SHEG) was selected as the more suitable dependent variable and the obtained equation was the following:

$$\mathrm{SHEG} = 0.589\mathrm{TG} + 0.519\mathrm{EG} - 0.410\mathrm{PEC} > 10^4\ \mathrm{Da} \qquad R^2 = 0.73$$

Total (TG) and extracellular (EG) β-glucosidase activities seem to have the greatest influence on enzymatically active humic carbon evolution as a statistically significant relation between SHEG (dependent variable) and TG and EG (independent variables) was found. The relationship between SHEG and TG suggested the contribution of microbial activity in the accumulation and storage of stable enzyme activities. As expected also the PEC $> 10^4$ Da influenced the dynamic of SHEG.

A stepwise linear regression analysis was also performed considering separately the results of the different management systems. The obtained equations gave evidence that in the organic amended soils the total organic carbon significantly affected the β-glucosidase activity preservation (relation between SHEG and TOC) suggesting the capability of soil to preserve and accumulate the added organic carbon in a stable humic fraction able to preserve the extracellular enzyme activity. On the contrary, in the mineral management systems, TOC seems not influence the dynamic of this biochemical parameter; this could be interpreted as lower capability of mineral managed soils to preserve both humic carbon and metabolic energy, thus lowering their quality with time.

Organic management systems:

$$\mathrm{SHEG} = 1.19\mathrm{EG} + 0512\mathrm{TG}^{-1}.7\mathrm{PEC} > 10^4\ \mathrm{Da} + 0.409\mathrm{TOC} \qquad R^2 = 0.92$$

Mineral management systems:

$$\mathrm{SHEG} = 0.645\mathrm{TG} + 0.473\mathrm{EG} \qquad R^2 = 0.89$$

5 Conclusions

IEF technique resulted an efficient tool to isolate and purify the humic-β-glucosidase enzyme complexes which, even though they represent only a small part of soil humic substances, clearly describe the evolution of organic carbon in almond orchard soils under organic and mineral management practices; in addition, information provided by the IEF well integrated with the conventional biochemical parameters and discriminate soil plots on the basis of slope and plant influence. The relation found between SHEG, that is the activity preserved onto the humic substances (humic-bound β-glucosidase activity), and parameters linked to metabolic processes clearly evidenced the contribute of total organic carbon (TOC) in determining accumulation and preservation of extracellular enzyme activity.

The study of these active humic-β-glucosidase enzyme complexes can be considered relevant indicators for assessing amount and role of humic carbon stored in degraded soils, following regeneration practices.

References

Bandick AK, Dick RP (1999) Field management effects on soil enzyme activities. Soil Biol Biochem 31:1471–1479

Benítez E, Nogales R, Masciandaro G, Ceccanti B (2000) Isolation by isoelectric focusing of humic urease complexes from earthworm (*Eisenia fetida*)-processed sewage sludges. Biol Fertil Soils 31:489–493

Benítez E, Sainz H, Nogales R (2005) Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. Bioresour Technol 96:785–790

Canali S, Trinchera A, Pinzari F, Benedetti A (1998) Study of compost maturity by means of humification parameters and isoelectrofocusing technique. In: Proceedings of the 16th world congress of soil science. Montpellier, France, Paper no. 778 (CD-ROM)

Ceccanti B, Masciandaro G (2003) Stable humus-enzyme nucleus: the last barrier against soil desertification. In: Lobo MC, Ibáñez JJ (eds) Preserving soil quality and soil biodiversity-the role of surrogate indicators. CSIC-IMIA, Madrid, pp 77–82

Ceccanti B, Nannipieri P (1979) Concerning the reliability of the isoelectric focusing technique to separate the soil humic substances. In: Frigerio A, Renoz L (eds) Recent developments in chromatography and electrophoresis, vol 10. Elsevier, Amsterdam, pp 225–232

Ceccanti B, Nannipieri P, Cervelli S, Sequi P (1978) Fractionation of humus-urease complex. Soil Biol Biochem 10:39–45

Ceccanti B, Alcañiz-Baldellou JM, Gispert-Negrell M, Gassiot-Matas M (1986) Characterization of organic matter from two different soils by pyrolysis-gas chromatography and isoelectric focusing. Soil Sci 142:83–90

Ceccanti B, Bonmatí-Pont M, Nannipieri P (1989) Microdetermination of protease activity in humic bands of different sizes after analytical isoelectic focusing. Biol Fertil Soils 7:202–206

Ceccanti B, Doni S, Macci C, Cercignani G, Masciandaro G (2008) Characterization of stable humic-enzyme complexes of different soil ecosystems through analytical isoelectric focussing technique (IEF). Soil Biol Biochem 40:2174–2177

Cheng Q, Ma W, Cai Q (2008) The relative importance of soil crust and slope angle in runoff and soil loss: a case study in the hilly areas of the Loess Plateau, North China. GeoJ 71:117–125

Ciavatta C, Govi M (1993) Use of insoluble polyvinylpyrrolidone and isoelectric focusing in the study of humic substances in soils and organic wastes. J Chromatogr 643:261–270

Feinstein AR (1996) Multivariable analysis. Yale University Press, New Haven

Govi M, Ciavatta C, Gessa C (1994) Evaluation of the stability of the organic matter in slurries, sludges and composts using humification parameters and isoelectric focusing. In: Senesi N, Miano TM (eds) Humic substances in the global environment and implications on human health. Elsevier Science, London, pp 1311–1316

Horner JD, Gosz JR, Cates RG (1988) The role of carbon based plant secondary metabolites in decomposition in terrestrial ecosystems. Am Nat 132:869–883

Indorante SJ, Follmer LR, Hammer RD, Koenig PG (1990) Particle-size analysis by a modified pipette procedure. Soil Sci Soc Am J 54:560–563

Knight TR, Dick RP (2004) Differentiating microbial and stabilized β-glucosidase activity relative to soil quality. Soil Biol Biochem 36:2089–2096

Lähdesmäki P, Piispanen R (1992) Soil enzymology: role of protective colloid systems in the preservation of exoenzyme activities in soil. Soil Biol Biochem 24:1173–1177

Masciandaro G, Ceccanti B (1999) Assessing soil quality in different agro-ecosystems through biochemical and chemical structural properties of humic substances. Soil Till Res 51:129–137

Nannipieri P, Ceccanti B, Conti C, Bianchi D (1982) Hydrolases extracted from soil: their properties and activities. Soil Biol Biochem 14:257–263

Newman EI (1985) The rhizosphere: carbon sources and microbial populations. In: Fitter AH, Atkinson D, Read DJ, Usher MB (eds) Ecological interactions in soil. Blackwell Scientific Publications, Oxford, pp 107–121

Palm CA, Rowland AP (1997) A minimum dataset for characterization of plant quality for decomposition. In: Cadish G, Giller KE (eds) Driven by nature plant litter quality and decomposition. CAB international, Wallingford, pp 379–392

Pascual JA, Hernández T, García C, Ayuso M (1998) Enzymatic activities in an arid soil amended with urban organic wastes: laboratory experiment. Bioresour Technol 64:131–138

Perucci P (1992) Enzyme activity and microbial biomass in a field soil amended with municipal refuse. Biol Fertil Soils 14:54–60

Ruggiero P, Radogna VM (1988) Humic acid-tyrosinase interactions as a model of soil humic-enzyme complex. Soil Biol Biochem 20:353–359

Saviozzi A, Levi-Minzi R, Cardelli R, Riffaldi R (2001) A comparison of soil quality in adjacent cultivated, forest and native grassland soils. Plant Soil 233:251–259

Smith SV, Renwick WH, Buddemeier RW, Crossland CJ (2001) Budgets of soil erosion and deposition for sediments and sedimentary organic carbon across the conterminous united states. Global Biogeochem Cycles 15:697–707

Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenyl phosphate in assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Yeomans JC, Bremner JM (1988) A rapid and precise method for routine determination of organic carbon in soil. Commun Soil Sci Plan 19:1467–1476

Zornoza R, Mataix-Solera J, Guerrero C, Arcenegui V, Mataix-Beneyto J (2009) Storage effects on biochemical properties of air-dried soil samples from southeastern spain. Arid Land Res Manag 23:213–222

Enzymatic Activity and Carbon Mineralization in Mexican Tepetates Cultivated Under Different Management Practices

Silvia Pajares, Juan F. Gallardo and Jorge D. Etchevers

Abstract *Tepetates* (hardened volcanic tuffs) occupy large extensions in the Central Mexican Highlands and some of them have been modified for agriculture. In their native condition *tepetates* contain traces of C, N, and available P. The objective of this work was to study how different agricultural practices affect the labile organic C availability and the enzymatic activity in cultivated *tepetates*. Experimental plots, cultivated since 1986, were subjected to three agricultural management systems during 2002–2005: Traditional (Tt); Improved (Ti); and Organic (To). In 2002 two new plots were subjected to the traditional system (Rt), and organic system (Ro). Two non-cultivated *tepetates* were chosen as reference (Tv and Td). In 2005 soil samples were collected at 0–10 cm depth and soil organic C (SOC), total N (Nt), dehydrogenase, β-glucosidase, phosphatase, urease and protease activities, mineralized C (Cm), and potentially mineralizable C (C_0) were determined. In 4 years the recently cultivated *tepetates* (Rt and Ro) increased the values of these variables in relation to the non-cultivated *tepetates* (Tv and Td), indicating that the incorporation of *tepetates* to the agriculture improved their biochemical properties. Enzymatic activity, Cm and C_0 increased with the years of cultivation and they were significantly higher with Ti and To, suggesting higher SOC availability for microbiology with these managements, likely due to a higher supply of organic substrates because fertilizers with crop residues (Ti) and organic

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manures (To) were added. Results also demonstrated that an appropriate management of *tepetates* produces a significant increase of SOC and Nt, leading to an improvement of their biochemical properties.

1 Introduction

Tepetates are hardened volcanic tuffs derived from geo-pedological processes that have been partially altered by weathering (Etchevers et al. 2006). There are two main types of *tepetate*s: the fragipan and the duripan (Hidalgo 1996); the former one can be broken up for making soil and be cultivated.

The *tepetates* occupy large extensions in the Central Mexican Highlands, principally in the States of Mexico and Tlaxcala, covering an area of 30,700 km^2 (Zebrowski 1992). They involve between 30 and 40% of the soils dedicated to the agriculture and have an important effect on the available resources to the rural population (Quantin et al. 1998). However, the intensive management of these soils has a negative effect on the quality and quantity of soil organic matter (SOM), which affects *tepetates* properties and thus their fertility (Etchevers et al. 1992). Therefore, the rehabilitation and improvement of these volcanic substrates are essential as preliminary steps for sustainability and a better quality of life in these Mexican areas.

In their native condition *tepetates* contain an insufficient amount of nutrients to sustain plant growth; in fact they contain only traces of C, N, and available P (Etchevers and Brito 1997). These almost sterile materials can be incorporated into agriculture after breaking up the native hard volcanic sediment and implementing suitable agricultural management systems, mainly by addition of organic material.

SOM can be divided in several pools. However a simple approach is considering only two major pools based on the relative susceptibility to biological decomposition (Tate 1987): (a) an easily decomposable pool (labile fractions of SOM); and (b) a more resistant pool (humic substances). The labile fractions of SOM consist of partially decomposed roots and non-harvested crop residues, and have a rapid turnover in soil (Masciandaro and Ceccanti 1999). Humic substances are complex polymeric organic compounds comprising polyphenols, proteins, active enzymes, lipids, and polysaccharides, which are more resistant to decomposition than the non-humic material (Ceccanti et al. 1986).

Soil quality is defined as the soil capacity to function suitably within natural or managed ecosystem boundaries (Karlen et al. 1997). In most soils the microbial populations and their activities are fundamental to maintain soil quality; they mediate the processes of SOM turnover and nutrient cycling (Doran and Parkin 1994). In the short to medium term, biological properties and labile fractions of SOM are much more sensitive to soil managements than soil physical and chemical properties; therefore, they give accurate and immediate information about changes in soil quality (Caravaca et al. 2002; Gil-Sotres et al. 2005). Little information exists about biological indicators of Mexican volcanic soils, which in general have suffered different degrees of degradation (Álvarez et al. 2000).

The mathematical description of C mineralization dynamics in incubation studies is of great interest for the prediction of soils ability to provide potentially mineralisable C (C_0) and for understanding the total balance of SOM (Fernández et al. 2007). The first-order model by Stanford and Smith (1972) is the most widely used, although many other models have been also postulated. These models include linear (Levi et al. 1990) and non-linear regression equations (Smith et al. 1980; Murwira et al. 1990), and first-order equations, including parameters related to groups of substrates of various degrees of stability (Murayama et al. 1990; Molina et al. 1983). These mathematical models provide useful indices and allow testing the hypotheses concerning the mechanisms that involve C mineralization.

Soil enzyme activities are related to soil microbial activities and thus can be used as indicators of soil functioning, simultaneously with other microbial and biochemical properties (Nannipieri et al. 2003). In fact enzymes activities have been widely used as early and sensitive indicators of management-induced changes in soil fertility and quality (Dick et al. 1996; Nannipieri et al. 1996; Pascual et al. 2000).

The objective of this work was to study how different agricultural management practices applied to cultivated *tepetates* affect the labile organic C and soil enzymatic activity.

2 Materials and Methods

The study site was located in Santiago de Tlalpan (Tlaxcala, Mexico; 19° 20' N and 98° 20' W) at 2600 m.a.s.l., in the eastern part of the piedmont of the "*Sierra Nevada*" mountains. The climate in the region is temperate and sub-humid. The mean annual temperature is 14°C and the mean annual precipitation is 760 mm and is concentrated in summer.

Six *tepetate* terraces of 1300 m^2 each were implemented for the present research. They were established in 1986 and 1990 by breaking up the parent material (tuff) and were cultivated for research purposes in 1990–1991 (Werner 1992) and in 1995–1996 (Fetcher et al. 1977). These terraces were then cultivated traditionally by farmers up to 2001 (rotation of wheat-barley-maize and then only maize) with a low fertilization rate: 45 and 15 kg ha^{-1} of N and P, respectively.

During the 2002–2005 period these six terraces were used for the present investigation. In 2002 each terrace was subdivided in two plots of 650 m^2 each and three types of agricultural management systems were tested (Table 1): Traditional (Tt: 4 plots cultivated using low inorganic fertilizer input, and full exportation of crop residues at the end of the agricultural cycle); b) Improved (Ti: 4 plots cultivated using moderate inputs of inorganic fertilizer according to the crop demand, and mulching with crop residues for soil protection); and c) Organic (To: 4 plots cultivated using organic inputs as nutrient sources every year, i. e., manure and compost). A crop rotation was used in every management in order to improve crop yields in *tepetates* (Table 1). At the beginning of the experiment (2002) four new plots were broken up in the same study site with similar substrate to that of the

Table 1 Agricultural management systems in the experimental plots of cultivated *tepetates*

Year	Treatments	Chemical Fertilization N–P–K (kg ha^{-1}) or Organic Manures and Composts (Mg ha^{-1})				Crops				Crop Residues (Mg dm ha^{-1})
		2002	2003	2004	2005	2002	2003	2004	2005	2002–2005
1986	Tt	23-00-00	23-00-00	80-00-00	62-23-00	H+V	A	Z+P	T	6.1
1986	Ti	60-100-34	23-60-00	90-40-00	82-23-00	H+V	A+V	Z+P	T	10.4
1986	To	17 (FC)	15 (FM)	1.9 (DC)	3.0 (DC)	H+V+AB	A+V	Z+P	T	7.8
2002	Rt	23-46-00	23-00-00	81-00-00	62-23-00	H	A	Z+P	T	2.6
2002	Ro	21 (FM)	15 (FM)	2.6	(DM)	4.3 (DC)				H+V+AB
A+V	Z+P	T	8.4							

DC dry compost, *DM* dry manure, *FM* fresh manure, *FC* fresh compost, *dm* dry matter, *H Vicia faba L., A Avena strigosa Schreb., Z Zea mais L., P Phaseolus vulgaris L., V Vicia villosa Roth., T Triticum aestivum L., AB* alive barrier, *Tt* Traditional system, *Ti* Improved system, *To* Organic system, *Rt* broken up in 2002 and cultivated with Tt, *Ro* broken up in 2002 and cultivated with To.

previous plots. Two of the newly-fragmented plots were subjected to the traditional system (Rt) and the others two to the organic system (Ro), being the crops established there the same as in the older plots. The crops of each particular year were chosen by the farmer, depending on his needs. Tillage of the experimental plots was done according to the traditional technology of local farmers (mules or tractor). A *tepetate* with natural vegetation (Tv) and another without vegetation (Td), located close to the agricultural plots, were used as reference. The experimental design was random blocks with nested replicates.

In December 2005 two composite soil samples (with a minimum of 10 simple samples each) were collected at 0–10 cm depth (the most sensitive soil layer) with a cylindrical auger from each plot. The samples were mixed and sieved to a 2 mm mesh, and the macroscopic SOM was separated from the mineral soil before carrying out chemical and biological analyses. Part of the samples was air dried for chemical analysis, while the other was stored at 4°C (previously measuring its humidity) for biochemical analysis.

Soil organic C (SOC) was analyzed by dry combustion, using a TOC Shimadzu analyzer, and total N (Nt) was measured by the micro-Kjeldahl procedure (Bremner 1996). Dehydrogenase activity (DHa) was determined by the reduction of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium chloride to iodonitrotetrazolium formazan (INTF) after incubating the soil 24 h at 22°C in darkness (Casida et al. 1964, modified). Acid phosphatase (PHa) and β-glucosidase (GLa) were determined incubating the soil 1.5 h to 37°C with *p*-nitrophenyl phosphate disodium salt and *p*-nitrophenyl glucopyranoside as substrates, respectively (Tabatabai 1994). Urease (URa) activity was calculated in function of the ammonium released after incubating the samples 1.5 h to 37°C using urea as substrate (Nannipieri et al. 1980). Casein hydrolyzing-protease (PRa) activity was analyzed through the amino-acids released after incubating the samples during 2 h to 50°C using casein as substrate (Ladd and Butler 1972).

Soil basal respiration was determined by static incubation as follows: 50 g of soil samples, moistened to 55% of its water-holding capacity, were placed in hermetically sealed flasks and incubated for 30 days at 25°C, and the CO_2 evolved

was collected in 10 mL 0.5 M NaOH and periodically (1, 2, 4, 7, 14, 21 and 30 days) titrated with 0.5 M HCl (Hernández and García 2003). Mineralizable C (Cm) was determined as the quotient between the total CO_2–C emitted during the incubation period and the total incubation time. C_0 was calculated by fitting to the obtained data of soil respiration to a first-order exponential equation by non-linear regression analysis (Murwira et al. 1990):

$$Ct = C_0(1 - e^{-kt})$$

Where Ct is the amount of C mineralized in the time. The mineralization rate constant (k) and the value of C_0 were calculated from this exponential function. C_0 shows the total content of potentially mineralisable C of these soils.

All assays were carried out by triplicate. Data were tested for normal distribution and subjected to one-way ANOVA ($P < 0.05$) according to the type of management. A Pearson correlation analysis was performed among the different parameters.

3 Results

SOC and Nt contents were very low in all plots (from 0.8 in Td to 5.0 mg C g^{-1} in To; and from 0.16 in Td to 0.53 mg N g^{-1} in To; Table 2). The recently cultivated *tepetates* (Rt and Ro) showed intermediate values between the not-cultivated *tepetates* (Td and Tv) and the oldest plots (To, Ti and Tt). The C/N ratio ranged substantially among treatments (from 5.0 in Td to 9.3 in To), being significantly lower in the non-cultivated *tepetates* (Tv and Td) and higher in the *tepetates* cultivated for more time (To, Ti and Tt). The DHa activity ranged from 18 to 132 μg TPF g^{-1} h^{-1} (Table 2), with the lowest value in Tv and the highest values in To and Ti. With regards to total hydrolytic enzyme activity, the highest activities of these enzymes were found in To and Ti and the lowest in Td. GLa activity ranged from 100 in To to 13 μg PNP g^{-1} h^{-1} in Td; PHa was higher in To and Ti (2794 and 2671 μg PNP g^{-1} h^{-1}, respectively) and lower in Td (144 μg PNP g^{-1} h^{-1}); URa and PRa activities were also higher in To and Ti treatments (26 and 28 μg NH_4–N g^{-1} h^{-1} and 63 and 56 μg tyrosine g^{-1} h^{-1}, respectively) and lower in Td (2.6 μg NH_4-N g^{-1} h^{-1} and 16.1 μg tyrosine g^{-1} h^{-1}, respectively).

The cumulative Cm had an exponential relationship with time during the incubation period of 30 d (Fig. 1). For all the samples the maximum Cm occurred the first and second day of incubation, above all with the To and Ti treatments. The flows of CO_2–C in the second day ranged from 17.1 in Td to 61.2 mg CO_2–C kg^{-1} d^{-1} in To (data not showed). A gradual decrease of Cm with time was observed for all the samples, but the treatments kept their differences, being significantly higher in To and Ti and lower in the non-cultivated *tepetates* (above all in Td). At the end of the incubation period the accumulated Cm ranged from 187 in Td to 528 mg CO_2–C kg^{-1} in To.

The calculated parameters for the C mineralization, according to the first-order model, are shown in the Table 3. All values of the coefficient of determination (R^2)

Table 2 Chemical and biological properties of cultivated and non-cultivated *tepetates*

Managements	Tt	Ti	To	Rt	Ro	Tv	Td
SOC (mg g^{-1})	3.4^{bc}	4.2^{ab}	5.0^{a}	1.6^{d}	3.1^{c}	1.3^{d}	0.8^{e}
Nt (mg g^{-1})	0.44^{bc}	0.50^{ab}	0.53^{a}	0.29^{d}	0.41^{c}	0.22^{e}	0.16^{f}
C/N	7.5^{b}	8.4^{ab}	9.3^{a}	5.4^{c}	7.6^{b}	5.9^{c}	5.0^{c}
DHa (μg TPF g^{-1} h^{-1})	103^{b}	122^{a}	132^{a}	60.5^{c}	103^{b}	18.4^{e}	37.8^{d}
GLa (μg PNP g^{-1} h^{-1})	56.9^{c}	86.3^{b}	100^{a}	39.1^{d}	51.0^{c}	32.7^{d}	13.0^{e}
PHa (μg PNP g^{-1} h^{-1})	2056^{b}	2671^{a}	2794^{a}	247^{d}	570^{c}	230^{d}	144^{e}
URa (μg NH_4-N g^{-1} h^{-1})	18.9^{b}	28.2^{a}	26.0^{a}	8.1^{c}	17.5^{b}	8.3^{c}	2.6^{d}
PRa (μg tyrosine g^{-1} h^{-1})	45.0^{b}	55.9^{a}	63.4^{a}	38.4^{c}	48.3^{b}	21.3^{d}	16.1^{e}

Soil organic C (SOC), total N (Nt), dehydrogenase activity (DHa), β-glucosidase activity (GLa), phosphatase activity (PHa), urease activity (URa), protease activity (PRa). *Tt* Traditional, *Ti* Improved, *To* Organic, *Rt* broken up in 2002 and cultivated with Tt, *Ro* broken up in 2002 and cultivated with *To*, *Tv Tepetate* with vegetation, *Td Tepetate* without vegetation. Different letters in each row indicate statistically different values (Tukey's test, $P < 0.05$)

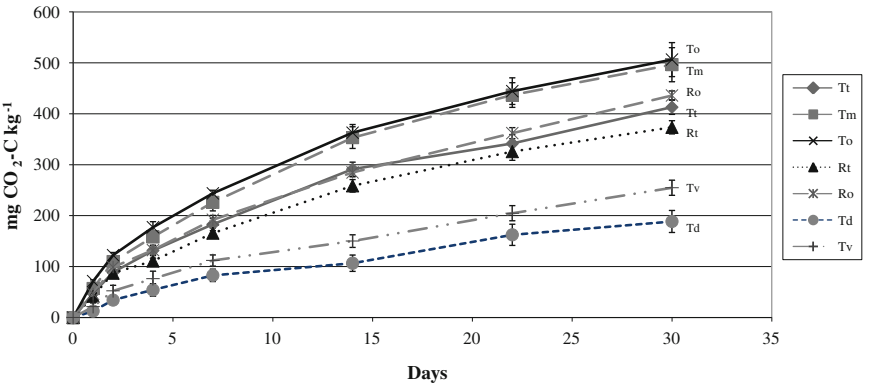

Fig. 1 Evolution of cumulative mineralisable C during the incubation of samples from cultivated and non-cultivated *tepetates*

Table 3 Parameters estimated according to the first-order model for C mineralization in cultivated and non-cultivated *tepetates*

Managements	Tt	Ti	To	Rt	Ro	Tv	Td
C_0 (mg CO_2–C kg^{-1})	448^{b}	573^{a}	548^{a}	402^{c}	464^{b}	282^{d}	224^{e}
k (d^{-1})	0.079^{b}	0.074^{b}	0.088^{a}	0.079^{b}	0.076^{b}	0.066^{c}	0.058^{c}
1/k (d)	13	13	11	13	13	15	18
C_0k (mg CO_2–C kg^{-1} d^{-1})	35^{b}	43^{a}	48^{a}	32^{b}	35^{b}	19^{c}	13^{c}
Cm (mg CO_2–C kg^{-1})	345^{b}	440^{a}	437^{a}	317^{b}	348^{b}	193^{c}	153^{c}
R^2	0.991	0.992	0.987	0.990	0.985	0.9783	0.983

Pontentially mineralisable C (C_0), mineralisation rate constant (k), mean residence time (1/k), initial potential rate of C mineralisation (C_0k), mineralisable C (Cm), coefficient of determination (R^2). *Tt* Traditional, *Ti* Improved, *To* Organic, *Rt* broken up in 2002 and cultivated with Tt, *Ro* broken up in 2002 and cultivated with *To*, *Tv* Tepetate with vegetation, *Td* Tepetate without vegetation. Different letters in each row indicate statistically different values (Tukey's test at $P < 0.05$)

were higher than 0.97; then, the selected kinetic model offers a good description of the C mineralization rate in the different plots studied. This type of model was found as the most suitable of several kinetic models in describing the composition process of many types of organic materials (Saviozzi et al. 1993). The lowest value of C_0 was found in Td (224 mg CO_2–C kg^{-1}) and the highest one in Ti (573 mg CO_2–C kg^{-1}). The rate constant for C mineralization (k) showed a relatively narrow range among treatments (from 0.058 in Td to 0.088 d^{-1} in To). Turnover times for C pools ($1/k$), calculated from the k, were relatively rapid and ranged from 11 in To to 18 d in Td. The initial potential rate of C mineralization (C_0k) had the lowest values in the non-cultivated *tepetates* (13 in Td and 19 mg CO_2–C kg^{-1} d^{-1} in Tv) and the highest values in To and Ti (48 and 43 mg CO_2–C kg^{-1} d^{-1}, respectively).

4 Discussion

SOC and Nt contents were very low in all plots (Table 2), which is characteristic in this type of hardened volcanic substrates (Etchevers et al. 1992). The highest values were observed in To (with organic fertilizers) and Ti (where crop residues were left and higher amounts of chemical fertilizers were added than in Tt), indicating the positive effects of these treatments on the SOM. SOC and Nt did not increase at the same rate in Tt due to the excessive tillage, the low addition of fertilizers, and the removal of crop residues with this management. In four years the recently cultivated *tepetates* managed with Ro increased the values of SOC (about 3 times) and Nt (about 2 times) in relation to the non-cultivated *tepetates* (Tv and Td). According to Álvarez et al. (2000), the addition of manure and compost in *tepetates* recently incorporated to the agriculture allows to increase the SOC and maintain the agricultural productivity in these substrates. The lowest values of these two variables were found in Td, because it is a hardened substrate without vegetation and, therefore, did not receive significant additions of organic residues. The C/N ratio (an indicator of the intensity of SOM mineralization) increased with the years of cultivation, being higher in the oldest plots, mainly in To, due to the high accumulation of SOC with this treatment. However, the soil C/N ratio was very low in the non-cultivated *tepetates* and in the Rt management, indicating scarce SOC contents (Etchevers et al. 2006).

The DHa activity is an indicator of microbial metabolism, being in relation to the quantity and quality of the SOM (Alef 1995). In addition, DHa has been proposed as a valid biomarker of soil management under different agronomic practices and climate (Ceccanti et al. 1994). The lowest DHa activity was found in the non-cultivated *tepetates* (Tv and Td), with scarce SOM content, indicating a low presence of associated microorganisms (García et al. 1997). By contrast higher DHa values were found in cultivated *tepetates* with higher SOM content (To and Ti treatments), which showed the positive effects of a higher SOM content on the metabolic activity of the microbial population in these soils. Therefore, DHa is a good indicator of microbial activity of these soils.

The highest value of GLa with the To treatment was probably due to a soil enrichment with fresh materials of cellulolytic nature that act as substrate for this

enzyme (Eivazi and Zakaria 1993). The lowest value of GLa in Td is explained by the scarce content of organic substrates for the microorganisms.

The significantly higher PHa activity in To and Ti could be related to a higher content of soil organic P with these treatments (Pascual et al. 2000). The presence of this enzyme is important, since it can hydrolyze the organic P of SOM to release inorganic P available for plants (García et al. 1994). This is important, as the quantity of organic compounds is very limited in the non-cultivated *tepetates* (especially in Td, with the lowest value of PHa) (Etchevers et al. 1992).

The highest URa and PRa activities with To and Ti treatments were also due to a higher availability of organic N compounds with these managements. The PRa activity determined by casein hydrolysis corresponds fundamentally to proteases not bound to soil organo-mineral colloids, thus the use of casein allows the PRa activity determination from the active microbial biomass and from the enzymes associated to cellular residues (Bonmatí et al. 1998). According to this, the PRa activity seems also to be a good indicator of quality of these substrates.

In summary, the activities of these enzymes were increased by inorganic and organic fertilization in concordance to which has been shown by other authors after the addition to soils of different organic fertilizers (Kandeler and Eder 1993; Pascual et al. 2000). The enzymatic activity also increased significantly after 4 years of cultivation (Rt and Ro) compared to the non-cultivated *tepetates* (Tv and Td), mainly with the Ro treatment. Therefore, conversion of uncultivated *tepetates* to agriculture improves their microbial activity.

The Cm is an indicator of the overall activity of the microbial pool. The Cm during the first week of incubation came from the active fraction of SOM (Fig. 1), probably originating from labile residues of particulate organic matter (Zagal et al. 2002). After this initial period Cm decreased with time, reflecting depletion of labile SOM. According to the Cm values after 30 d of incubation, it is possible to distinguish three groups of plots: (a) the non-cultivated *tepetates* (Td and Tv, with the lowest values of Cm); (b) the recently cultivated *tepetates* (Rt and Ro) and those plots managed with Tt (with middle values of Cm); (c) and the plots managed with To and Ti treatments (with the highest values of Cm). The highest Cm values observed with Ti and To treatments show the positive effect that these two managements had in the induction of biological activity in *tepetates*, which was promoted by the increment of C availability and energy for the microbial population (Elliot et al. 1994). Such availability would be consequence of an abundant supply of organic substrates by crop residues (Ti) and organic manures (To) that would maintain the quality and quantity of SOM, favoring the soil microbial activity. In cultivated *tepetates* the traditional managements (Tt and Rt) had lower Cm values than the other treatments, which causes a lower microbial development than with the other managements (Schnürer et al. 1985). The lowest values of Cm in Td and Tv were due to the depletion of readily decomposable substrates. It has been suggested that organic substrate availability is the principal limiting-factor for soil respiration (Wang et al. 2003).

The C mineralization rates (k) differed among the cultivated and non-cultivated *tepetates,* even though all the values were within a relatively narrow range. This

Table 4 Correlation coefficients among different biochemical variables

	SOC	Nt	Cm	DHa	GLa	PHa	URa	PRa
SOC	1							
Nt	0.960**	1						
Cm	0.799**	0.804**	1					
DHa	0.902**	0.840**	0.820**	1				
GLa	0.908**	0.880**	0.825**	0.911**	1			
PHa	0.884**	0.866**	0.640*	0.876**	0.880**	1		
URa	0.820**	0.905**	0.724**	0.905**	0.937**	0.837**	1	
PRa	0.870**	0.860**	0.740**	0.890**	0.832**	0.721**	0.790**	1

Same acronyms as indicated before. *Correlation coefficients are significant at $P < 0.05$ (two tailed), **correlation coefficients are significant at $P < 0.01$ (two tailed)

relatively narrow range of k values among these treatments suggests that microbial respiration metabolized organic compounds that were similar or had the same degree of availability. In general, a higher k value was observed in the cultivated *tepetates* (from 0.074 to 0.088 d^{-1}), while the decomposition rate of the non-cultivated *tepetates* had lower values (from 0.058 to 0.066 d^{-1}), since the SOM content in these substrates is very low.

The mean residence time of SOM ($1/k$) was relatively short among treatments. The same order of magnitude was observed by Riffaldi et al. (1996) in a short term laboratory incubation of agricultural soils from Italy. The $1/k$ value in the cultivated *tepetates* was lower than in the non-cultivated *tepetates*, indicating that the agricultural management practices favor the microbial activity and accelerate the C mineralization. The C_0k was higher with Ti and To treatments and lower in Td and Tv *tepetates*. Therefore, the C_0k shows an estimation of the potential of C mineralization in these substrates equal or better than C_0 or k parameters individually examined. Several researchers (Murwira et al. 1990; Saviozzi et al. 1993) have suggested that this product (C_0k) is more accurate to explain and understand the SOM quality than each parameter separately.

These results reveal a higher microbial decomposition of SOM in the cultivated *tepetates* managed with To and Ti in relation to the basal levels of the non-cultivated *tepetates*. However, the SOC accumulation obtained with these two treatments is significantly higher than that of the non-cultivated *tepetates*, even more when these volcanic substrates are managed traditionally, because the former had much higher annual inputs of C than the other managements (Table 2).

In order to gain an overall understanding of the results a correlation analysis was performed (Table 4). There were significant high correlations among the variables, which indicate a high relation between chemical and biological processes. A very significant correlation was found between SOC, Nt, and Cm, which indicates that SOC and Nt are determinant factors of microbial activity. According to Wang et al. (2003) the availability of organic C substrate is the main determinant of soil respiration. There was also a significant correlation between Cm and DHa ($r = 0.820$, $P < 0.01$), suggesting that intra-cellular enzyme activity can be

used as an indicator of soil microbial activity. There was a very significant association between the studied enzyme activities and the SOC and Nt, indicating that these activities are conditioned by the available energy (García et al. 1994; Caravaca et al. 2002). A high positive correlation existed among the overall total enzyme activities (URa, PRa, GLa and PHa), suggesting an equilibrium among the principal nutrients cycles. In summary, these correlations showed that the studied biochemical indices can provide good indications of SOM quality under different agronomic management systems in this degraded soil.

5 Conclusions

An appropriate input of C and fertilizers to tepetates caused an increase of soil organic C and N contents, leading to an improvement of their biochemical properties and hence to a better quality of these volcanic substrates, in particular when an organic treatment is applied or *tepetates* are fertilized appropriately with crop residues. Four years of organic or improved managements were enough to produce a significant increment of organic matter in these *tepetates*, which demonstrates the positive effect of these managements on the recovery of these substrates.

The enzymatic activity and C mineralization increased with the years of cultivation and were affected by the managements applied to the *tepetates*. They were significantly higher with Ti and To treatments, suggesting higher SOC availability for microbes with both managements, due to a higher supply of labile organic substrates by crop residues (Ti) and manures (To).

The higher values of biochemical variables obtained when the two latter treatments were applied are related to higher SOC contents, showing the positive effect of these managements in the induction of microbial activity of *tepetates*. Therefore, these variables could be considered good early indicators of SOC accumulation in these degraded volcanic substrates.

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References

Alef K (1995) Dehydrogenase activity. In: Alef K and Nannipieri P (eds) Methods on Applied Soil Microbiology and Biogeochemistry. Academic Press, London, pp 228–231

Álvarez D, Ferrera R, Etchevers JD (2000) Actividad microbiana en tepetates con incorporación de residuos orgánicos. Agrociencia 34:523–532

Bonmatí M, Ceccanti B, Nannipieri P (1998) Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biol Biochem 30:2113–2125

Bremner JM (1996) Nitrogen-Total. In: Sparks DL (ed) Methods of soil analysis. part 3 chemical methods. American Society of Agronomy-Soil Science Society of America, Madison, WI, USA, pp 1085–1121

Caravaca F, Masciandaro G, Ceccanti B (2002) Land use in relation to soil chemical and biochemical properties in a semiarid mediterranean environment. Soil Till Res 68:23–30

Casida L, Klein DA, Santoro R (1964) Soil dehydrogenase activity. Soil Sci 98:371–378

Ceccanti B, Alcañiz JM, Gispert M, Gassiot M (1986) Characterization of organic matter from two different soils by pyrolysis-gas chromatography and isoelectrofocusing. Soil Sci 142:83–90

Ceccanti B, Pezzarossa B, Gallardo JF, Masciandaro G (1994) Bio-tests as markers of soil utilization and fertility. Geomicrobiol J 11:309–316

Dick RP, Breakwill D, Turco R (1996) Soil enzyme activities and biodiversity measurements as integrating biological indicators. In: Doran JW, Jones AJ (eds) Handbook of methods for assessment of soil quality. Soil Science Society of America, Madison, WI, USA, pp 247–272

Doran JW, Parkin TB (1994) Defining and assessing soil quality. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (eds) Defining soil quality for a sustainable environment. Soil Science Society of America Special Publication n° 35, Madison, WI, USA, pp 3–21

Eivazi F, Zakaria A (1993) β-Glucosidase activity in soils amended with sewage sludge. Agric Ecosyst Environ 43:155–161

Elliot ET, Burke IC, Monz CA, Frey SD (1994) Terrestrial carbon pools: preliminary data from the corn belt and great plains regions. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (eds) Defining soil quality for a sustainable environment. Soil Science Society of America Special Publication N° 35, Madison, WI USA, pp 179–191

Etchevers JD, Brito H (1997) Levantamiento nutrimental de los tepetates de méxico y tlaxcala. In: Zebrowski C, Quantin P, Trujillo G (eds) Suelos volcánicos endurecidos. ORSTOM, Quito, Perú, pp 202–212

Etchevers JD, Cruz L, Mares J, Zebrowski C (1992) Fertilidad de los tepetates. I fertilidad actual y potencial de los tepetates de la vertiente occidental de la sierra nevada (méxico). Terra 10:379–384

Etchevers JD, Hidalgo C, Prat C, Quantin P (2006) Tepetates of Mexico. In: Lal R (ed) Encyclopedia of soil science. Marcel Dekker, New York, USA, pp 1745–1748

Fernández JM, Plaza C, Hernández D, Polo A (2007) Carbon mineralization in an arid soil amended with thermally-dried and composted sewage sludges. Geoderma 137:497–503

Fetcher U, Vera A, Werner G (1997) Erosión hídrica en un suelo volcánico endurecido (tepetate t3) en el bloque de Tlaxcala, México. In: Zebroski C, Quantin P, Trujillo G (eds) Suelos volcánicos endurecidos. ORSTOM, Quito, pp 351–358

García C, Hernández T, Costa F, Ceccanti C (1994) Biochemical parameters in soil regenerated by the addition of organic wastes. Waste Manage Res 12:454–466

García C, Hernández T, Costa F (1997) Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun Soil Sci Plan 28:123–134

Gil-Sotres F, Trasar C, Leirós MC, Seoane S (2005) Different approaches to evaluating soil quality using biochemical properties. Soil Biol Biochem 37:877–887

Hernández T, García C (2003) Estimación de la respiración microbiana del suelo. In: García C, Gil F, Hernández T, Trasar C (eds) Técnicas de análisis de parámetros bioquímicos en suelos medida de actividades enzimáticas y biomasa microbiana. Editorial Mundi-Prensa, Madrid, Spain, pp 311–346

Hidalgo C (1996) Étude d'horizons indurés á comportement de fragipan, appelés tepetates, dans les sols volcaniques de la vallée de Mexico Contribution à la connaissance de leurs caractères et de leur formation. ORSTOM, Paris, France

Kandeler E, Eder G (1993) Effect of cattle slurry in grassland on microbial biomass and on activities of various enzymes. Biol Fertil Soils 16:246–254

Karlen DL, Mausbach MJ, Doran JW, Cline RG, Harris RF, Schuman GE (1997) Soil quality: a concept, definition, and framework for evaluation. Soil Sci Soc Am J 61:4–10

Ladd JN, Butler JH (1972) Short-term assay of soil proteolytic enzyme activities using proteins and dipeptide derivates as substrates. Soil Biol Biochem 4:19–30

Levi R, Riffaldi R, Saviozzi A (1990) Carbon mineralization in soil amended with different organic materials. Agric Ecosyst Environ 31:325–335

Masciandaro G, Ceccanti B (1999) Assessing soil quality in different agro-ecosystems through biochemical and chemico-structural properties of humic substances. Soil Till Res 51:129–137
Molina JA, Clap CE, Shaffer MJ, Chichester FW, Larson WE (1983) A model of nitrogen and carbon transformation in soil: description, calibration and behavior. Soil Sci Soc Am J 47:85–91
Murayama S, Asakawa Y, Ohno Y (1990) Chemical properties of subsurface peats and their decomposition kinetics under field conditions. Soil Sci Plant Nutr 36:129–140
Murwira HK, Kirchmann y H, Swift MJ (1990) The effect of moisture on the decomposition rate of cattle manure. Plant Soil 122:197–199
Nannipieri P, Ceccanti B, Cervelli S, Matarese E (1980) Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016
Nannipieri P, Sequi P, Fusi P (1996) Humic and enzyme activity. In: Piccolo A (ed) Humic substances in terrestrial ecosystems. Elsevier, Amsterdam, The Netherlands, pp 293–328
Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. Eur J Soil Sci 54:655–670
Pascual JA, García C, Hernández T, Moreno JL, Ros M (2000) Soil microbial as a biomarker of degradation and remediation processes. Soil Biol Biochem 32:1877–1883
Quantin P, Prat C, Zebrowski C (1998) Soil restoration and conservation: the "tepetates"-indurate volcanic soils-in mexico. In: Harper D, Brown T (eds) The sustainable management of tropical catchments. John Wiley & Sons, New York, USA, pp 109–119
Riffaldi R, Saviozzi A, Levi R (1996) C mineralization kinetics as influenced by soil properties. Biol Fertil Soils 22:293–298
Saviozzi A, Levi R, Riffaldi R (1993) Mineralization parameters from organic materials added to soil as a function of their chemical composition. Bioresour Technol 45:131–135
Schnürer J, Clarholm M, Rosswall T (1985) Microbial biomass and activity in an agricultural soil with different organic matter contents. Soil Biol Biochem 17:611–618
Smith JL, Schnabel RR, Mc Neal BL, Campbell GS (1980) Potential errors in the first-order model for estimating soil nitrogen mineralization potentials. Soil Sci Soc Am J 44:996–1000
Stanford G, Smith SJ (1972) Nitrogen mineralization potential of soil. Soil Sci Soc Am J 36:465–472
Tabatabai MA (1994) Soil enzymes. In: Weaver RW, Angle JS, Bottomly PS, Bezdicek D, Smith S, Tabatabai MA, Wollum A (eds) Methods of soil analysis. Part 2. Chemical and microbiological properties. ASA-SSSA, Madison WI, USA, pp 775–833
Tate RL III (1987) Ecosystem management and soil organic matter level. In: Tate RL III (ed) Soil organic matter: biological and ecological effects. Wiley & Sons, New York, USA, pp 260–280
Wang WJ, Dalal RC, Moody PW, Smith J (2003) Relationships of soil respiration to microbial biomass, substrate availability and clay content. Soil Biol Biochem 35:273–284
Werner G (1992) Suelos volcánicos endurecidos (tepetates) en el estado de Tlaxcala: Distribución, rehabilitación, manejo y conservación. Terra 10:318–331
Zagal E, Rodríguez N, Vidal I, Quezada L (2002) Actividad microbiana en un suelo de origen volcánico bajo distinto manejo agronómico. Agricultura Técnica 62:297–309
Zebrowski C (1992) Los suelos volcánicos endurecidos de américa latina. Terra 10:15–23

Enzyme Diversity in Andosols of the Canary Islands (Spain)

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Abstract The loss of biodiversity usually accompanies the degradation of the terrestrial ecosystems. Besides the loss of species, such a phenomenon affects the functional diversity of the ecosystem, that is, the diversity of the ecological processes taking place inside it, and the performance of the main soil functions. This work includes the results obtained at Andosols located at nine experimental plots in the National Parks of Garajonay and El Teide (Canary Islands, Spain). The measurement of the enzyme diversity by means of the Shannon seems to provide a good estimation of the variety of functions and ecological processes in which the soil microbiota is involved. Its temporal stability can allow obtaining a good approximation to its value from single measures, in contrast to the usual long monitoring time needed in the studies of the soil enzyme activities.

1 Introduction

There is an increasing concern regarding whether the worldwide increasing loss of biodiversity will affect the quality and health of the soils, that is, their capacity to

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function as vital living systems, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health (Doran and Zeiss 2000; Griffiths et al. 2001; Hunt and Wall 2002; Nannipieri et al. 2003).

Soil microbial communities are often highly integrated so that each species contributes a distinct set of enzymes (Chapin et al. 2000). Due to this high degree of specificity, the loss of a species or group of species may have cascading effects and affect core ecological processes such as primary production, decomposition, nutrient cycling and water fluxes (Myers 1996; Schlapfer and Schmid 1999; Wardle et al. 2000; Hättenschwiler et al. 2005).

Nevertheless, not all microbial species are equally relevant since many of them are functionally redundant and play an analogous role at the ecological processes. The loss of a certain species might thus be compensated for by changes in abundance of other species, such that soil processes are unaffected (Walker 1992; Lawton and Brown 1994; Andrén et al. 1995; Hunt and Wall 2002). The concept of species itself is often unclear or diffuse at the microbial world (Ogunseitan 2005; Achtman and Wagner 2008). Because of that, some modern approaches focus on directly assessing the functional diversity of the soil microbiota, instead of measuring the less-useful, conventional taxonomic diversity (Zak et al. 1994; Marx et al. 2001; Emmerling et al. 2002; Mungai et al. 2005; Lupwayi et al. 2007).

The microbiological and biochemical properties of the soils such as the measurement of the microbial activity (soil respiration, enzyme activities) and the estimation of the microbial biomass, are among the most important indicators of the quality and health of the soil, due to their relation to the dynamics of the organic matter and the nutrient turnover (Dick et al. 1996; Pankhurst et al. 1997; Nannipieri et al. 2002). The soil enzyme activities exhibit a high seasonal variability, so that the results of individual measurements are not useful as indicators of soil quality. Because of this variability, a time monitoring is often needed and enzymes measurements are often combined into soil quality indexes to provide more reliable information about the soil biochemical functioning (Trasar-Cepeda et al. 1998; Nannipieri et al. 2002).

A few authors (Bending et al. 2002; Tscherko et al. 2003; Caldwell 2005; Rodríguez-Loinaz et al. 2008) have proposed the use of widely-used ecological biodiversity measures such as the Shannon diversity index (Shannon and Weaver 1949) in evaluating soil enzyme functional diversity. The formula of the Shannon index (H') is:

$$H' = -\sum_{i=1}^{i=k} p_i \ln p_i$$

At conventional biodiversity measurements, p_i represents the relative weight of each species to the total population or biomass of the ecosystem. As used with enzyme measurements, p_i is the relative weight of the ith enzyme activity to the sum of enzyme activities, previously standardized by dividing by an arbitrary reference value such as the maximum activity (Rodríguez-Loinaz et al. 2008) or the activity at a reference site (Tscherko et al. 2003).

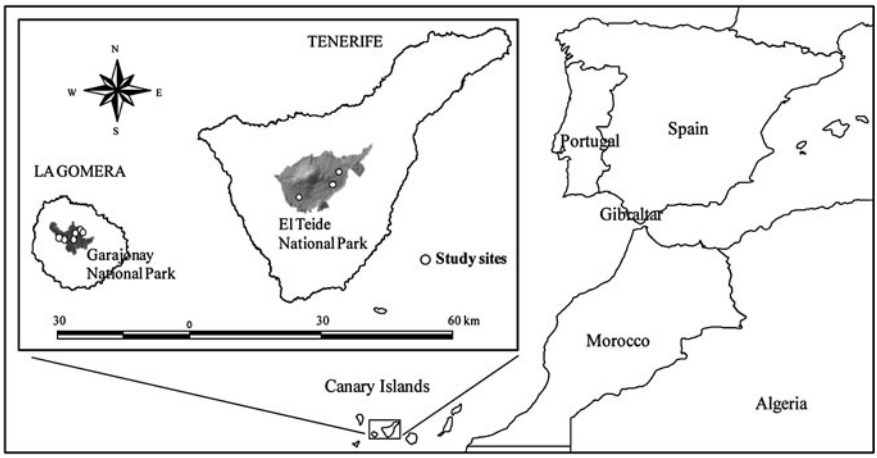

Fig. 1 Location of the study sites

Andosols have been recognized to have a high environmental quality (Arbelo et al. 2002; Óskarsson et al. 2004; Rodríguez Rodríguez et al. 2004) because of the thickness and high degree of humification of their surficial organic layers. However, the microbiological processes in these soils are not still well-understood, and few efforts concerning biological quality indexes have been reported (Armas et al. 2007).

The aims of the present study are: (1) to study the microbiological activity of Andosols at characteristics ecosystems of the Canary Islands, focusing on its variation in relation to processes of degradation of the soils and the whole ecosystem (2) to characterize the temporal variation of the microbiological activity of the soils in these areas, paying attention to the relation to climatic or phenological patterns, and (3) to study the functional diversity of the soil as seen on the measurements of the enzyme activity and its value as an indicator of degradation processes.

2 Materials and Methods

This work was carried out in nine experimental plots in the National Parks of Garajonay (La Gomera) and El Teide (Tenerife) (Fig. 1) comprising the main natural ecosystems in each area (del Arco Aguilar et al. 2006) (Table 1).

The Garajonay area hosts the largest representation of the laurel forest, typical in the windward midland areas of the Canary Islands. The most common soils are deep, humus-rich Andosols, developed over Miocene-Pliocene volcanic rocks (Rodríguez Rodríguez et al. 2002, 2004). The experimental plots include mature, healthy samples of the main forest types in the area: tree heath forest, laurel forest,

Table 1 Main characteristics of the study sites

Plant community	Predominant plant species	Soil type[a]
Garajonay Area		
Tree heath forest (*mature*)	*Erica arborea*, *Ilex canariensis*	Umbric Andosols
Tree heath forest (*regressive*)	*Erica arborea*, *Myrica faya*	Umbric Andosols
Laurel forest (*mature*)	*Laurus novocanariensis*, *Myrica faya*	Fulvic Andosols
Laurel forest (*devitalized*)	*Ilex canariensis*, *Erica arborea*	Fulvic Andosols
Riparian laurel forest (*mature*)	*Persea indica*, *Laurus novocanariensis*	Mollic Andosols
Riparian laurel forest (*devitalized*)	*Persea indica*, *Laurus novocanariensis*	Melanic Andosols
El Teide area		
Broom scrub (*mature*)	*Spartocytisus supranubius*, *Descourainia bourgaeuana*	Haplic Andosols
Scabious shrubland (*pioneer*)	*Pterocephalus lasiospermus*, *Erysimum scoparium*	Leptic Andosols
Pine afforestation (*transgressive*)	*Pinus canariensis*, *Pterocephalus lasiospermus*	Vitric Andosols

[a] According to IUSS Working Group (2006)

riparian forest, as well as nearly sites at a regressive successional stage, conspicuously degraded, and affected by spontaneous clearing processes, respectively.

El Teide Park is located at a high-mountain area, and its typical soils are young, slightly developed Andosols, located over Holocene volcanic materials. A nearly-mature legume scrub, a pioneer plant community on recent volcanic ashes, and a Canary pine afforestation conducted above the natural altitudinal boundaries of the Canary pine, are studied.

Soil samples were collected seasonally at 0–10 cm depth for a period of one year in Garajonay and two years at El Teide. Winter sampling was not carried out at El Teide area since the soils are usually frozen in this season.

The activities of various enzymes: dehydrogenase (DH; Camiña et al. 1997), β-D-glucosidase (GL; Eivazi and Tabatabai 1988), phosphomonoesterase (PH; Tabatabai and Bremner 1969) at optimum pH (ranging 5.5–6.0), and urease (UR; Tabatabai and Bremner 1972), were measured. CM-cellulase activity (CE; Schinner and von Mersi 1990) was determined at El Teide sites for a closer study of the depolymerization processes, since plant residues tend to accumulate over the soil in this area (Castroviejo 1989) whereas they are quickly decomposed at the soil surface in Garajonay (Armas 2010). The microbial biomass carbon (Microbial-C) content (Vance et al. 1987) and the amount of CO_2 mineralised during incubation for ten days (Guitián and Carballas 1976) were also analyzed. The litter-fall was collected and weighted in each site over the periods of study. The carbon and nitrogen contents of the litter (C and N inputs) were determined using a LECO elemental auto analizer.

The Shannon index (H') was calculated from the β-D-glucosidase, urease and phosphatase activities, related to the carbon, phosphorus and nitrogen cycles respectively, and dehydrogenase, usually considered as a general indicator of the

microbiological activity. In addition, the Shannon index was also calculated excluding the typically intracellular dehydrogenase activity (H'_{ex}), to assess the contribution of the extracellular stabilized enzyme activities. The ratios between the measured enzyme activities and the microbial biomass were calculated as further indicators of the intracellular/extracellular location of the enzyme activities (Landi et al. 2000).

Temporal variation and site influence were analysed by means of ANOVA/LSD tests using *SPSS for Windows version 17*. A Principal Component Analysis (PCA) of the microbiological and biochemical properties of the soils in each area was conducted using *Canoco for Windows version 4.5* (Ter Braak and Šmilauer 2002).

3 Results

The results obtained at the Garajonay area (Table 2) show high contents of microbial biomass, higher in the mature/healthy sites than in the corresponding degraded ones. A clear seasonality is observed, with increasing values in autumn and spring coinciding with the highest litter-fall inputs (Fig. 2).

The soil respiration exhibits its lowest values at the degraded tree heath and devitalized laurel forests. Nonetheless the soils under devitalized riparian forest show similar values, even higher, than the corresponding healthy community. No clear seasonality is observed but a general correlation to the soil water content ($r = 0.766$, $P = 0.000$) (Fig. 3).

In general, the enzyme activities show high values in the devitalized riparian forest and very low ones in the devitalized laurel and degraded tree heath forests. No regular seasonal variation is observed but a diffuse relationship is detected linking the litter N inputs and the urease activity (Fig. 4). The enzyme activity: microbial biomass quotients show a greater seasonality than the raw activity values, being highest in April-January and lowest in July–October.

The Shannon index (H') of the Garajonay soils ranges between 1.28 and 1.36 and is significantly lower in the degraded tree heath and laurel forests. The H'_{ex}/H' ratio values are in a close range between 0.77 and 0.80, which indicates the high stability of the extracellular contribution to the total enzyme diversity. A correlation between the enzyme diversity and the total plant biomass of the ecosystem ($r = 0.976$, $P = 0.001$) is observed (Fig. 5).

The first axis of the PCA (Fig. 6) shows the strong correlation between the microbiological properties of the Garajonay soils, and discriminates the soils of the degraded tree heath and laurel forests from those showing greater microbiological activity. The second axis separates the seasons: the values registered during autumn and summer are located in the positive semiaxis, associated to the highest contents of microbial biomass and the biggest litterfall, whereas the winter and spring values are located in the negative axis, coinciding with the greatest content of labile C forms and the highest CO_2 flux suggesting a predominance of the mineralization process during those seasons.

Table 2 Microbial biomass C, respiration, enzyme activities and functional diversity index H' of the soils of the Garajonay area (mean±standard error). Means within a row followed by the same lower case letter are not significantly different at $P = 0.05$ LSD level

Parameter[#]	$F_{d.f.=3}$	Sig.	July	October	January	April	$F_{d.f.=5}$	Sig.	Riparian laurel forest		Laurel forest		Tree heath forest	
									Healthy	Devitalized	Healthy	Degraded	Mature	Regressive
Microbial-C	6.83	0.004	87.0 ± 6.8^{a}	122.8 ± 22.6^{b}	61.5 ± 10.4^{a}	80.3 ± 16.8^{a}	6.83	0.002	142.5 ± 24.5^{a}	103.1 ± 16.8^{b}	80.5 ± 25.6^{bc}	70.2 ± 0.2^{c}	78.9 ± 7.3^{bc}	52.1 ± 4.1^{c}
Microbial-C/Total-C	3.28	0.050	0.86 ± 0.10^{a}	1.32 ± 0.22^{b}	0.77 ± 0.18^{a}	0.95 ± 0.19^{ab}	3.77	0.021	1.60 ± 0.16^{a}	0.93 ± 0.10^{b}	1.05 ± 0.40^{b}	0.70 ± 0.09^{b}	0.70 ± 0.09^{b}	0.88 ± 0.10^{b}
Mineralisable C	0.92	0.455	35.3 ± 6.1^{a}	40.7 ± 7.4^{a}	33.0 ± 6.2^{a}	40.9 ± 10.3^{a}	16.7	0.000	49.9 ± 7.3^{a}	58.6 ± 7.8^{ab}	39.0 ± 3.3^{b}	20.2 ± 1.7^{c}	41.0 ± 4.8^{b}	16.0 ± 2.5^{c}
Dehydrogenase	2.41	0.107	24.3 ± 4.9^{a}	24.8 ± 6.6^{a}	27.7 ± 6.0^{a}	37.8 ± 8.3^{b}	11.9	0.000	35.8 ± 4.1^{ab}	46.5 ± 8.3^{a}	30.0 ± 5.7^{bc}	5.8 ± 1.7^{d}	31.0 ± 7.6^{bc}	22.8 ± 1.3^{c}
Urease	0.85	0.486	0.62 ± 0.13^{a}	0.76 ± 0.23^{a}	0.58 ± 0.15^{a}	0.57 ± 0.14^{a}	27.5	0.000	0.77 ± 0.12^{a}	1.03 ± 0.25^{a}	0.60 ± 0.08^{a}	0.09 ± 0.01^{c}	0.86 ± 0.13^{a}	0.45 ± 0.04^{b}
β-D-glucosidase	1.17	0.356	0.56 ± 0.20^{a}	0.46 ± 0.19^{a}	0.39 ± 0.13^{a}	0.44 ± 0.16^{a}	28.9	0.000	0.31 ± 0.01^{c}	1.09 ± 0.20^{a}	0.61 ± 0.13^{b}	0.07 ± 0.01^{d}	0.57 ± 0.09^{b}	0.12 ± 0.03^{d}
Phosphatase	1.11	0.376	0.79 ± 0.19^{a}	0.76 ± 0.22^{a}	0.74 ± 0.21^{a}	0.82 ± 0.20^{a}	60.7	0.000	0.67 ± 0.02^{b}	1.47 ± 0.13^{a}	0.78 ± 0.11^{b}	0.21 ± 0.01^{d}	1.15 ± 0.14^{a}	0.39 ± 0.04^{c}
DH/Microbial-C	3.66	0.037	0.28 ± 0.06^{ab}	0.21 ± 0.05^{a}	0.48 ± 0.12^{bc}	0.51 ± 0.11^{c}	7.96	0.001	0.26 ± 0.02^{a}	0.48 ± 0.10^{a}	0.54 ± 0.18^{a}	0.08 ± 0.03^{b}	0.43 ± 0.13^{a}	0.45 ± 0.06^{a}
UR/Microbial-C	1.86	0.180	7.21 ± 1.45^{ab}	6.06 ± 1.36^{a}	9.25 ± 1.87^{b}	7.89 ± 1.99^{ab}	8.87	0.000	5.72 ± 0.97^{b}	9.82 ± 1.36^{a}	8.81 ± 1.55^{ab}	1.32 ± 0.30^{c}	11.2 ± 2.08^{a}	8.72 ± 0.50^{ab}
GL/Microbial-C	1.05	0.400	6.13 ± 2.04^{a}	3.59 ± 1.21^{a}	6.76 ± 2.63^{a}	5.99 ± 2.04^{a}	16.2	0.000	2.34 ± 0.29^{b}	10.6 ± 1.57^{a}	9.92 ± 2.91^{a}	0.97 ± 0.12^{c}	7.62 ± 1.72^{a}	2.27 ± 0.50^{b}
PH/Microbial-C	5.03	0.013	8.84 ± 1.80^{ab}	6.49 ± 1.58^{a}	12.0 ± 2.94^{b}	11.5 ± 2.88^{b}	18.2	0.000	5.03 ± 0.70^{c}	14.8 ± 1.49^{a}	12.9 ± 3.49^{ab}	3.16 ± 0.50^{d}	15.0 ± 2.53^{a}	7.33 ± 0.29^{c}
H'	0.49	0.694	1.33 ± 0.01^{a}	1.35 ± 0.01^{a}	1.31 ± 0.04^{a}	1.32 ± 0.02^{a}	3.52	0.026	1.34 ± 0.01^{a}	1.36 ± 0.01^{a}	1.36 ± 0.00^{a}	1.28 ± 0.03^{b}	$1.35 \pm 0,01^{a}$	$1.28 \pm 0,04^{b}$
H'_{ex}	0.68	0.577	1.05 ± 0.01^{a}	1.06 ± 0.01^{a}	1.03 ± 0.03^{a}	1.04 ± 0.03^{a}	3.36	0.031	1.05 ± 0.01^{a}	1.07 ± 0.02^{a}	1.07 ± 0.01^{a}	0.99 ± 0.03^{b}	1.08 ± 0.01^{a}	0.99 ± 0.04^{b}

[#] Microbial-C in g C m^{-2} 0–10 cm, Microbial-C/Total-C in %, [#] Mineralisable C in g CO_2-C m^{-2} 0–10 cm 10 $days^{-1}$, Dehydrogenase in mol INTF m^{-2} 0–10 cm h^{-1}, Urease in mol NH_3 m^{-2} 0–10 cm h^{-1}, β-D-glucosidase and phosphatase in mol PNP m^{-2} 0–10 cm h^{-1}, DH/Microbial-C in mol INTF kg C^{-1} h^{-1}, UR/Microbial-C in mol NH_3 kg C^{-1} h^{-1}, GL/Microbial-C in mol PNP kg C^{-1} h^{-1}, PH/Microbial-C in mol PNP kg C^{-1} h^{-1} H' and H'_{ex} in bits $unit^{-1}$

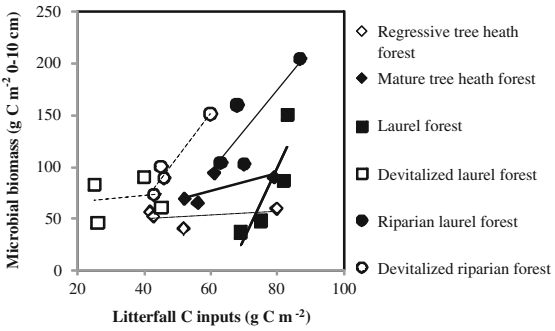

Fig. 2 Relationship between litterfall C inputs and soil microbial biomass C in the Garajonay area

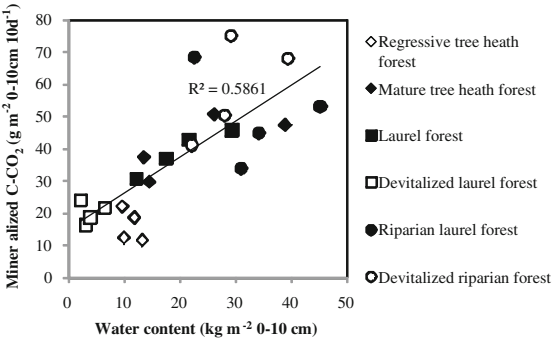

Fig. 3 Relationship between soil water content and respiration in the Garajonay area

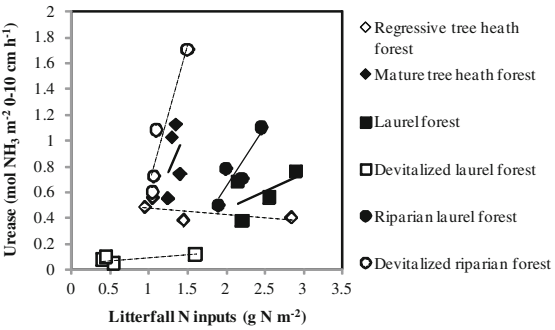

Fig. 4 Relationship between litterfall N inputs and soil urease activity in the Garajonay area

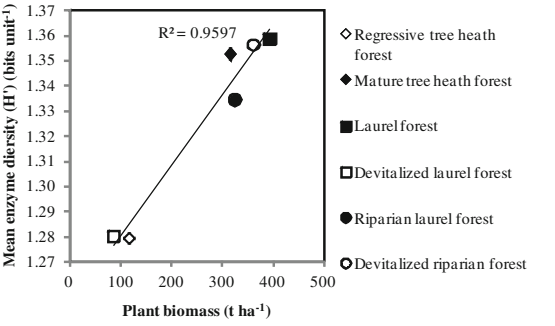

Fig. 5 Relationship between the Shannon functional diversity and the ecosystem plant biomass in the Garajonay area. Source of the plant biomass data: Rodríguez Rodríguez et al. (2002, 2004)

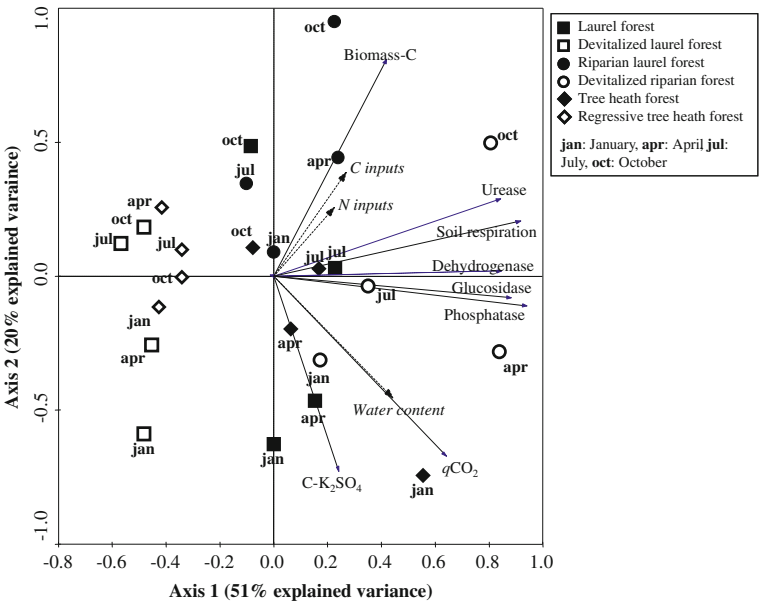

Fig. 6 Principal Component Analysis (PCA) of the soil microbiological and biochemical properties in the Garajonay area

The soils of the El Teide area (Table 3) show a highly irregular microbiological activity, although some temporal patterns can be observed: microbial biomass and urease activity were significantly greater during the first year, and in general terms, the activity is higher in summer. The maximum microbiological activity values and Shannon index scores are found at the mature scrub, intermediate under the pioneer shrub, and minimum under the pine afforestation. The relative weight of the dehydrogenase activity in the enzyme diversity is very constant (H'_{ex}/H' between 0.74–0.76), but needed to allow the differences between the sites to be significant.

The PCA of the soils of El Teide (Fig. 7) allows the relationships between the studied parameters to be inferred. Cellulase activity appears to be tightly related to the soil respiration, the highest urease activities are associated with the greatest soil humidity and microbial biomass, and the dehydrogenase, phosphatase, and, to a lesser extent, glucosidase activities, are correlated to the content of labile C forms. The first PCA axis separates the three sites according to their degree of microbiological activity, whereas the second axis discriminates the samples of the two years. The seasonal irregularity of the enzyme activities can be seen on the dispersion of the samples of the distinct seasons all over the diagram.

Table 3 Microbial Biomass C, respiration, enzyme activities and functional diversity index H' of the soils of the El Teide area (mean±standard error). Means within a row followed by the same lower case letter are not significantly different at $P = 0.05$ LSD level

Parameter[#]	$F_{d.f.=2}$	Sig.	March	July	November	$F_{d.f.=1}$	Sig.	Year		$F_{d.f.=2}$	Sig.	Broom scrub	Scabious shrubland	Pine afforestation
								1	2					
Microbial-C	1.68	0.228	13.2 ± 6.6^{a}	8.3 ± 4.2^{a}	4.4 ± 1.4^{a}	23.1	0.000	15.3 ± 4.2	2.1 ± 1.0	4.44	0.036	13.1 ± 3.2^{a}	9.3 ± 4.3^{a}	3.5 ± 1.9^{b}
Microbial-C/ Total-C	2.44	0.129	2.20 ± 1.19^{a}	0.66 ± 0.29^{a}	0.35 ± 0.11^{a}	16.5	0.002	1.94 ± 0.77	0.20 ± 0.09	0.18	0.838	0.61 ± 0.26^{a}	1.01 ± 0.55^{a}	1.59 ± 1.20^{a}
Mineralisable C	2.03	0.174	6.7 ± 1.2^{a}	12.4 ± 2.3^{a}	9.0 ± 2.6^{a}	1.05	0.327	8.2 ± 1.4	10.5 ± 2.2	1.73	0.218	12.0 ± 2.6^{a}	9.3 ± 2.2^{a}	6.7 ± 1.6^{a}
Dehydrogenase	3.95	0.048	8.2 ± 1.4^{a}	10.4 ± 1.0^{b}	7.4 ± 1.5^{a}	1.10	0.315	9.3 ± 1.2	8.0 ± 1.0	13.4	0.001	11.3 ± 1.2^{a}	9.1 ± 0.9^{b}	5.5 ± 0.8^{c}
Urease	46.5	0.000	80 ± 20^{a}	280 ± 61^{b}	68 ± 23^{a}	56.7	0.000	210 ± 51	75 ± 25	1.54	0.254	158 ± 47^{a}	155 ± 49^{a}	113 ± 42^{a}
β-D-glucosidase	0.86	0.448	33.3 ± 6.2^{a}	45.0 ± 9.9^{a}	31.9 ± 10.5^{a}	0.02	0.897	36.7 ± 8.2	36.8 ± 6.7	6.02	0.015	45.2 ± 6.2^{a}	48.3 ± 8.7^{a}	16.7 ± 5.6^{b}
Phosphatase	0.59	0.568	53.3 ± 18.7^{a}	60.2 ± 16.1^{a}	56.7 ± 15.9^{a}	0.15	0.710	60.1 ± 16.7	53.3 ± 9.0	24.2	0.000	100.0 ± 12.7^{a}	50.2 ± 6.9^{b}	20.0 ± 2.6^{c}
CM-Cellulase	0.04	0.958	2.93 ± 0.63^{a}	3.42 ± 1.00^{a}	3.96 ± 1.57^{a}	0.12	0.732	3.33 ± 1.04	3.55 ± 0.76	3.02	0.087	5.52 ± 1.34^{a}	2.41 ± 0.39^{ab}	2.38 ± 0.85^{b}
DH/Microbial-C	2.01	0.177	3.56 ± 1.72^{a}	54.7 ± 28.7^{a}	25.5 ± 23.6^{a}	22.5	0.000	1.18 ± 0.38	54.7 ± 22.5	1.38	0.290	49.7 ± 29.7^{a}	7.79 ± 6.35^{a}	26.4 ± 23.5^{a}
UR/Microbial-C	2.33	0.139	18.9 ± 6.1^{a}	802 ± 399^{a}	358 ± 333^{a}	1.11	0.313	26.2 ± 7.8	760 ± 324	2.02	0.176	123 ± 108^{a}	352 ± 335^{a}	703 ± 418^{a}
GL/Microbial-C	2.01	0.176	18.0 ± 9.4^{a}	338 ± 190^{a}	162 ± 154^{a}	25.0	0.000	3.98 ± 1.32	341 ± 149	0.97	0.407	29.6 ± 24.2^{a}	166 ± 154^{a}	322 ± 194^{a}
PH/Microbial-C	2.28	0.145	22.3 ± 10.7^{a}	307 ± 155^{a}	140 ± 128^{a}	22.7	0.000	6.11 ± 1.73	306 ± 121	0.33	0.723	55.3 ± 43.2^{a}	145 ± 127^{a}	269 ± 161^{a}
CE/Microbial-C	1.20	0.334	1.9 ± 1.2^{a}	19.2 ± 9.1^{a}	8.4 ± 7.4^{a}	19.3	0.001	0.52 ± 0.25	19.2 ± 7.0	1.12	0.358	4.5 ± 3.8^{a}	8.2 ± 7.5^{a}	16.8 ± 9.1^{a}
H'	2.32	0.141	1.22 ± 0.02^{a}	1.27 ± 0.05^{a}	1.23 ± 0.04^{a}	2.56	0.136	1.26 ± 0.04	1.22 ± 0.03	4.26	0.040	1.30 ± 0.03^{a}	1.24 ± 0.04^{ab}	1.18 ± 0.04^{b}
H'_{ex}	0.28	0.764	0.90 ± 0.04 ^{a}	0.96 ± 0.08^{a}	0.93 ± 0.06^{a}	0.56	0.470	0.96 ± 0.05	0.91 ± 0.04	1.05	0.380	1.00 ± 0.04^{a}	0.92 ± 0.05^{a}	0.88 ± 0.07^{a}

[#] *Units* Microbial-C in g C m^{-2} 0–10 cm, Microbial-C/Total-C in %, Mineralisable C in g C–CO_2 m^{-2} 0–10 cm 10 $days^{-1}$, Dehydrogenase in mmol INTF m^{-2} 0–10 cm h^{-1}, Urease in mmol NH_3 m^{-2} 0–10 cm h^{-1}, β-D-glucosidase and phosphatase in mmol PNP m^{-2} 0–10 cm h^{-1}, CM-cellulase in mmol glucose m^{-2} 0–10 cm h^{-1}, DH/Microbial-C in mol INTF kg C^{-1} h^{-1}, UR/Microbial-C in mol NH_3 kg C^{-1} h^{-1}, GL/Microbial-C in mol PNP kg C^{-1} h^{-1}, PH/Microbial-C in mol PNP kg C^{-1} h^{-1}, CE/Microbial-C in mol glucose kg C^{-1} h^{-1} H' and H'_{ex} in bits $unit^{-1}$

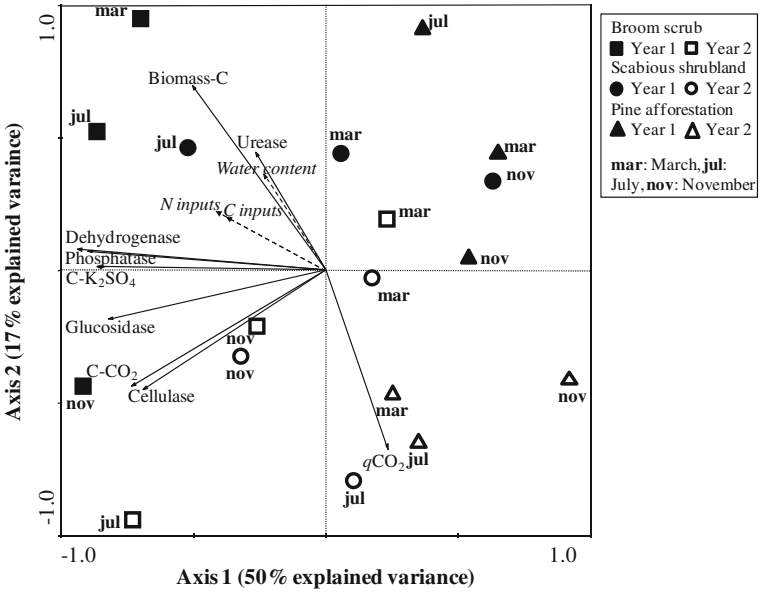

Fig. 7 Principal Component Analysis (PCA) of the soil microbiological and biochemical properties in the El Teide area

4 Discussion

The results show high and relatively stable values of the soil enzyme activities in the Garajonay area, and very low and changing values in El Teide. In general terms, significant variations of the enzyme activities are seen as related to phenological and climatic patterns and the plots representing different stages in the plant succession.

The results obtained in certain particular cases must be stressed. The soil under devitalized riparian forest shows a high microbiological dynamism which cannot be attributed to a degradation process. In this site, a progressive clearing of the tree canopy is observed, probably leading to its eventual transformation into a mesophytic laurel forest. The reason for such a phenomenon is unclear (Rodríguez Rodríguez et al. 2002, 2004), but is probably related to the water stress due to a descent of the phreatic level. Seemingly, the unusually high microbiological activity in this soil points to an accelerated transition process involving the main biogeochemical fluxes.

In turn, the soil under pine afforestation exhibits a poor microbiological functioning despite the high plant biomass of the ecosystem. Pine forests are not natural in the summit areas in the Canary Islands (Gieger and Leuschner 2004; del Arco Aguilar et al. 2006), and the deficient microbiological performance of the soil reflects a poor adaptation to the high-mountain conditions.

Table 4 Coefficients of temporal variation (%) of the soil enzyme activities and the functional diversity index H' (mean±standard error). Means within a row followed by the same lower case letter are not significantly different at $p = 0.05$ LSD level. (n.d. not determined)

	F	d.f.	Sig.	DH	UR	GL	PH	CE	H'
Garajonay	7.55	4	0.001	35.9 ± 7.0^{a}	31.7 ± 4.1^{ab}	30.9 ± 6.3^{ab}	18.4 ± 3.5^{b}	—	2.9 ± 0.9^{c}
El Teide	19.4	5	0.000	29.0 ± 3.2^{b}	95.9 ± 14.7^{a}	52.0 ± 12.8^{b}	36.1 ± 4.3^{b}	62.0 ± 14.0^{ab}	6.7 ± 1.0^{c}

The high seasonal and interannual variation observed emphasizes the need for a time-consuming temporal monitoring of the enzyme activities. Multivariate statistical analysis and elaboration of integrative indexes must also be carried out to get an adequate understanding of the soil microbiological functioning. Enzyme diversity (H') exhibits a lesser variation along the year (Table 4) than the individual enzyme measurements, often varying only moderately around a typical value characteristic of each soil, and seems to have a clear relation to the degradation processes affecting the ecosystem.

5 Conclusions

As enzymes related to different biogeochemical processes are analyzed, the Shannon index reflects the evenness of the biochemical performance of the soil. High index scores are expected in healthy soils where the activity of the enzymes is in equilibrium, whereas low scores point to degraded soils and the malfunctioning or uncoupling of one or several biogeochemical fluxes.

The measurement of the enzyme diversity by means of the Shannon Index seems to provide a good estimation of the variety of functions and ecological processes in which the soil microbiota is involved. Its temporal stability can allow obtaining a good approximation to its value from a single or few measures, in contrast to the usual long monitoring time needed in the studies of the soil enzyme activities.

References

Achtman M, Wagner M (2008) Microbial diversity and the genetic nature of microbial species. Nat Rev Microbiol 6:431–440

Andrén OJ, Bengtsson J, Clarholm M (1995) Biodiversity and species redundancy among litter decomposers. In: Collins HP, Robertson GP, Klug MJ (eds) The Significance and regulation of soil biodiversity. Kluwer, Dordrecht, The Netherlands, pp 141–151

Arbelo CD, Rodríguez Rodríguez A, Guerra JA, Mora JL (2002) Calidad del suelo y sucesión vegetal en Andosoles forestales de las Islas Canarias. Edafología 9:31–38

Armas Herrera CM (2010) Caracterización físico-química y dinámica del carbono orgánico en los suelos de algunos ecosistemas de las Islas Canarias. Unpublished Ph.D. Thesis. Departamento de Edafología y Geología, Universidad de La Laguna, Tenerife, Spain

Bending GD, Tuner MK, Jones JE (2002) Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities. Biol Fertil Soils 34:1073–1082

Caldwell BA (2005) Enzyme activities as a component of soil biodiversity: a review. Pedobiologia 49:637–644

Camiña F, Trasar-Cepeda C, Gil-Sotres F, Leirós C (1997) Measurement of dehydrogenase activity in acid soils rich in organic matter. Soil Biol Biochem 30:1005–1011

Castroviejo M (1989) El parque nacional del teide. In: Araña V, Coello J (eds) Los volcanes y la caldera del parque nacional del teide (tenerife, islas canarias). ICONA, Madrid, Spain, pp 13–36

Chapin III FS, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM, Reynolds HL, Hooper DU, Lavorel S, Sala OE, Hobbie SE, Mack MC, Díaz S (2000) Consequences of changing biodiversity. Nature 405:234–242

del Arco Aguilar MJ, Wildpret de la Torre W, Pérez de Paz PL, Rodríguez Delgado O, Acebes Ginovés JR, García Gallo A, Martín Osorio MV, Reyes Betancort JA, Salas Pascual M, Díaz MA, Bermejo Domínguez JA, González González R, Cabrera Lacalzada MV, García Ávila S (2006) Mapa de vegetación de canarias. GRAFCAN, Santa Cruz de Tenerife, Spain

Dick RP, Breakwell DP, Turco RF (1996) Soil enzyme activities and biodiversity. measurements as integrative microbiological indicators. In: Doran JW, Jones AJ (eds) Methods for assessing soil quality. Soil Science Society of America, Madison, WI, USA, pp 247–271

Doran JW, Zeiss MR (2000) Soil health and sustainability: managing the biotic component of soil quality. Appl Soil Ecol 15:3–11

Eivazi F, Tabatabai MA (1988) Glucosidases and galactosidases in soils. Soil Biol Biochem 20:601–606

Emmerling C, Schloter M, Hartmann A, Kandeler E (2002) Functional diversity of soil organisms–a review of recent research activities in Germany. J Plant Nutr Soil Sci 165:408–420

Gieger T, Leuschner C (2004) Altitudinal change in needle water relations of pinus canariensis and possible evidence of a drought-induced alpine timberline on mt. teide, tenerife. Flora 199:100–109

Griffiths BS, Ritz K, Wheatley R, Kuan HL, Boag B, Christensen S, Ekelund F, Sørensen SJ, Muller S, Bloem J (2001) An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. Soil Biol Biochem 33:1713–1722

Guitián F, Carballas T (1976) Técnicas de análisis de suelos. Pico Sacro Editorial, Santiago de Compostela, Spain

Hättenschwiler S, Tiunov AV, Scheu S (2005) Biodiversity and litter decomposition in terrestrial ecosystems. Annu Rev Ecol Evol Syst 36:191–218

Hunt HW, Wall DH (2002) Modelling the effects of loss of soil biodiversity on ecosystem function. Glob Change Biol 8:33–50

IUSS Working Group WRB (2006) World reference base for soil resources. 2nd edition. World Soil Resources Reports No. 103. FAO, Rome

Landi L, Renella G, Moreno JL, Falchini L, Nannipieri P (2000) Influence of cadmium on the metabolic quotient, L-:D-glutamic acid respiration ratio and enzyme activity:microbial biomass ratio under laboratory conditions. Biol Fertil Soils 32:8–16

Lawton JH, Brown VK (1994) Redundancy in ecosystems. In: Schulze E-D, Mooney HA (eds) Biodiversity and ecosystem function. Springer, New York, USA, pp 255–270

Lupwayi NZ, Hanson KG, Harker KN, Clayton GW, Blackshaw RE, O'Donovan JT, Johnson EN, Gan Y, Irvine RB, Monreal MA (2007) Soil microbial biomass, functional diversity and enzyme activity in glyphosate-resistant wheat-canola rotations under low-disturbance direct seeding and conventional tillage. Soil Biol Biochem 39:1418–1427

Marx M-C, Wood M, Jarvis SC (2001) A microplate fluorometric assay for the study of enzyme diversity in soils. Soil Biol Biochem 33:1633–1640

Mungai NW, Motavalli PP, Kremer RJ, Nelson KA (2005) Spatial variation of soil enzyme activities and microbial functional diversity in temperate alley cropping systems. Biol Fertil Soils 42:129–136

Myers N (1996) Environmental services of biodiversity. Proc Natl Acad Sci USA 93:2764–2769

Nannipieri P, Kandeler E, Ruggiero P (2002) Enzyme activities and microbiological and biochemical processes in soil. In: Burns RG, Dick R (eds) Enzymes in the environment: activity, ecology and applications. Marcel Dekker, New York, USA, pp 1–33

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. Eur J Soil Sci 54:655–670

Ogunseitan O (2005) Microbial diversity: form and function in prokaryotes. Blackwell, Oxford, UK

Óskarsson H, Arnalds Ó, Gunmundsson J, Gudbergsson G (2004) Organic carbon in icelandic andosols: geographical variation and impact of erosion. Catena 56:225–238

Pankhurst CE, Doube BM, Gupta VVSR (1997) Biological indicators of soil health. CAB International, Wallingford, UK

Rodríguez Rodríguez A, Fernández López AB, Arbelo CD, Notario JS, Vargas GE, Mora JL, Guerra JA, Armas CM, Amigó R (2002) Plan complementario edafológico del programa de seguimiento ecológico en el parque nacional de garajonay. Departamento de Edafología y Geología, Universidad de La Laguna, Tenerife, Spain

Rodríguez Rodríguez A, Arbelo CD, Notario JS, Mora JL, Guerra JA, Armas CM (2004) Contenido y formas de carbono orgánico en Andosoles forestales: aproximación a su dinámica. Edafología 11:67–102

Rodríguez-Loinaz G, Ornaindia M, Amezaga I, Mijangos I, Garbisu C (2008) Relationship between vegetation diversity and soil functional diversity in native mixed-oak forests. Soil Biol Biochem 40:49–60

Schinner F, von Mersi W (1990) Xylanase, CM-cellulase and invertase activity in soil: an improved method. Soil Biol Biochem 22:511–515

Schlapfer F, Schmid B (1999) Ecosystem effects of biodiversity: a classification of hypotheses and exploration of empirical results. Ecol Appl 9:893–912

Shannon CE, Weaver W (1949) The mathematical theory of communication. University of Illinois Press, Urbana IL USA

Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Tabatabai MA, Bremner JM (1972) Assay of urease activity in soils. Soil Biol Biochem 4:479–487

Ter Braak CFJ, Šmilauer P (2002) CANOCO reference manual and canodraw for windows user's guide to canoco for windows. software for canonical community ordination (version 4.5). Centre for Biometry, Wageningen, The Netherlands

Trasar-Cepeda C, Leirós MC, Gil-Sotres F, Seoane S (1998) Towards a biochemical quality index for soils: an expression relating several biological and biochemical properties. Biol Fertil Soils 26:100–106

Tscherko D, Rustemeier J, Richter A, Wanek W, Kandeler E (2003) Functional diversity of the soil microflora in primary succession across two glacier forelands in the Central Alps. Eur J Soil Sci 54:685–696

Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C. Soil Biol Biochem 19:703–707

Walker BH (1992) Biodiversity and ecological redundancy. Conserv Biol 6:18–23

Wardle DA, Huston MA, Grime JP, Berendse F, Garnier E, Lauenroth WK, Setala H, Wilson SD (2000) Biodiversity and ecosystem function: an issue in ecology. Bull Ecol Soc Am 81:235–239

Zak JC, Willig MR, Moorhead DL, Wildman HG (1994) Functional diversity of microbial communities: a quantitative approach. Soil Biol Biochem 26:1101–1108

Biochemical Characterisation of Minimally Disturbed Soils Under Mediterranean Conditions

Patricia Jiménez, Graciela Marando, Ramón Josa, Maria Julià, Marta Ginovart and Manuel Bonmatí

Abstract Urease, BAA-hydrolase, β-glucosidase and β-galactosidase enzyme activities, total and extractable carbohydrate content, organic C, basal respiration and microbial biomass carbon were determined in soils with minimal anthropogenic disturbance in Catalonia (NE Spain). Superficial layer samples of ten soils were collected in spring 2006 from different zones including a wide range of plant cover, climatic conditions and lithologic characteristics. Data were submitted to variance analysis and Student Newman Keuls test and total and partial correlation coefficients were calculated. Cluster analysis was performed to identify similarity between variables and soil locations.The studied biochemical properties were highly variable between sites. Seven of the 10 variables differed about 10-fold between lowest and highest values while soil respiration showed the lowest variation range (5-fold) and total carbohydrate content and β-galactosidase activity the highest variation range (25-fold and 35-fold respectively). The cluster analysis showed two groups of variables that were useful to biochemically characterize the ten studied soils. One group (organic C, total and extractable carbohydrates, β-glucosidase activity and microbial biomass carbon) represented the organic matter composition and microbial content and microbial activity; and another group (Urease, BAA-protease and β-galactosidase) probably represented the stabilized enzyme activity. Overall, the studied biochemical parameters were found

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to be suitable for reflecting the main differences (pH, salinity and $CaCO_3$ content) between soil type and location.

1 Introduction

Soil biological attributes refer to living organisms and their derived material; they include many soil components and processes related to organic matter cycling, enzyme activities and soil fauna and flora. These soil attributes are particularly fitting as indicators of soil quality because they respond to change, both natural and human-induced (Gregorich et al. 1997). Several soil quality indices including biological parameters are exhibited in the literature as a simple or multiparametric expression. The indicators most widely used for constructing soil quality indices are organic matter, microbial biomass C, microbial respiration and enzyme activities (Gil-Sotres et al. 2005; Bastida et al. 2008a, b). These soil quality indices are developed to describe native soils or to reflect any soil disturbance such as agricultural management, degradation or contamination.

Recently Bastida et al. (2008b) have published a review of some soil quality indices and according to these authors, more than 25 groups around the world are working in this topic. In Spain many recent publications provide knowledge about the usefulness and limitations of biological quality indices in soils under diverse geographic conditions (García et al. 1994; Trasar-Cepeda et al. 1998, 2000, 2008; Leirós et al. 1999, 2000; Gil-Sotres et al. 2005; Armas et al. 2007; Zornoza et al. 2007a, 2007b, 2008). Some of these authors (Trasar-Cepeda et al. 1998, 2000, 2008; Zornoza et al. 2007a, 2008) suggest the use of native soils as a reference for the highest soil quality because these soils represent a natural ecosystem that has reached equilibrium among key soil properties.

In this work, we characterised ten native soils with the aim of providing information about the biochemical properties of minimally anthropogenic disturbed soils of Catalonia and to study the behaviour of these parameters when describing the differences between soil type and location. Our results are part of a project of the Spanish Group of Soil Enzymology (SGSE), which seeks to elaborate a database of biochemical properties of native soils with different geographic conditions in Spain by using analytical methods standardized by the group (García et al. 2003).

2 Materials and Methods

2.1 Study Sites and Soil Sampling

Soil samples were taken from ten localities in Catalonia (NE Spain): Serra Litoral (LT), Serra del Corredor (CR), Serra del Montnegre (MN), Plana de Vic (VC), Serra de l'Ordal (OR), Conca d'Odena, Igualada (IG), La Panadella plateau (PN),

Serres dels Camps, Balaguer (BL), Serra de la Picarda, La Granja d'Escarp (LG) and Segre alluvial plane (SG). An overview of the sites, land uses employed in each site and soils characteristics are given in Tables 1 and 2. We focused on native soils under natural and autochthonous vegetation, as close as possible to potential vegetation, which had not been disturbed by human action for an undetermined number of decades. In all localities, forest and abandoned agricultural soils were distributed over wide zones in a landscape mosaic. Thus, to validate soil results, we selected four land uses: undisturbed (or low intensity disturbed), forest (f), abandoned agricultural (ab), dry grasslands (dg) and steppe (s).

A plot of 100 m^2 was defined in each site and the sample was composed of 20–25 homogeneously mixed sub samples from the A horizon (0–10 cm) after litter removal. Samples were taken on two consecutive days in spring 2006. The samples were immediately sieved to obtain fine earth (< 2 mm) and homogenized and one part was stored at 4°C prior to biochemical analysis (within 15 days of sampling). Another part was air dried for one week and stored at room temperature for the analysis of chemical and physical soil properties. A part of these samples were finely ground before analysis of inorganic carbon, organic carbon and nitrogen contents. For the analytical assays, the mean value of three or four replicates per sample was used and data was expressed in dry weight basis.

2.2 Analytical Methods

Texture analysis was determined by the Bouyoucos method (Gee and Bauder 1986). Electrical conductivity was measured in a 1:5 aqueous solution and pH in a 1:2.5 water or KCl 1 M suspension. Total carbonates were measured by Shimadzu TOC-V-Series SSM 5,000A and total N was determined using Kjeldhal's method. Soil organic carbon (SOC) was determined by potassium dichromate oxidation with Walkley–Black procedure (Nelson and Sommers 1982).

Content of total carbohydrates (TCH) was determined as reported by Cheshire and Mundie (1966) and extractable (soluble in 0.5 M K_2SO_4) carbohydrates (ECH) by Badalucco et al. (1992). Microbial biomass carbon (MBC) was determined by the fumigation-extraction method (Vance et al. 1987) and basal respiration (BR) as the CO_2 produced during 7 days of incubation at 28°C, as reported by Hernández and García (2003). The microbial metabolic quotient, qCO_2 and the ECH/TCH ratio of each soil were also calculated. For the determination of enzyme activities we used analytical methods that had been previously standardized by the SGSE group (García et al. 2003). The β-D-glucosidase (GLU) and β-D-galactosidase (GAL) activities were determined as reported by Tabatabai (1982) with calibration plots of *p*-nitrophenol prepared by using individual soil, so as to take into account the relative adsorption of *p*-nitrophenol (*p*NP) by each soil (Vuorinen 1993). Urease activity (UR) was determined by the method of Tabatabai and Bremner (1972) modified by Nannipieri et al. (1978)

Table 1 Sites and soils characteristics

Soil sample	Location	Parent material	Soil type[c]	Habitat type	Soil use type	T°C (2006)[a]	P mm (2006)[b]
LT	Litoral	Granodiorite	Cambisol	MF Qi	f	15.5	504
CR	Corredor	Shale	Ranker	MF Qs	f	13.5	461
MN	Montnegre	Granodiorite	Ranker	MF Qs	f	13.5	461
VC	Vic	Marls	Cambisol	AG	dg	12.3	564
OR	Ordal	Limestone	Luvisol	MF Pf	f	15.3	404
IG	Igualada	Marls	Cambisol	DG	ab	12.9	311
PN	Panadella	Limestone	Xerosol	MF Qi	f	12.9	311
BL	Balaguer	Gypseous marls	Xerosol	RGa	dg	14.9	237
LG	La Granja	Marls	Cambisol	SS	s	15.6	137
SG	Segre	Alluvial deposits	Fluvisol	RP	f	15.6	137

[a] 2006 average air temperature
[b] 2006 average precipitation
[c] FAO—Unesco, 1974
MF mediterranean forests, *Qi* catalo-provençal lowland holm-oak woodlands, *Qs* catalan cork-oak woodlands, *AG* aphyllanthes grasslands, *Pf* Iberian aleppo pine forests, *SS* sparto steppes, *RPr*iparian poplar galleries, *DG* dry calcareous grasslands, *RGa* rosemary garrigues, *f* forests *dg* dry grasslands, *ab* abandoned lands, *s* steppe

Table 2 Characteristics of the soils from the selected sites under study

Soil sample	Texture	Sand %	Silt %	Clay %	pH (1:2.5)	EC (1:5)	$CaCO_3$ %	Total N %	Organic C %
LT	LSa	85.8	5.7	8.5	6.95	0.064	–	0.16	3.07
CR	L	37.7	38.9	23.3	6.45	0.129	–	0.37	6.20
MN	SaL	63.9	21.7	14.4	6.45	0.092	–	0.54	7.23
VC	L	48.5	28.1	23.4	8.50	0.163	37	0.32	4.15
OR	C	3.7	34.0	62.3	8.00	0.191	–	0.35	7.81
IG	CL	28.6	33.6	37.8	8.50	0.159	64	0.17	2.31
PN	SaCL	45.2	19.9	34.9	7.80	0.243	–	0.94	10.74
BL	SaL	60.1	33.2	6.6	8.15	2.000	12	0.11	1.21
LG	CL	36.4	29.9	33.7	8.40	1.377	35	0.08	0.85
SG	SaL	72.2	15.5	12.4	8.65	0.121	33	0.11	1.82

Sa sand, *L* loam, *C* clay, *EC* electrical conductivity at 25°C in dS m^{-1}

and N-α-benzoil-L-argininamide (BAA)-protease activity (BAAP) by the method of Ladd and Butler (1972) modified by Bonmatí et al. (1998).

2.3 Statistical Analyses

A one-way ANOVA was carried out with all properties to assess the differences among soils. The comparison of means was made according SNK procedure (at the level of $\alpha = 0.05$). We calculated coefficients of correlations among the means of

the different biochemical parameters and also partial correlation analysis was carried out to measure the strength of relationship between two variables controlling for the effect of organic carbon content or microbial biomass content. In both correlation studies, sample PN was omitted because its extreme values would have skewed the results. Finally, hierarchical agglomerative Cluster Analysis was performed with the means of the variables in each soil using squared Euclidean distances as a measure of similarity. Clusters were illustrated by a dendogram tree diagram. The standardized values of the variables from the same cluster were plotted into a sunray plot with ten axes representing the ten soils. All the parameters were standardized for the cluster analysis of observations (soil locations) and also represented by a dendogram.

3 Results

3.1 Microbial and Biochemical Characteristics of Soils

The mean of the variables in the ten soils and their coefficient of variation (CV) are summarized in Table 3, whereas the results of the analysis of variance are shown in Table 4. Total carbohydrates (TCH), extractable carbohydrates (ECH) and β-galactosidase activity (GAL), with coefficient of variation higher than 90%, were the parameters presenting the highest dispersion, whereas basal respiration (BR) and β-glucosidase activity (GLU), showing a CV $< 65\%$, were the least dispersed measured variables (Table 3).

Six of the nine measured variables differed about 10-fold between lowest and highest values, while BR showed the lowest range (5-fold) and TCH and β-galactosidase activity the highest range (25-fold and 35-fold respectively). The ANOVA analyses showed that all the parameters were significantly ($P < 0.001$) influenced by soil location. By a comparison of the calculated F values, the ten variables seem to be classified into three groups: TCH, ECH and soil organic C (SOC) had the highest variability, while BR and microbial biomass C (MBC) had the lowest variability and the four hydrolyzing enzyme activities had a medium variability value. The SNK between-means-differences showed that organic C was the only variable that differentiated the ten soils. Generally, PN, MN and CR soils (being those with the higher SOC content) showed the highest values of all the studied parameters, whereas LG, BL and SG soils showed the lowest values.

The value range of our variables was similar to that of other native soils under Mediterranean conditions (Bastida et al. 2006; Zornoza et al. 2007a) but it was higher than that of denuded soils (García et al. 1994; Bastida et al. 2008a).

We found a between-site variation similar to that reported by (García et al. 1994) in 12 different soils of Murcia (SE Spain). Generally, other authors recorded lower CV because they compared soils that shared more similarities. Other authors also

Table 3 Mean and Coefficient of Variation (CV) of the studied parameters in the ten soils ranked in descending order of CV

Parameter[#]	Mean	CV %
β-D-galactosidase	0.35	97
Extractable carbohydrates	0.44	95
Total carbohydrates	7.31	93
BAA-protease	2.70	80
Urease	2.56	73
Soil organic C	4.54	73
Microbial Biomass C	813	70
β-D-glucosidase	1.71	62
Basal respiration	36.57	49
qCO_2	2.32	40
Extractable/Total carbohydrates	0.067	30

[#] Units: Soil Organic C in %, Extractable and Total carbohydrates in mg glucose g^{-1} dry soil, β-D-glucosidase and β-D-galactosidase in μmol *p*NP g^{-1} dry soil h^{-1}, Urease and BAA-protease in μmol NH_3 g^{-1} dry soil h^{-1}, Microbial biomass C in mg kg^{-1} dry soil and basal respiration in mg CO_2–C kg^{-1} dry soil day^{-1}, qCO_2 microbial metabolic quotient μg CO_2–C mg^{-1} MBC h^{-1}

found lower variation of β-glucosidase activity when this activity was compared with the other measured enzyme activities (García et al. 1994; Trasar-Cepeda et al. 1998, 2000, 2008; Zornoza et al. 2007b).

3.2 Correlation Between Properties

All the reported variables were significantly ($P < 0.001$ in most cases) and positively correlated with each other (Table 5) excepting both BR and BAA-protease activity, which did not show significant correlations either with SOC or TCH. The results of partial correlation analysis are summarized in Table 6. Any correlation between TCH and the rest of variables could be explained by their dependence on SOC content. The correlation between β-glucosidase activity and ECH disappeared when the effect of organic C was controlled; this also happened with β-glucosidase activity and other enzyme activities when MBC was held constant.

The correlations between biochemical parameters found in our study were in consonance with the general findings of other authors (García et al. 1994; Bastida et al. 2006, 2008a; Trasar-Cepeda et al. 2000; Zornoza et al. 2007b); some of these authors (García and Hernández, 1997; Trasar-Cepeda et al. 1998) found BAA-protease activity less correlated with soil organic C than the other enzyme activities.

Table 4 Results of one-factor ANOVA (soil location) for the studied parameters

Prm[#]	F value	Soil location									
		LT	CR	MN	VC	OR	IG	PN	BL	LG	SG
SOC	3,835***	3.07^{f}	6.20^{d}	7.23^{c}	4.15^{e}	7.81^{b}	2.31^{g}	10.74^{a}	1.21^{i}	0.85^{j}	1.82^{h}
ECH	2,685***	0.22^{f}	0.57^{c}	0.69^{b}	0.25^{e}	0.48^{d}	0.27^{e}	1.50^{a}	0.17^{g}	0.11^{h}	0.16^{g}
TCH	1,688***	4.13^{e}	8.60^{c}	11.87^{b}	5.19^{d}	11.64^{b}	3.33^{f}	23.00^{a}	1.81^{h}	0.99^{i}	2.51^{g}
E/T	57***	0.05^{ef}	0.07^{d}	0.06^{de}	0.05^{fg}	0.04^{g}	0.08^{c}	0.07^{d}	0.09^{b}	0.11^{a}	0.06^{de}
GLU	717***	1.34^{f}	2.37^{c}	2.84^{b}	1.89^{d}	1.73^{e}	1.15^{g}	3.83^{a}	0.53^{h}	0.39^{i}	1.06^{g}
GAL	473***	0.33^{d}	0.89^{b}	0.98^{a}	0.24^{e}	0.16^{f}	0.16^{f}	0.56^{c}	0.05^{g}	0.03^{g}	0.12^{f}
UR	888***	1.96^{e}	5.20^{b}	6.21^{a}	2.18^{d}	1.63^{f}	2.07^{de}	3.72^{c}	1.16^{g}	0.71^{h}	0.80^{h}
BAAP	601***	3.52^{b}	5.46^{a}	5.52a	2.25^{c}	0.82^{e}	1.64^{d}	5.64^{a}	0.83^{e}	0.45^{f}	0.90^{e}
MBC	78***	439^{e}	946^{c}	1344^{b}	802^{cd}	592de	741^{cd}	2170^{a}	415^{e}	338^{e}	343^{e}
BR	204***	27.0^{de}	67.8^{a}	62.5^{b}	29.7^{d}	31.7^{d}	52.9^{c}	23.5^{e}	26.5^{de}	13.9^{f}	30.2^{d}
qCO_2	13***	2.6^{bc}	3.0^{ab}	1.9b^{c}	1.5^{c}	2.3^{bc}	3.1^{ab}	0.5^{d}	2.7^{b}	1.9^{bc}	3.8^{a}

[#] *Prm* parameter, Units: *SOC* soil organic C in %, *ECH* extractable carbohydrates in mg glucose g^{-1} dry soil, *TCH* total carbohydrates in mg glucose g^{-1} dry soil, *E/T* ECH/TCH, *GLU* β-D-glucosidase in μmol *p*NP g^{-1} dry soil h^{-1}, *GAL* β-D-galactosidase in μmol *p*NP g^{-1} dry soil h^{-1}, *UR* urease in μmol NH_3 g^{-1} dry soil h^{-1}, *BAAP* baa-protease in μmol NH_3 g^{-1} dry soil h^{-1}, *MBC* microbial biomass C in mg kg^{-1} dry soil, *BR* basal respiration in mg CO_2–C kg^{-1} dry soil day^{-1}, qCO_2 microbial metabolic quotient in μg CO_2–C mg^{-1} MBC h^{-1}

*** Significant at $P < 0.001$. Means within a row followed by the same lower case letter are not significantly different at $P = 0.05$ SNK

Table 5 Correlation matrix coefficients (Pearson coefficient value) between the different soil properties determined (n = 9, sample PN omitted)

Parameter[#]	ECH	TCH	GLU	GAL	UR	BAAP	MBC	BR
SOC	0.919***	0.991***	0.878**	0.685*	0.701*	ns	0.717*	ns
ECH		0.940***	0.910***	0.868**	0.896**	0.757*	0.873**	0.810**
TCH			0.870**	0.700*	0.723*	ns	0.741*	ns
GLU				0.884**	0.893**	0.836**	0.898**	0.770*
GAL					0.983***	0.965***	0.863**	0.837**
UR						0.936***	0.933***	0.875**
BAAP							0.799*	0.786*
MBC								0.837**

[#] *SOC* soil organic C, *ECH* extractable carbohydrates, *TCH* total carbohydrates, *GLU* β-D-glucosidase, *GAL* β-D-galactosidase, *UR* urease, *BAAP* baa-protease, *MBC* microbial biomass C, *BR* basal respiration
Significant at *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; *ns* not significant

Table 6 Partial correlation coefficients (r value) for the relationships between the different soil properties determined (n = 9, sample PN omitted) controlling the SOC or the MBC effect

Parameter [#]	Carbohydrates	Microbial biomass	Enzymes
Carbohydrates	ECH-TCH, SOC ns	MBC-TCH, SOC ns	
		MBC-ECH, SOC 0.777*	
Enzymes	GLU-ECH, SOC ns	GLU- MBC, SOC 0.805*	GLU-GAL, SOC 0.810*
	GAL-ECH, SOC 0.829**	GAL-MBC, SOC 0.733*	GLU-UR, SOC 0.812*
	UR-ECH, SOC 0.896**	UR-MBC, SOC 0.866**	UR -GAL, SOC 0.968***
	GLU-TCH, SOC ns		GLU-GAL, MBC ns
	GAL-TCH, SOC ns		GLU-UR, MBC ns
	UR-TCH, SOC ns		GLU-BAAP, MBC ns
			UR -GAL, MBC 0.977***
			BAAP-GAL, MBC 0.906**
			UR- BAAP, MBC 0.882**

[#] *SOC* soil organic C, *ECH* extractable carbohydrates, *TCH* total carbohydrates, *GLU* β-D-glucosidase, *GAL* β-D-galactosidase, *UR* urease, *BAAP* baa-protease, *MBC* microbial biomass C, *BR* basal respiration
Significant at *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; *ns* not significant

3.3 Cluster Analysis

Cluster analysis was used to identify the similarity between the nine measured variables (Fig. 1). The analysis provided three groups of variables (similarity level 92%) with high internal homogeneity.

The first group was made up of three enzyme activities (β-galactosidase, urease and BAA-protease activity), the second was composed of five variables (ECH, TCH, β-glucosidase activity, MBC and SOC) whereas BR was separated from the others two groups. Sunray plots (Fig. 2) provided a graphical comparison of cluster variables behaviour when describing differences among the ten soils. Plot

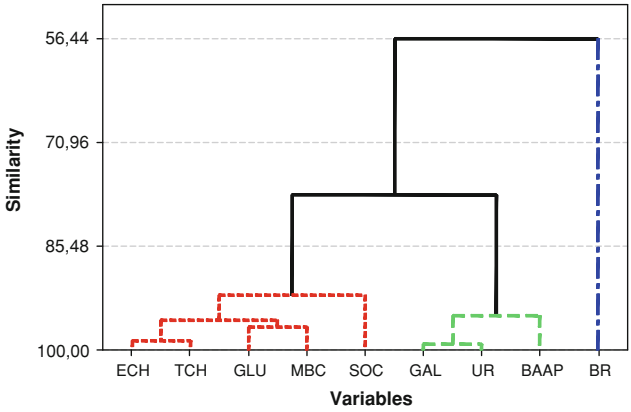

Fig. 1 Dendogram showing clustering of variables. *SOC* Soil Organic C, *ECH* Extractable carbohydrates, *TCH* Total carbohydrates, *GLU* β-D-glucosidase, *GAL* β-D-galactosidase, *UR* urease, *BAAP* BAA-protease, *MBC* Microbial biomass C, *BR* Basal respiration

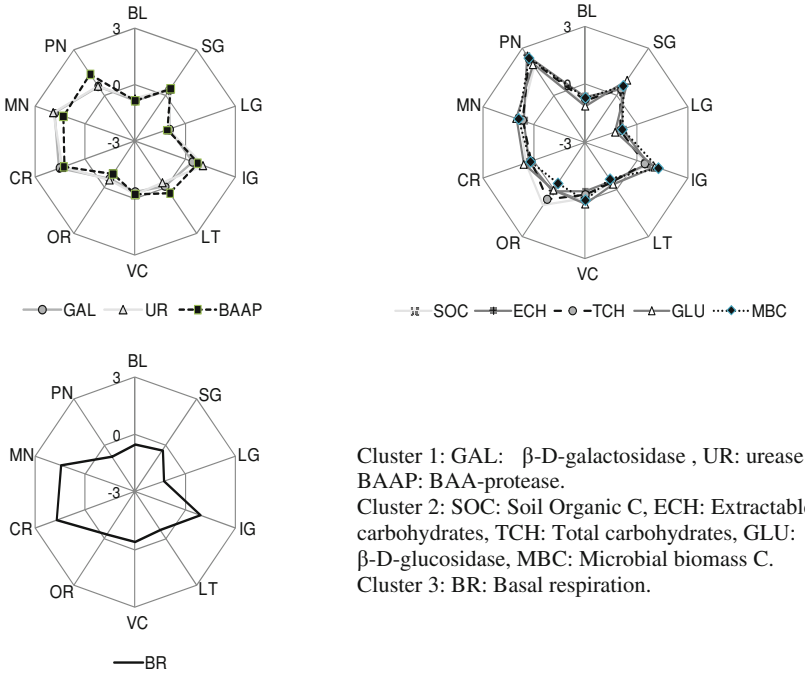

Fig. 2 Sunray plots of the standardized parameters with the ten soil locations according to the cluster analysis of variables

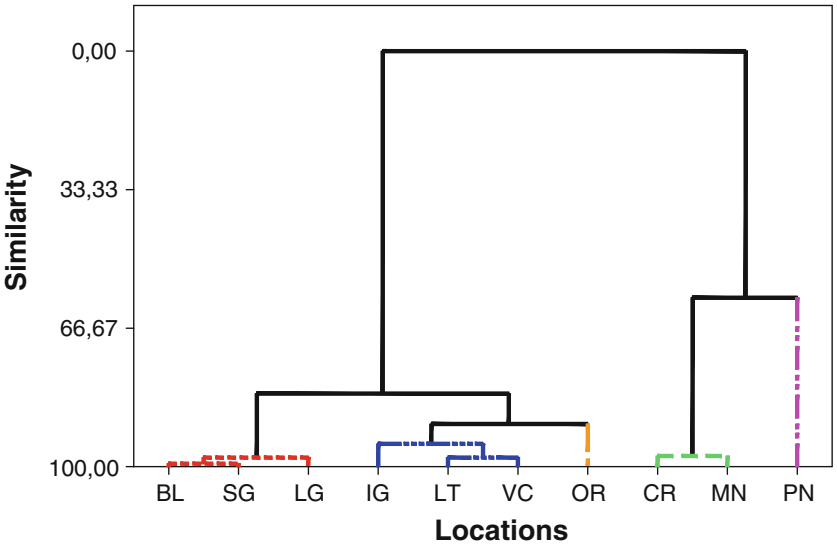

Fig. 3 Dendogram showing clustering of soil locations

displays were different for members of different clusters, whereas variables belonging to the same cluster described the soils in the same way.

When using the same similarity level (94%) than in cluster analysis of variables, cluster analysis of soil locations (observations) rendered dendogram shown in Fig. 3. The dendrogram showed five groups of soils that shared common characteristics. The first group includes BL, SG and LG soils, the second one CR and MN, the third IG, LT and VC soils and the last two groups contain only one soil, either OR or PN.

4 Discussion

4.1 Biochemical Properties Behaviour

In general, the CV values are in consonance with those obtained by analysing ANOVA F values. However, in the case of SOC and GLU, the fact that the analytical variability was so low (data not shown) made the between-site differences very high. The cluster analysis identified a group of five variables, four of which (TCH, ECH, SOC and GLU) were among those five having the higher effectiveness in distinguishing the different soils according to ANOVA analysis (the fifth was MBC). This group could represent the organic matter composition and microbial content and its activity. Another group of variables was composed of the remaining enzyme activities (GAL, UR and BAAP) probably because they

were highly correlated among them even when MBC was held constant, this probably indicating that the activities mostly corresponded to stabilized enzymes. The BR variable could not be grouped with the others, probably because it is the only variable highly dependent on ready available organic matter content (Wang et al. 2003).

4.2 Between-Site Variation

The five groups that appear in the dendogram are, on the whole, in correspondence with the field observations and the soil physicochemical properties. The first group includes soils developed on non-calcareous material (MN and CR) with pH values lower than 6.5 and with a habitat type corresponding to wet conditions. In the second group we can find only one soil (PN) developed from calcareous rock and located in a dry area but with wet microclimate conditions which allow the development of a deep organic horizon. The third group (BL, SG and LG) corresponds to the most arid part of the area; of this group, BL and LG are gypsum or saline soils and SG is a fluvisol exposed to yearly flooding. Theoretically, the forth group (IG, LT and VC) would consist only on calcareous soils belonging to the Central Depression, which means that the presence of LT soils is anomalous; the LT soil is usually found in more humid conditions and has a non-calcareous parent material which is typical of that described in the first group. The inclusion of LT soil in the fourth group can be explained by the specific meteorological conditions of 2006 year in this area (hotter and higher potential evapotranspiration value) and the physical characteristic of this soil (86% sand).

Close to this previous cluster we could find the last group integrated just for OR soil that was also developed on calcareous rock. The position of OR soil in the dendogram, between calcareous and non-calcareous groups, can be explained by the soil formation process because this luvisol is a typical Mediterranean red soil with a decarbonated A horizon over a B carbonated horizon.

5 Conclusions

The studied biochemical properties were found highly variable between sites, nevertheless two groups of variables were useful to biochemically characterize the ten studied soils; one group, which includes the higher effectiveness in distinguishing the different soils (SOC, ECH, TCH, BMC and GLU) was directly related with their organic matter and microbial content and their activity and the other group would be represented by stabilized enzyme activities (GAL, UR and BAAP). Overall, the studied biochemical parameters were found to be suitable for reflecting the main differences (pH, salinity and $CaCO_3$ content) between soils; nevertheless, the exceptional dry conditions of the sampling year and the local

microclimate or the flooding frequency of some sampling zones increased the overall variability.

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References

Armas CM, Santana B, Mora JL, Notario JS, Arbelo CD, Rodríguez-Rodríguez A (2007) A biological quality index for volcanic andisols and aridisols (Canary Islands, Spain): Variations related to the ecosystem degradation. Sci Total Environ 378:238–244

Badalucco L, Gelsomino A, Dell'Orco S, Grego S, Nannipieri P (1992) Biochemical characterization of soil organic compounds extracted by 0.5 M K_2SO_4 before and after chloroform fumigation. Soil Biol Biochem 24:569–578

Bastida F, Moreno JL, Hernández T, García C (2006) Microbiological degradation index of soils in semiarid climate. Soil Biol Biochem 38:3463–3473

Bastida F, Barbera GG, García C, Hernández MT (2008a) Influence of orientation, vegetation and season on soil microbial and biochemical characteristics under semiarid conditions. Appl Soil Ecol 38:62–70

Bastida F, Zsolnay A, Hernández MT, García C (2008b) Past, present and future of soil quality indices: A biological perspective. Geoderma 147:159–171

Bonmatí M, Ceccanti B, Nannipieri P (1998) Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biol Biochem 30:2113–2125

Cheshire MV, Mundie CM (1966) The hydrolytic extraction of carbohydrates from soil by sulphuric acid. J Soil Sci 17:372–381

Unesco FAO- (1974) Soil Map of the World 1:5000000, Vol I, Legend. Unesco, Paris, France

García C, Hernández T, Costa F (1994) Microbial activity in soils under mediterranean environmental conditions. Soil Biol Biochem 26:1185–1191

García C, Hernández T (1997) Biological and biochemical indicators in derelict soils subject to erosion. Soil Biol Biochem 29:171–177

García C, Gil F, Hernández MT, Trasar C (2003) Técnicas de análisis de parámetros bioquímicos en suelos: Medida de actividades enzimáticas y biomasa microbiana. Editorial Mundi-Prensa, Madrid, Spain

Gil-Sotres F, Trasar-Cepeda C, Leirós MC, Seoane S (2005) Different approaches to evaluating soil quality using biochemical properties. Soil Biol Biochem 37:877–887

Gee GM, Bauder JW (1986) Particle-size analysis. In: Klute A (ed) Methods of soil analysis. part 1. Physical and mineralogical methods. American society of agronomy, Madison, WI USA, pp 383–411

Gregorich EG, Carter MR, Doran JW (1997) Biological attributes of soil quality. In: Gregorich EG, Carter MR (eds) Soil quality for crop production and ecosystem health. Elsevier, Amsterdam, The Netherlands, pp 81–113

Hernández T, García C (2003) Estimación de la respiración microbiana del suelo. In: García C, Gil F, Hernández MT, Trasar C (eds) Técnicas de análisis de parámetros bioquímicos en suelos: Medida de actividades enzimáticas y biomasa microbiana. Editorial Mundi-Prensa, Madrid, Spain, pp 311–345

Ladd JN, Butler JH (1972) Short-term assays of soil proteolytic activities using proteins and dipeptide derivates as substrate. Soil Biol Biochem 4:19–30

Leirós MC, Trasar-Cepeda C, García-Fernández F (1999) Defining the validity of a biochemical index of soil quality. Biol Fertil Soil 30:140–146

Leirós MC, Trasar-Cepeda MC, Seoane S, Gil Sotres F (2000) Biochemical properties of acid soils under climax vegetation (Atlantic oakwood) in area of the european temperate-humid zone (Galicia, NW Spain): General parameters. Soil Biol Biochem 32:733–747

Nannipieri P, Johnson RL, Paul EA (1978) Criteria for measurement of microbial growth and activity in soil. Soil Biol Biochem 10:223–229

Nelson DW, Sommers LE (1982) Total carbon, organic carbon and organic matter. In: Page AL, Miller RH, Keeney DR (eds) Methods of Soil Analysis Part 2: Chemical and Microbiological Properties. Soil Science Society of America, Madison, WI USA, pp 570–571

Tabatabai MA, Bremner JM (1972) Assay of urease activity in soil. Soil Biol Biochem 4:479–487

Tabatabai MA (1982) Soil Enzymes. In: Page AL, Miller RH, Keeney DR (eds) Methods of Soil Analysis. Part 2. Chemical and microbiological properties. Soil Science Society of America, Madison, WI USA, pp 903–947

Trasar-Cepeda C, Leirós MC, Gil-Sotres F, Seoane S (1998) Towards a biochemical quality index for soils: An expression relating several biological and biochemical properties. Biol Fertil Soil 26:100–106

Trasar-Cepeda C, Leirós MC, Gil Sotres F (2000) Biochemical properties of acid soils under climax vegetation (Atlantic oakwood) in area of the European temperate-humid zone (Galicia, NW Spain): Specific parameters. Soil Biol Biochem 32:747–755

Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Hydrolytic enzyme activities in agricultural and forest soils. Some implications for their use as indicators of soil quality. Soil Biol Biochem 40:2146–2155

Zornoza R, Mataix-Solera J, Guerrero C, Arcenegui V, García-Orenes F, Mayoral AM, Mataix-Beneyto J, Morugán A (2007a) Evaluation of soil quality using multiple lineal regression based on physical, chemical and biochemical properties. Sci Total Environ 378:233–237

Zornoza R, Guerrero C, Mataix-Solera J, Guerrero C, Arcenegui V, Mayoral AM, Morales J, Mataix-Beneyto J (2007b) Soil properties under natural forest in the Alicante province of Spain. Goederma 142:334–341

Zornoza R, Mataix-Solera J, Guerrero V, Arcenegui V, Mataix-Beneyto J, Gómez I (2008) Validating the effectiveness and sensitivity of two soil quality indices based on natural forest soils under Mediterranean conditions. Soil Biol Biochem 40:2079–2087

Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass. Soil Biol Biochem 19:703–707

Vuorinen AH (1993) Requirement of *p*-nitrofenol standard for each soil. Soil Biol Biochem 25:295–296

Wang WJ, Dalal RC, Moody PW, Smith CJ (2003) Relationships of soil respiration to microbial biomass, substrate availability and clay content. Soil Biol Biochem 35:273–284

Seasonal Evolution of Soil Dehydrogenase Activity at Two Different Depths in an Eucalyptus Stand and a Cultivated Field

José Manuel Mirás Avalos and Patricia Sande Fouz

Abstract Dehydrogenase activity is a good indicator of changes in fertility, quality and microbiological status of the soil, which may vary seasonally. The aim of this study was to evaluate the seasonal evolution of this activity in two different soils (a cultivated field and an Eucalyptus stand) located in a periurban area of Coruña (NW Spain). From April 2004 to April 2005, a total of 38 soil samples were collected in the forest site and 152 samples in the cultivated field (two replicates at the top and the bottom of the hillslope at two depths: 0–5 cm and 5–10 cm). General soil properties were analyzed by routine methods, soil respiration under laboratory conditions and dehydrogenase activity using soluble tetrazolium salts. This activity ranged from $5\ 10^{-5}$ to 0.033 μg TPF g^{-1} soil 24 h^{-1} among individual samples in the forest, whereas it varied from 0.005 to 0.023 μg TPF g^{-1} soil 24 h^{-1} in the cultivated field. No differences with depth were observed in the cultivated field; whereas in the forest soil, higher activities were found in the top layer, which combined better conditions for microbial development. Dehydrogenase activity was affected by position and season in the cultivated field; higher values were detected in the top of the hillslope. In the forest site, higher values were observed in the top layer during spring and fall; due to favourable temperature and moisture conditions. In the cultivated field, dehydrogenase activity was correlated to soil basal respiration and soil pH, though, in the forest soil, no significant correlations were observed.

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1 Introduction

The term "soil quality" has been coined to describe the combination of chemical, physical, and biological characteristics that enables soils to perform a wide range of functions (Doran and Jones 1996). The assessment of soil quality requires quantification of critical soil attributes. This assessment should be made in high and low productivity areas to establish their specific ranges of values. Thus, changes occurring over time can be measured to evaluate effects of different practices, land uses or degradation stages.

Visser and Parkinson (1992) suggested that the most useful biological and biochemical properties for detecting the deterioration of soil quality should be those most closely related to nutrient cycles. These properties include soil respiration, microbial biomass, nitrogen mineralization capacity and enzymatic activities, which are especially significant in soil quality assessments because of their major contribution to the soil ability to degrade organic matter (Schloter et al. 2003) and because they are intimately involved in the nutrient cycling, affecting fertilizer efficiency and reflecting in part the microbial activity in the soil (Jordan et al. 1995).

Land use activities, particularly those related to agricultural practices and forestry can have a considerable impact on the size and activity of soil microbial communities and on the biological health of soils (Kirchner et al. 1993). Therefore, comparing enzymatic activities in disturbed and undisturbed sites may improve the knowledge on the effects of soil management on the dynamics of microbial communities and their activities, allowing to assess the soil quality and fertility status and the sustainability of the land management (Matinizadeh et al. 2008).

Dehydrogenase activity (DhA) is considered a key parameter for a rapid determination of changes in soil fertility. It is an indicator of the microbial redox system, hence, it is regarded as a good estimator of the soil oxidative activities and a general indicator of soil microbial activity (Nannipieri et al. 1990). This idea is supported by the correlation observed between dehydrogenase activity and soil respiration (von Mersi and Schinner 1991; Leirós et al. 2000), although this does not imply that this activity constitute an estimation of the number of microorganisms (Casida et al. 1964). It can also be regarded as a measure of the intensity of microbial metabolism in soil (Schloter et al. 2003).

Moreover, soil DhA has been proposed as a biochemical indicator of soil degradation (García and Hernández 1997; García et al. 1997) since the microorganisms responsible for this activity are the most active in the soil microbial community; being one of the first activities affected when soil is subjected to erosion. Not only degradation processes affect soil dehydrogenase activity, but also fertilizer application (Melero et al. 2007; Lalfakzuala et al. 2008) and tilling (Diosma et al. 2003).

Accordingly, the primary objective of this study was to investigate soil DhA in a cultivated field and a forest stand in a periurban area of A Coruña (NW Spain) as

affected by seasonal variation, soil depth and soil properties. A secondary objective of this study was to compare dehydrogenase activities in both sites.

2 Materials and Methods

The studied sites are located at A Zapateira, a periurban area of A Coruña (NW Spain). Two different soils were surveyed: a cultivated one and a soil under *Eucalyptus* vegetation. Both fields presented a high slope (16%, approximately) and were over a granite bedrock, but they were not neighbouring soils although both were classified as Umbrisols (FAO-ISRIC-ISSS, 1998). Long-term (30 years) mean annual temperature and rainfall for this area are 14.4°C and 1,008 mm, respectively. Yearly rainfall distribution is uneven with water surplus in the winter months and water deficits in summer. During the study period, mean annual temperature was 13.64°C and total rainfall was 876 mm. The cultivated topsoil horizon is sandy-loam textured with 61.78% sand, 19.58% silt and 18.64% clay. The soil from the *Eucalyptus* stand is also sandy-loam textured with 65.92% sand, 16.77% silt and 17.31% clay.

The cultivated field was conventionally tilled following a common rotation system in the region consisting of winter cereal and potatoes. Duplicate soil samples, each of which a composite of several soil cores, were collected on 19 successive dates between April 2004 and April 2005. In the cultivated field, two different depths were sampled (0–5 and 5–10 cm) in the upper and lower side of the hillslope. Thus, a total of 152 individual samples were analyzed over the study period. In the case of the forest soil, samples were collected on the same dates and depths as those of the cultivated field but no replicates were considered, hence, a total of 38 individual samples corresponded to the forest soil.

Soil wet samples were sieved through a 2 mm sieve. The general properties of the soil such as pH (H_2O), pH (KCl), organic carbon and nitrogen contents, and texture were determined by routine methods (MAPA 1994). Briefly, soil pH was measured in water (1:2.5, w/v) and in a 0.1 N KCl solution (1:2.5, w/v); particle size distribution was determined by the pipette method; soil water content was measured gravimetrically. Organic carbon and nitrogen contents were measured by dry combustion using a ThermoQuest Flash EA1112 Analyzer.

Soil basal respiration was estimated in laboratory conditions as described by Hernández and García (2003). In summary, soil sample (50 g) was put into hermetic lock jars with flasks containing 10 mL of 0.1 N NaOH to react with the CO_2 produced by microbial respiration, in a culture chamber for a four-day incubation period at 28°C. The biological activity was determined using three replicates per sample. Once the incubation was complete, unreacted NaOH was back-titrated with 0.1 N HCl after $BaCl_2$ addition to precipitate carbonates. Results are expressed as mg CO_2–C g^{-1} dry soil 4 d^{-1}.

Soil DhA determination is based on the use of soluble tetrazolium salts as artificial electron-acceptors. The evaluation of this enzymatic activity was carried

out by extraction and colorimetric determination of the final product, triphenylformazan (TPF) in methanol (Casida et al. 1964) with the modifications described by Mirás Avalos et al. (2007). Dehydrogenase activity was assessed by duplicate. Results are expressed as μg TPF g^{-1} soil 24 h^{-1}.

Significant differences between soil dehydrogenase activity as a function of topographic location (only in the case of the cultivated field), depth and season were analyzed using the ANOVA test. Pearson's r correlation coefficient was used to assess the significance of correlations between variables. Mean comparison was performed using the Student's *t* test. In all cases, differences were considered significant at $\alpha = 0.01$. Statistical analyses were carried out using R software, version 2.7.2 (R Development Core Team 2008).

3 Results

Average pH (H_2O) values were higher in the lower zone of the cultivated field (4.89) than in the upper zone (4.82). The same occurred in the case of pH (KCl) average values (Table 1). Other soil properties that showed the same behaviour, namely their average values were greater in the lower than in the upper zone of the cultivated site, were soil water content (15.62% and 14.91%, respectively), nitrogen content (0.22% and 0.18%, respectively), carbon content (2.6% and 2.09%, respectively) and C/N ratio (12.03 and 12, respectively).

On the contrary, soil basal respiration and dehydrogenase activity presented a different pattern. In the case of basal respiration, average values were similar in both areas (Table 1). Dehydrogenase activity was slightly higher in the upper side of the cultivated field (0.014 μg TPF g^{-1} soil 24 h^{-1}) than in the lower side (0.012 μg TPF g^{-1} soil 24 h^{-1}).

In the case of the forest soil, the main differences on the variables were observed between depths. Average pH (H_2O) values were higher in the 5–10 cm layer than in the 0–5 cm layer (Table 1). The same occurred with average pH (KCl) values. Nevertheless, the rest of the variables showed the opposite behaviour and their average values were higher in the top layer than in the 5–10 cm layer (Table 1). Soil basal respiration was significantly higher in the 0–5 cm layer (0.41 mg CO_2–C g^{-1} dry soil 4 d^{-1}) than in the 5–10 cm layer (0.13 mg CO_2–C g^{-1} dry soil 4 d^{-1}) (Table 1). A similar behaviour was observed in the case of DhA, being the average value for the 0–5 cm layer (0.014 μg TPF g^{-1} soil 24 h^{-1}) significantly higher than that of the 5–10 cm layer (0.007 μg TPF g^{-1} soil 24 h^{-1}).

An ANOVA was performed for all the variables and factors using the ensemble of data from the cultivated and the forest soils. Results from this analysis showed that season, depth and location on the hillslope affected the values of the variables considered (p-values < 0.01).

When only the cultivated field dataset was taken into account, ANOVA showed significant differences for DhA among positions in the hillslope (p-value $= 0.005$), being higher those activities at the upper side of the hillslope. In addition, season

Table 1 Statistical summary for the different analysed variables at cultivated upper and lower zones and at the 0–5 and 5–10 cm layers of the forest soil. SD = Standard deviation

	Cultivated upper zone			Cultivated lower zone			Forest soil (0–5 cm)			Forest soil (5–10 cm)		
Parameter	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
pH (H_2O)	38	4.82	0.22	38	4.89	0.27	18	3.88	0.27	19	3.91	0.23
pH (KCl)	38	4.06	0.17	38	4.31	0.21	18	3.12	0.16	19	3.49	0.20
Soil respiration	38	0.07	0.03	38	0.06	0.03	18	0.41	0.19	19	0.13	0.06
Water content	38	14.91	5.33	38	15.62	5.40	18	30.73	13.41	19	21.74	8.42
N content	38	0.18	0.06	38	0.22	0.05	18	1.21	0.32	19	0.81	0.21
C content	38	2.09	0.67	38	2.60	0.58	18	22.78	7.25	19	12.44	3.58
DhA	38	0.014	0.004	38	0.012	0.004	18	0.014	0.008	19	0.007	0.004
C/N	38	12.00	0.85	38	12.03	0.99	18	17.80	2.72	19	15.85	1.34

Units: Water content in %, N content in %, C content in %, Soil respiration in mg CO_2–C g^{-1} dry soil 4 d^{-1} and DhA in μg TPF g^{-1} dry soil 24 h^{-1}

Table 2 Statistical significances obtained from the ANOVA for the DhA

Factor	All datasets	Cultivated soil	Forest soil
Season	**	***	ns
Depth	*	ns	**
Localization	**	**	–
Season × depth	ns	ns	ns
Season × localization	ns	ns	–
Depth × localization	***	ns	–
Season × depth × localization	ns	ns	–

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, *ns* non-significant

proved to have a significant effect on the values of dehydrogenase activity (p-value < 0.001). However, no differences were observed regarding depths (p-value $= 0.782$). No interaction effects among depth, season and position were observed (Table 2). Regarding the forest soil, ANOVA showed significant differences with depth (p-value $= 0.003$), whereas season did not have an effect on the values of dehydrogenase activity (p-value $= 0.623$). No interaction effects between these two factors were detected (Table 2). The rest of the variables showed a similar behaviour. Then, the following datasets were distinguished: cultivated soil from the upper hillslope, cultivated soil from the lower hillslope, forest soil from the 0–5 cm layer, and forest soil from the 5–10 cm layer.

Accounting for the seasons, the highest average value for the forest soil was observed in autumn at the 0–5 cm layer, whereas that of the cultivated field was observed in winter at the upper zone of the hillslope (Table 3). The highest values of DhA in the 5–10 cm layer of the forest soil were observed in winter (Table 3). In the case of the lower zone of the cultivated hillslope, DhA average values increased from spring to winter (Table 3). Mean comparisons showed significant

Table 3 Average seasonal values of DhA (μg TPF g^{-1} soil 24 h^{-1}) for each topographical position at the cultivated field and for each depth in the forest soil

Season	Cultivated upper zone	Cultivated lower zone	Forest (0–5 cm)	Forest (5–10 cm)
Spring	0.0116 (±0.0025)[a]	0.0096 (±0.0035)[a]	0.0149 (±0.0092)[a]	0.0064 (±0.0041)[b]
Summer	0.0138 (±0.0031)[a]	0.0108 (±0.0039)[b]	0.0116 (±0.0065)[a,b]	0.0050 (±0.0034)[c]
Autumn	0.0135 (±0.0051)[a]	0.0130 (±0.0032)[a]	0.0175 (±0.0128)[a]	0.0071 (±0.0051)[b]
Winter	0.0160 (±0.0056)[a]	0.0144 (±0.0051)[a]	0.0118 (±0.0019)[b]	0.0103 (±0.0049)[b]

Standard deviation values are showed in parenthesis. Means within a row followed by the same lower case letter are not significantly different at $P = 0.01$ level

differences between DhA values and differences within the seasons among the sample locations. The lowest values were always observed in the 5–10 cm layer of the forest soil (Table 3).

Dehydrogenase activity in the upper and lower parts of the cultivated soil followed a similar temporal evolution (Fig. 1). The top layer (0–5 cm) of the forest soil presented greater values of this enzymatic activity whereas the layer from 5 to 10 cm showed the lowest values. Regarding the cultivated field, a similar seasonal pattern was observed between DhA and soil water content. On the contrary, no similar pattern with soil basal respiration was observed. A correlated effect between DhA and temperature was not clear (data not shown). Greater DhA values were measured when minimum temperatures were higher than 10°C, both in the upper and the lower side of the slope.

In the case of the forest, a similar seasonal evolution was observed between the DhA and soil basal respiration in the 0–5 cm layer (Fig. 2). Although peaks of DhA were observed when basal respiration was higher, no significant correlation was detected between these two variables, this may be due to the low amount of data. In the case of the 5–10 cm layer, seasonal patterns for these variables were different. In addition, DhA and soil water content showed a rather similar seasonal evolution in the top layer (0–5 cm), however, their behaviour was different in the 5–10 cm layer (Fig. 3). In the case of the top layer, some peaks of DhA were detected when soil water content was high. No similar patterns with climatic variables were observed (data not shown). Considering the cultivated soil data separately, significant linear correlations were found between DhA and soil respiration, pH (H_2O) and pH (KCl). In the case of the forest soil, significant correlations were observed between DhA and soil respiration, C and N content (Table 4).

Considering the four groups of data separately, significant linear correlations between DhA and the general soil properties were found only for the following combinations: pH (H_2O) in the cultivated upper hillslope; respiration, pH (H_2O) and pH (KCl) in the cultivated lower hillslope. For the top layer of the forest soil, a significant correlation was observed between DhA and soil carbon content. No significant relationships between DhA and soil general properties were found in the 5–10 cm layer of the forest soil. No significant correlations were observed

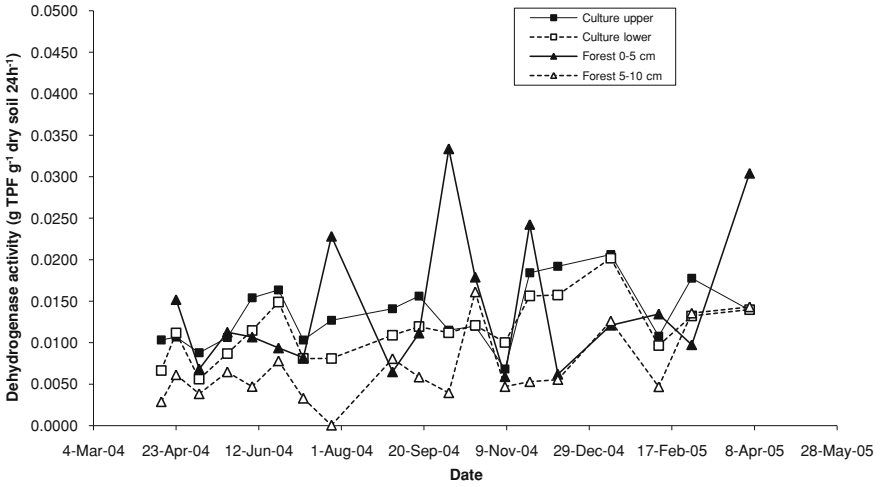

Fig. 1 Seasonal evolution of DhA in the four distinguished groups of sites: *upper* and *lower parts* of the cultivated soil, 0–5 and 5–10 cm layers of the forest soil

between soil DhA and climatic variables such as rainfall and temperature for any of the datasets (data not shown).

4 Discussion

In our study, the forest site presented higher values of soil basal respiration and total C and N contents when compared to the cultivated field. However, dehydrogenase activities were similar in both sites; this differed from that observed by other authors who found greater dehydrogenase activities in undisturbed sites compared to disturbed ones (Matinizadeh et al. 2008).

Significant seasonal differences in soil basal respiration were observed in the cultivated field, in accordance with temperature and soil water contents. Also differences in soil dehydrogenase activities along the seasons were detected in the cultivated site. However, these differences were not significant in the case of the forest soil, probably due to the high contents in organic matter observed in this site.

The decrease observed in DhA with depth in the forest soil may be related to the greater organic carbon content found in the top layer, similarly explained by other authors (Matinizadeh et al. 2008) who also suggested the higher abundance of soil microorganisms in the top layer as a factor for this decrease. Especially, forest soil surfaces tend to have a higher organic matter content compared to other systems such as agricultural and pasture soils (Lavahun et al. 1996). These results were in accordance with those previously observed by other researchers under different conditions (Aon et al. 2001; Taylor et al. 2002; Zaman et al. 2002). In addition, pH in the 5–10 cm layer was higher than that measured at the topsoil; this should have influenced dehydrogenase activity.

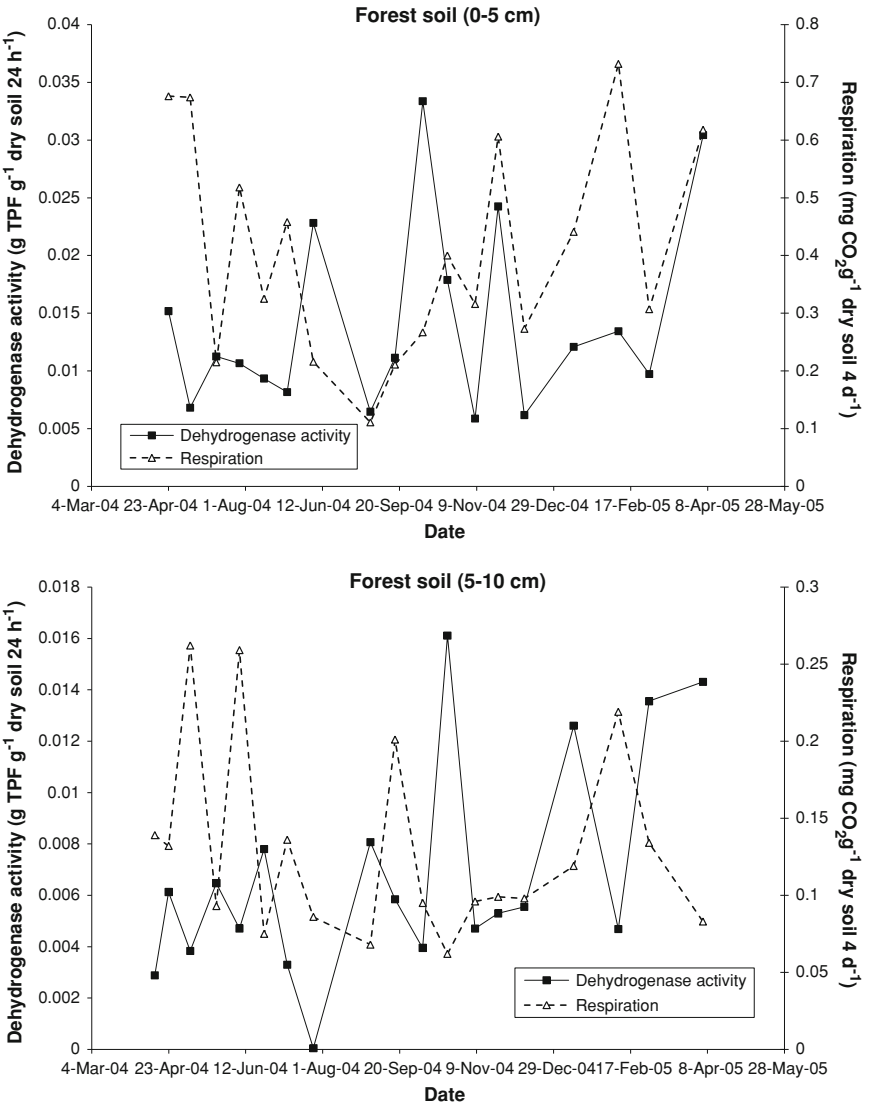

Fig. 2 Seasonal evolution of DhA and soil basal respiration at the two studied depths in the forest soil. *Upper panel* 0–5 cm layer and *lower panel* 5–10 cm layer

Moreover, differences between dehydrogenase activities were observed between cultivated and forest sites. The 5–10 cm layer of the forest soil always showed the lowest values for this activity. However, cultivated sites and forest top layer did not present significant differences in most of the seasons. This fact seems to indicate that DhA of the cultivated field is favoured by fertilizer application, as

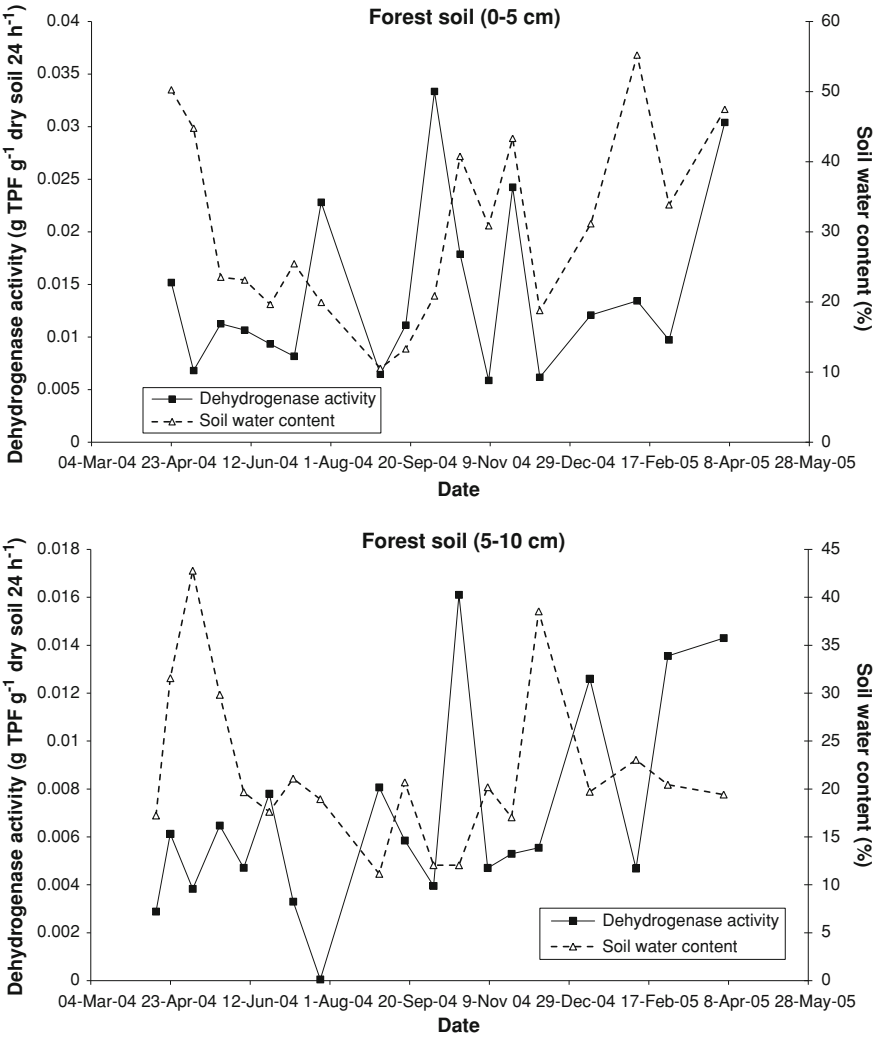

Fig. 3 Seasonal evolution of DhA and soil water content at the two studied depths in the forest soil. *Upper panel* 0–5 cm layer and *lower panel* 5–10 cm layer

suggested by Acosta and Paolini (2005). Nevertheless, cultivation techniques do not alter the soil microbial activity over short periods of time (Diosma et al. 2003; Melero et al. 2007) and it has been found that DhA depends more on the type of soil than on the management practices (Beyer et al. 1993; Burket and Dick 1998).

The lowest values of soil basal respiration in the cultivated field were related to low soil water contents. DhA values were not directly related to soil water contents in both the cultivated and the forest sites. However, a similar seasonal dynamics was observed between both variables.

Table 4 Correlation coefficients for the DhA versus the rest of the variables considered in this study and for all the groups of data

	Soil basal respiration	pH (H_2O)	pH (KCl)	Humidity	N	C
Cultivated soil	0.25**	0.60**	0.18*	−0.09	−0.10	−0.05
Forest soil	0.41**	0.27	−0.30	0.29	0.47**	0.53**
Cultivated upper side	0.17	0.61**	0.20	−0.11	−0.08	−0.04
Cultivated lower side	0.29**	0.70**	0.49**	−0.04	0.03	0.12
Forest soil (0–5 cm)	0.17	0.42	−0.13	0.25	0.45	0.48*
Forest soil (5–10 cm)	−0.33	0.21	0.45*	−0.24	−0.35	−0.34

$^{**}P < 0.01$, $^{*}P < 0.05$, *ns* non-significant

Relatively high DhA values during winter months were the consequence of the combination of soil water contents close to saturation and mild temperatures, which is illustrated by the fact that mean temperatures between December 2004 and February 2005 were above 10°C and averaged minimum monthly temperatures were above 7°C. A significant correlation between maximum temperatures and soil basal respiration was found in the lower zone of the cultivated field but not in the upper zone. However, this relation was not observed for the dehydrogenase activity in the present study.

No similar seasonal patterns between DhA and climatic variables have been observed. However, it was shown that a certain temperature range affected dehydrogenase activities in the cultivated field, namely that higher dehydrogenase values were observed when minimum temperatures were above 10°C. A similar pattern was observed in the forest soil.

In the cultivated field, the correlation between soil pH and DhA was statistically significant in both locations of the hillslope. In the lower side of the cultivated field, a significant correlation with soil basal respiration was found. However, no correlation with soil water content was detected, although a similar seasonal pattern was observed between both variables. These results seem to indicate that DhA, basal respiration and soil water content are interrelated; however, another factor may act in their seasonal dynamics.

The lack of correlation between soil general properties and DhA in the case of the forest stand may be regarded as a consequence of the low amount of data considered in this study. In fact, a negative relation of DhA with soil pH may be suggested.

5 Conclusions

- Dehydrogenase activity in the cultivated field was higher at the top than at the bottom of the hillslope. However, no differences with depth were found, likely due to similar carbon and water contents between the surveyed depths. In the forest soil, DhA was higher in the top layer.

- This activity was strongly influenced by soil pH in the cultivated field. Seasonal differences in DhA seemed to be caused by pH, nitrogen and carbon contents variations during the year and not by climatic factors.
- The lack of correlation between soil general properties and DhA in the case of the forest stand may be regarded as a consequence of the low amount of data considered in this study. Under the studied conditions, a negative relation between soil dehydrogenase activity and pH is suggested. Temperature and humidity are suspected to affect soil DhA.
- The surveyed cultivated area can be rated as a field with moderate to low soil biological activity.

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References

Acosta Y, Paolini J (2005) Actividad de la enzima deshidrogenasa en un suelo Calciorthirds enmendado con residuos orgánicos. Agron Trop 55:217–232

Aon MA, Cabello MN, Sarena DE, Colaneri AC, Franco MG, Burgos JL, Cortaza S (2001) Spatio-temporal patterns of soil microbial and enzymatic activities in an agricultural soil. Appl Soil Ecol 18:239–254

Beyer L, Wachendorf C, Elsner DC, Knabe R (1993) Suitability of dehydrogenase activity assay as an index of soil biological activity. Biol Fertil Soils 16:52–56

Burket JZ, Dick RP (1998) Microbial and soil parameters in relation to N mineralization in soils of diverse genesis under differing management systems. Biol Fertil Soils 27:430–438

Casida LE Jr, Klein PA, Santoro T (1964) Soil dehydrogenase activity. Soil Sci 98:371–376

Diosma G, Golik SI, Chidichimo HO, Balatti PA (2003) Nitrification potential, dehydrogenase activity and microbial biomass in an argiudol soil cultivated with wheat under two tillering methods. Span J Agric Res 1:111–119

Doran JW, Jones AJ (1996) Methods for assessing soil quality. Soil Science Society of America, Publication No. 49, Madison, WI USA

FAO-ISRIC-ISSS (1998) World reference base for soil resources. World Soil Resources Reports 84, FAO, Rome, Italy

García C, Hernández T (1997) Biological and biochemical indicators in derelict soils subject to erosion. Soil Biol Biochem 29:171–177

García C, Hernández T, Costa F (1997) Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun Soil Sci Plan 28:123–134

Hernández T, García C (2003) Estimación de la respiración microbiana. In: García C, Gil F, Hernández T, Trasar C (eds) Técnicas de Análisis de Parámetros Bioquímicos de Suelos: Medida de Actividades Enzimáticas y Biomasa Microbiana. Editorial Mundi-Prensa, Madrid, Spain, pp 311–346

Jordan D, Kremer RJ, Bergfield WA, Kim KY, Cacnio VN (1995) Evaluation of microbial methods as potential indicators of soil quality in historical agricultural fields. Biol Fertil Soils 19:297–302

Kirchner MJ, Wollum AG II, King LD (1993) Soil microbial populations and activities in reduced chemical input agro-ecosystem. Soil Sci Soc Am J 57:1289–1295

Lalfakzuala R, Kayang H, Dkhar MS (2008) The effects of fertilizers on soil microbial components and chemical properties under leguminous cultivation. American-Eurasian J Agric Environ Exp Sci 3:314–324

Lavahun MFE, Joergensen RG, Meyer B (1996) Activity and biomass of soil microorganisms at different depths. Biol Fertil Soils 23:38–42

Leirós MC, Trasar-Cepeda C, Seoane S, Gil-Sotres F (2000) Biochemical properties of acid soils under climax vegetation (Atlantic oakwood) in an area of the european temperate-humid zone (Galicia, NW Spain): general parameters. Soil Biol Biochem 32:733–745

MAPA (1994) Métodos Oficiales de Análisis de Suelos y Aguas para el Riego. In: Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Métodos Oficiales de Análisis. Tomo III. Plantas, productos orgánicos fertilizantes, suelos, aguas, productos fitosanitarios, fertilizantes inorgánicos, Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain, pp. 205–324

Matinizadeh M, Korori SAA, Teimouri M, Praznik W (2008) Enzyme activities in undisturbed and disturbed forest soils under oak (*Quercus brantii* var. persica) as affected by soil depth and seasonal variation. Asian J Plant Sci 7:368–374

Melero S, Madejón E, Herencia JF, Ruiz JC (2007) Biochemical properties of two textured soils (loam and clay) after the addition of two different composts during conversion to organic farming. Spanish J Agric Res 5:593–604

Mirás Avalos JM, Sande Fouz P, Vidal Vázquez E (2007) Actividad deshidrogenasa en dos posiciones topográficas de un suelo de cultivo. Cadernos do Lab Xeolóxico Laxe 32:151–163

Nannipieri P, Grego S, Cercanti B (1990) Ecological significance of the biological activity in soil. In: Bollag JM, Stotzky G (eds) Soil biochemistry, vol 6. Marcel Dekker, New York, pp 293–355

R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

Schloter M, Dilly O, Munch JC (2003) Indicators for evaluating soil quality. Agric Ecosyst Environ 98:255–262

Taylor JP, Wilson B, Mills MS, Burns RG (2002) Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. Soil Biol Biochem 34:387–401

Visser S, Parkinson D (1992) Soil biological criteria as indicators of soil quality: Soil microorganisms. Am J Altern Agric 7:33–37

von Mersi W, Schinner F (1991) An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol Fertil Soils 11:216–220

Zaman M, Cameron KC, Di HJ, Inubushi K (2002) Changes in mineral N, Microbial biomass and enzyme activities in different soil depths after applications of dairy shed effluent and chemical fertilizer. Nutr Cycl Agroecosyst 63:275–290

Effects of Air-Drying and Rewetting on Extractable Organic Carbon, Microbial Biomass, Soil Respiration and β-Glucosidase and β-Galactosidase Activities of Minimally Disturbed Soils Under Mediterranean Conditions

Graciela Marando, Patricia Jiménez, Ramón Josa, Maria Julià, Marta Ginovart and Manuel Bonmatí

Abstract The objectives of this study were: (1) to evaluate the effect of air-drying or air-drying and rewetting on microbial biomass carbon (MBC), extracted organic carbon (EOC), basal soil respiration (BSR), the evolution of the respiration process (ERP) and β-glucosidase and β-galactosidase activities in ten native soils with minimal anthropogenic disturbance in Catalonia (NE Spain); and (2) to determine whether air-drying or air-drying and rewetting are accurate sample pre-treatment procedures when the above properties are used to evaluate the quality of the soils. In order to assess the effect of air-drying on the MBC, BSR, ERP and the β-glucosidase and β-galactosidase activities of field-moist soils, the values of these parameters were determined after the field-moist soil samples had been air-dried for one week at room temperature. To evaluate the effect of air-drying plus rewetting on the β-glucosidase and β-galactosidase activities of field-moist soils, air-dried samples were rewetted to 60% of their water holding capacity and kept in the dark at 28°C for 7 days before the assays were performed. In the case of EOC, air-dried values were compared with those of air-dried plus rewetted samples. The results showed that air-drying caused a reduction in the MBC and an increase in the BSR of field-moist soil samples. Rewetting air-dried soils generally increased the activity of the two enzymes. The values of MBC and of β-glucosidase and β-galactosidase activities in air-dried soils had the same ranking as in field-moist

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soils, whereas air-drying plus rewetting led to a change in the ranking of both enzyme activities compared with field-moist samples. β-glucosidase was probably more protected by humic colloids than β-galactosidase.

1 Introduction

Soil quality can be defined as the usefulness of a soil for a specific purpose on a large time scale (Carter et al. 1997). A soil quality indicator is a measurable property that affects the capacity of a soil to perform a given function (Bastida et al. 2008). Soil microbial activity directly influences the stability of the ecosystem and fertility (Smith and Papendick 1993) and it is widely accepted that a good level of microbiological activity is essential for the maintenance of soil quality. Soil enzyme production as a result of microbial metabolism is a sensitive indicator of soil microbial activity and responds quickly to changes (Dick and Tabatabai 1993; Trasar-Cepeda et al. 1998; Ros et al. 2003). However, this rapid responsiveness and sensitivity is also a potential disadvantage in soil monitoring, because delays between collection and analysis may change the individual activity during storage.

Soils should be analysed for biological parameters immediately after sampling, but this is rarely possible due to the number of assays that are often performed. Air-drying greatly facilitates soil sample processing, and the use of this pre-treatment or air-drying plus rewetting should encourage the adoption of biological parameters as part of a soil quality index. However, these operations influence soil biological properties. Air-drying leads to the death of sensitive microorganisms or induces microorganisms to acquire a state of rest or resistance. It can also cause a change in the composition of the microbial community (Kieft et al. 1987; Van Gestel et al. 1993; Tate 2000; Wu and Brookes 2005). Rewetting dry soil may promote mineralization of C from sources which were not available for decomposition in field-moist soils (Van Gestel et al. 1993). This causes a higher level of basal respiration (respiration pulses) within the first few days of incubation (Kieft et al. 1987; Shan-Min et al. 1987; Fierer and Schimel 2003). Furthermore, after remoistening and prior incubation to allow the soil to stabilize, the biomass C may not accurately represent the biomass at the time of sampling (Sparling et al. 1986). However, taking into account the aforementioned advantages of these pre-treatments for soil processing, if air-drying or air-drying plus rewetting results in a consistent ranking of soils in the same order on a relative basis as fresh soils, the procedure should encourage the adoption of biological parameters as part of soil quality assessments (Lee et al. 2007; Zornoza et al. 2007) worked with degraded soils and showed that air-drying and rewetting had different effects on microbial biomass carbon (MBC), basal soil respiration (BSR), metabolic quotient (qCO_2) and water soluble Carbon (WSC), depending on the geographical situation, soil degradation status and sampling date. Bandick and Dick (1999) observed that the enzyme

assays for α-and β-glucosidase, amidase, arylsulfatase, and urease consistently led to the same ranking of treatments between field-moist and air-dried samples in soil plots when different crops and agricultural practices were compared in the NW of the USA. Eivazi and Tabatabai (1990) studied the effect of air-drying on four glycosidases of arable soils, and observed that β-glucosidase activity was 1.5–2 times higher and β-galactosidase activity was hardly altered in air-dried soils. Trasar-Cepeda et al. (2000), who investigated the best storage procedure for natural soils from NW Spain taken from the organic soil material and the Ah horizon, concluded that air-drying was the procedure that caused the greatest alteration in BBA-protease, urease, invertase and β-glucosidase activities. Hinojosa et al. (2004) studied the effect of contaminated soils rewetted to 80% of water holding capacity and then incubated at 21°C for 7 d. They observed that all enzyme activities, except β-glucosidase, were significantly greater for pre-treated rewetted and incubated soils. However, there have been no systematic studies to determine the effect of air-drying and air-drying plus rewetting on a range of microbial properties and Mediterranean soil types under climax vegetation.

Natural soils developed under local potential vegetation (climax vegetation) are usually considered as soil quality references with which to compare soils affected by environmental stress (Trasar-Cepeda et al. 2008).

The objectives of this study were: (1) to evaluate the effect of air-drying and/or air-drying and rewetting on 0.5 M K_2SO_4 extracted organic C (EOC), MBC, BSR, the evolution of the respiration process (ERP), which is the daily CO_2 evolved over seven days, and β-glucosidase and β-galactosidase activities in Mediterranean soils with minimal anthropogenic disturbance; and (2) to determine whether air-drying or air-drying plus rewetting soil samples is an accurate sample pre-treatment procedure when these properties are used to evaluate the quality of the studied soils.

2 Materials and Methods

2.1 Study Sites and Soil Sampling

Soil samples were taken from ten localities in Catalonia: Serra Litoral (LT), Serra del Corredor (CR), Serra del Montnegre (MN), Plana de Vic (VC), Serra de l'Ordal (OR), Conca d'Odena, Igualada (IG), La Panadella (PN), Serres dels Camps, Balaguer (BL), La Granja d'Escarp (LG) and the Segre alluvial plain (SG). An overview of the sites, the land uses at each site and the soil characteristics are given in Tables 1 and 2. We focused on native soils under natural and autochthonous vegetation that was as close as possible to potential vegetation. The soils had not been disturbed by human action for several decades. At all localities, forest and abandoned agricultural soils were distributed over wide zones in a landscape mosaic. Therefore, to validate soil results, we selected four land uses: undisturbed

Table 1 Pedological characteristics and location of the soils under study

Soil sample	Location	Parent material	Soil type[c]	Habitat type	Soil use type	T°C (2006)[a]	P mm (2006)[b]
LT	Litoral	Granodiorite	Cambisol	MF Qi	f	15.5	504
CR	Corredor	Shale	Ranker	MF Qs	f	13.5	461
MN	Montnegre	Granodiorite	Ranker	MF Qs	f	13.5	461
VC	Vic	Marls	Cambisol	AG	dg	12.3	564
OR	Ordal	Limestone	Luvisol	MF Pf	f	15.3	404
IG	Igualada	Marls	Cambisol	DG	ab	12.9	311
PN	Panadella	Limestone	Xerosol	MF Qi	f	12.9	311
BL	Balaguer	Gypseous marls	Xerosol	RGa	dg	14.9	237
LG	La Granja	Marls	Cambisol	SS	s	15.6	137
SG	Segre	Alluvial deposits	Fluvisol	RP	f	15.6	137

[a] 2006 Average air temperature
[b] 2006 Average precipitation
[c] FAO—Unesco, 1974
MF Mediterranean forests, *Qi* Catalo-provençal lowland holm-oak woodlands, *Qs* Catalan cork-oak woodlands *AG* Aphyllanthes grasslands, *Pf* Iberian aleppo pine forests *SS* Sparto steppes, *RP* Riparian poplar galleries, *DG* Dry calcareous grasslands *RGa* Rosemary garrigues, *f* Forests, *dg* Dry grasslands, *ab* Abandoned lands, *s* Steppe

Table 2 Physical and chemical characteristics of the soils under study

Soil Sample	Texture	Sand (%)	Silt (%)	Clay (%)	pH (1:2.5)	EC (1:5)	$CaCO_3$ (%)	Total N (%)	Organic C (%)
LT	LSa	85.8	5.7	8.5	6.95	0.064	–	0.16	3.07
CR	L	37.7	38.9	23.3	6.45	0.129	–	0.37	6.20
MN	SaL	63.9	21.7	14.4	6.45	0.092	–	0.54	7.23
VC	L	48.5	28.1	23.4	8.50	0.163	37	0.32	4.15
OR	C	3.7	34.0	62.3	8.00	0.191	–	0.35	7.81
IG	CL	28.6	33.6	37.8	8.50	0.159	64	0.17	2.31
PN	SaCL	45.2	19.9	34.9	7.80	0.243	–	0.94	10.74
BL	SaL	60.1	33.2	6.6	8.15	2.000	12	0.11	1.21
LG	CL	36.4	29.9	33.7	8.40	1.377	35	0.08	0.85
SG	SaL	72.2	15.5	12.4	8.65	0.121	33	0.11	1.82

Sa Sand, *L* Loam, *C* Clay *EC* Electrical conductivity at 25°C in dS m^{-1}

(or low intensity disturbed) forest, abandoned agricultural, dry grasslands and steppe. A plot of 100 m^2 was defined at each site and the sample was composed of 20–25 homogeneously mixed sub-samples from the A horizon (0–10 cm) after litter removal. Samples were taken on two consecutive days in spring 2006. They were immediately sieved to obtain fine earth (<2 mm), which was then homogenized and stored at 4°C. Determinations in field-moist samples were performed within the first 2 weeks after sampling.

2.2 Sample Pre-Treatments

To assess the effect of air-drying on MBC, BSR, ERP, EOC and the β-glucosidase and β-galactosidase activities of field-moist soils, the values of these parameters were determined after the field-moist soil samples had been air-dried for one week at room temperature. The values were then compared with those found in field-moist samples. To assess the effect of air-drying plus rewetting on EOC, β-glucosidase activity and β-galactosidase activity, air-dried samples were rewetted to 60% water holding capacity (WHC) and kept in the dark at 28°C for 7 days before the aforementioned parameters were assayed. The values were then compared with those found in field-moist soils.

As specified in the next sub-section, MBC, BSR and ERP determinations in both field-moist and air-dried samples were performed once they had been adjusted to 60% WHC and incubated for 7 days in the dark at 28°C. It therefore made no sense to elucidate the effect of air-drying plus rewetting on these three endpoints.

2.3 Analytical Methods

MBC was determined using the fumigation extraction procedure (Vance et al. 1987) in samples that had been pre-incubated for 7 days in the dark at 28°C after adjustment to 60% WHC. The ERP was determined in 100 g of soil that had been adjusted to 60% WHC and incubated for 7 days in the dark at 28°C in sealed jars containing a vial with 10 mL of 0.5 N NaOH to absorb CO_2. NaOH traps were removed every day of incubation and the quantity of CO_2–C evolved was determined by titration of NaOH with 0.5 N HCl (Hernández and García 2003). BSR was determined by calculating the CO_2 evolved between the 6th and 7th day of incubation in the ERP method. β-D-glucosidase and β-D-galactosidase activities were determined as the amount of *p*-nitrophenol (*p*NP) released from 1.5 g soil after incubation at 37°C for 1 h with the substrate *p*-nitrophenyl-β-glucopyranoside or *p*-nitrophenyl-β-galactopyranoside in MUB buffer (pH 6.5). The reaction was stopped by cooling to 2°C for 15 min; 1 mL of 2 M $CaCl_2$ and 4 mL of 0.1 M THAM-NaOH 0.1 M at pH 12 were then added and the mixture was shaking and filtered. The released *p*-nitrophenol was determined spectrophotometrically at 400 nm. Controls were made in the same way, but the substrate was added before the $CaC1_2$ and THAM (Tabatabai 1982). Calibration plots of *p*-nitrophenol (*p*NP) were prepared by including the soil in the standard solutions, to take into account the relative adsorption of *p*NP by each soil (Vuorinen 1993). EOC, a measure of readily available organic matter in soil (Joergensen and Brookes 1990), was obtained by extraction with 0.5 M K_2SO_4 in the proportion of 1:4 (w/v), quantification by oxidation with $K_2Cr_2O_7$ in a concentrated H_2SO_4 medium, and the titration of dichromate excess using $(NH_4)_2Fe(SO_4)_2$ (Yeomans and Bremner 1989).

All determinations were performed in triplicate and all values reported are averages of the three determinations expressed on an oven-dried soil basis.

2.4 Statistical Analysis

We considered the influence of two fixed main factors (pre-treatment and soil type) on the studied parameters. General Linear Models (GLM) were used to evaluate the influence of the different factors on the measured variables. Data were analyzed using Statistical Analysis System software, and the GLM procedure was performed using variance tests (SAS Institute 1990). Separation of means was made according to the Tukey-Kramer procedure (at the level of $\alpha = 0.05$).

3 Results

3.1 ANOVA Results

The results obtained from repeated measures ANOVA (Table 3) showed that the type of soils and the type of applied pre-treatment had significant effects on all the studied properties. Interaction between the two factors was also statistically significant for all the properties except MBC.

3.2 Air-Drying Effect on MBC, BSR and ERP of Field-Moist Soils

Values of MBC (μg C g^{-1} dry soil) in field-moist and air-dried soil samples are shown in Fig. 1. The calculated mean value of the MBC of air-dried soils (675.40) was found to be significantly lower than that of field-moist soils (813.19). As deduced from the ANOVA results, the relative differences in MBC values within air-dried samples were the same as those within field-moist samples.

Air-drying significantly increased the basal respiration of field-moist soils (Fig. 2). SBR values (μg CO_2–C g^{-1} dry soil h^{-1}) in air-dried samples were generally 2–3 times higher than in fresh samples. The exceptions were LT, LG and OR soils, in which SBR values were 1.2, 3.8 and 5 times higher, respectively, than in fresh samples.

The ERP results (Table 4) showed that, in general, air-drying significantly increased the daily production of CO_2. The CO_2 production on the first day of incubation was between 1.1 and 1.6 higher in air-dried samples, except in PN soil, in which it was 15 times higher. Air-drying modified both the ranking of BSR and the CO_2 pulse in field-moist samples.

Table 3 ANOVA table showing F values of microbial biomass carbon (MBC), basal soil respiration (BSR), β-glucosidase activity, β-galactosidase activity and extractable organic carbon (EOC) in soils

	MBC[a]	BSR[a]	β-glucosidase[a]	β-galactosidase[a]	EOC[a]
Pre-treatment (PT)	24.07**	776.75***	169.60***	281.47***	774.77***
Soils (S)	160.52***	205.44***	859.37***	1560.28***	603.66***
PT x S	1.46 ^{ns}	103.47***	21.42***	84.50***	44.46***

[a] F-value

*, **, and ***indicate significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively

ns non-significant

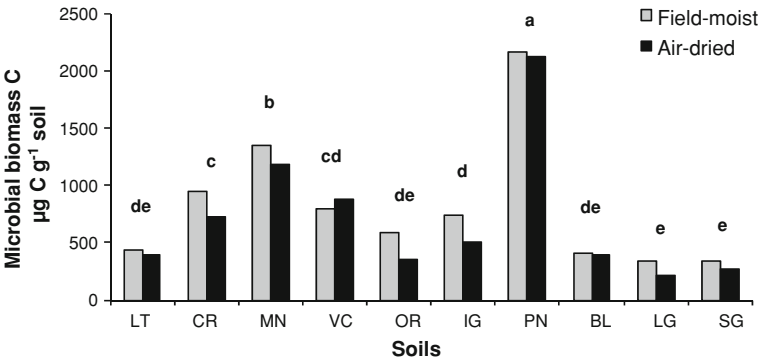

Fig. 1 Values of microbial biomass in field-moist and air-dried soils. For both pre-treatments means with different letters indicate significant differences among soils ($P \leq 0.05$)

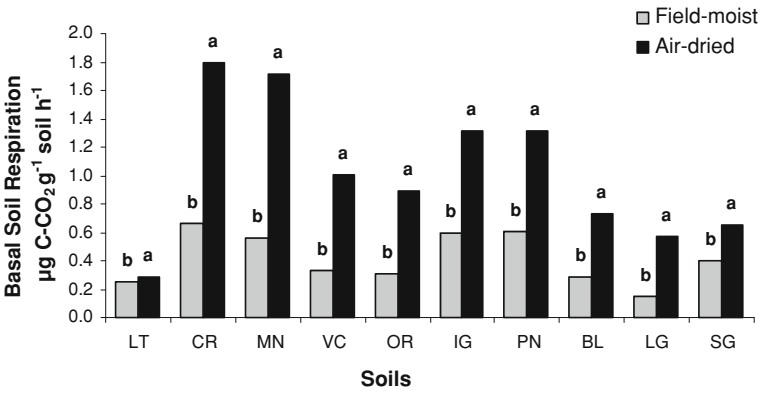

Fig. 2 Effects of air-drying on Basal Soil Respiration. For each soil means with different letters indicate significant differences between pre-treatments ($P \leq 0.05$)

Table 4 Effects of air-drying on the evolution of the respiration CO_2–C (μg g^{-1} soil h^{-1}) during 7 days of incubation

Soils	Days of incubation										Cumulative CO_2	
	1		2		3		5		7			
	Fm	AD	Fm	AD	Fm	AD	Fm	AD	Fm	AD	Fm	AD
LT	3.53^{aB}	5.61^{aA}	1.51^{bB}	3.61^{bA}	1.11^{cB}	2.32^{cA}	0.97^{cB}	1.18^{dA}	0.25^{dB}	0.29^{eA}	7.9	15.4
CR	8.87^{aB}	9.79^{aA}	3.18^{bB}	9.53^{aA}	3.26^{bB}	5.51^{bA}	2.45^{bB}	3.25^{cA}	0.67^{cB}	1.79^{dA}	19.8	36.4
MN	7.64^{aB}	10.49^{aA}	3.68^{bB}	7.25^{bA}	2.86^{cA}	3.35^{cA}	2.39^{cA}	2.47^{dA}	0.56^{dB}	1.14^{eA}	18.2	23.2
VC	3.31^{aB}	5.07^{aA}	1.51^{bB}	3.27^{bA}	1.47^{bB}	1.91^{cA}	1.37^{cA}	1.64^{cA}	0.33^{dB}	0.67^{dA}	8.7	16.2
OR	3.82^{aB}	5.28^{aA}	1.72^{bB}	3.36^{bA}	1.51^{cA}	2.50^{bcA}	1.28^{dA}	1.81^{cA}	0.31^{eB}	1.56^{cA}	9.3	17.3
IG	6.06^{aB}	7.57^{aA}	2.76^{bB}	3.52^{bA}	2.52^{bB}	3.15^{bA}	2.34^{bA}	2.18^{cB}	0.59^{cB}	1.31^{dA}	15.4	21.9
PN	1.33^{bB}	20.00^{aA}	0.64^{cB}	8.75^{bA}	1.86^{aB}	7.08^{cA}	1.19^{bB}	3.33^{dA}	0.61^{cB}	1.25^{eA}	6.8	47.3
BL	1.97^{aB}	2.41^{aA}	1.97^{aA}	1.64^{bB}	1.62^{bA}	1.05^{cB}	1.33^{cA}	0.83^{cB}	0.28^{dB}	0.73^{cA}	7.7	5.8
LG	1.55^{aB}	2.50^{aA}	0.47^{dB}	0.85^{bA}	0.62^{cB}	0.75^{bA}	0.95^{bA}	0.57^{bA}	0.15^{eA}	0.57^{cA}	4.1	6.3
SG	3.06^{aB}	4.91^{aA}	1.51^{bB}	2.14^{bA}	1.40^{cA}	1.76^{cA}	1.65^{cA}	1.40^{dA}	0.40^{dB}	0.65^{eA}	8.8	14.4

Fm Field-moist, *AD* Air-dried. Means within a row of same pre-treatment followed by the same lower case letter are not significantly different according Tukey test ($P = 0.05$). Means within a row of same day of incubation followed by the same capital letter are not significantly different according Tukey test ($P = 0.05$)

Table 5 β-glucosidase activity and β-galactosidase activity in field-moist, air-dried and air-dried plus rewetted soils ($P \leq 0.05$)

Soils	β-glucosidase			β-galactosidase		
	Field-moist	Air-dried	Rewetted	Field-moist	Air-dried	Rewetted
LT	1.34^{fB}	1.59^{dA}	0.92^{gC}	0.33^{dA}	0.29^{dA}	0.29^{dA}
CR	2.37^{cB}	2.26^{cB}	2.71^{cA}	0.89^{bB}	0.76^{bB}	1.83^{aA}
MN	2.84^{bB}	3.55^{bA}	3.54^{bA}	0.98^{aB}	1.05^{aB}	1.21^{bA}
VC	1.88^{dC}	2.20^{cB}	3.04^{cA}	0.24^{eB}	0.20^{eC}	0.31^{dA}
OR	1.73^{eA}	1.60^{dA}	1.34^{fB}	0.16^{fA}	0.18^{eA}	0.20^{efA}
IG	1.15^{gC}	1.55^{dB}	2.10^{dA}	0.16^{fB}	0.10^{fC}	0.24^{deA}
PN	3.83^{aB}	3.83^{aB}	4.71^{aA}	0.56^{cB}	0.33^{cC}	0.75^{cA}
BL	0.53^{hB}	0.55^{fB}	0.72^{gA}	0.05^{gB}	0.05^{gB}	0.13^{fgA}
LG	0.39^{iB}	0.46^{fB}	0.68^{gA}	0.03^{gB}	0.03^{gB}	0.10^{gA}
SG	1.06^{gC}	1.25^{eB}	1.69^{eA}	0.11^{fB}	0.10^{fC}	0.14^{fgA}

Means within a row followed by the same capital letter are not significantly different according Tukey test ($P \leq 0.05$). Means within a column followed by the same lower case letter are not significantly different according Tukey test

3.3 The Effects of Air-Drying and Air-Drying Plus Rewetting on the Enzyme Activities of Field-Moist Soils

The values of β-glucosidase and β-galactosidase activities (μmol *p*-nitrophenol g^{-1} dry soil h^{-1}) in field-moist, air-dried and air-dried plus rewetted soil samples are shown in Table 5. In LT, MN, VC, IG and SG soils, air-drying significantly increased soil β-glucosidase activity (1.2, 1.3, 1.2, 1.3 and 1.2 times higher respectively), whereas the treatment did not significantly modify the activity of the rest of the field-moist soils. Air-drying did not change the ranking of β-glucosidase activity values in fresh samples. However, it caused a decrease in the between-soils discrimination capacity (Table 5). Air-drying significantly decreased the β-galactosidase activity in VC, IG, PN and SG soils (1.2. 1.4, 1.4 and 1.2 times respectively) but did not significantly modify the activity found in the rest of field-moist soils. In contrast with β-glucosidase, β-galactosidase activity values consistently showed the same relative differences within air-dried and field-moist samples (Table 5). Air-drying plus rewetting generally caused a significant increase and also modified the ranking of both the enzyme activity values found in field-moist samples (Table 5).

3.4 Comparison of the Effects of Air-Drying With the Effects of Air-Drying Plus Rewetting on the EOC Values of Soils

In the case of EOC, due to an identification error, the values of air-dried samples could only be compared with those of air-dried plus rewetted (7 days, 60% WHC, 28°C) samples.

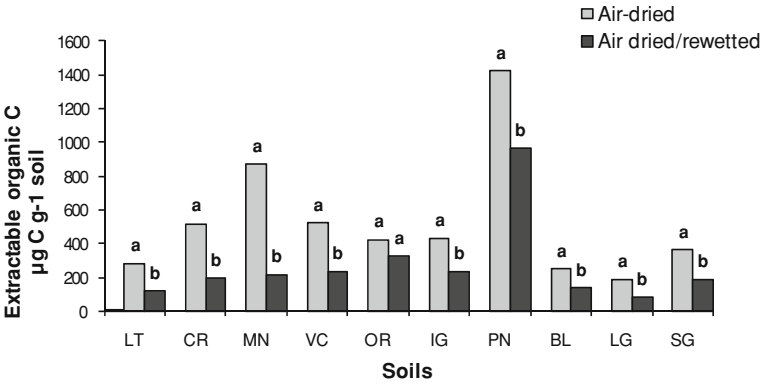

Fig. 3 Values of extractable organic C in air-dried and air-dried plus rewetted soils. For each soil means with different letters indicate significant differences among pre-treatment ($P \leq 0.05$)

Except in OR soil, in which the values were higher but not significantly different, the EOC values (µg C g^{-1} dry soil) of air-dried samples were significantly higher than those of air-dried plus rewetted samples (Fig. 3).

3.5 Simple Linear Regressions Between the Values of Air-Dried or Air-Dried Plus Rewetted Samples and Field-Moist Samples for the Enzyme Activities of Soils

Following the procedure proposed by Zornoza et al. (2006), we evaluated the slope obtained from simple linear regressions between air-dried or air-dried plus rewetted samples and field-moist samples for enzyme activities and microbial biomass. Our aim was to quantify deviations from a 1:1 relationship and find out which pre-treatment represented fewer shifts from the initial field-moist soil values.

β-glucosidase activities in air-dried samples were representative of those obtained in field conditions (slope 1.02; R^2 0.947, $P < 0.001$) (Fig. 4), whereas air-drying underestimated the β-galactosidase values (slope 0.94; R^2 0.932, $P < 0.001$) present in fresh samples. Overestimations of the field values for rewetting air-dried soils were found for β-glucosidase activity (slope 1.15; R^2 0.88, $P < 0.001$) and β-galactosidase activity (slope 1.49; R^2 0.865, $P < 0.001$).

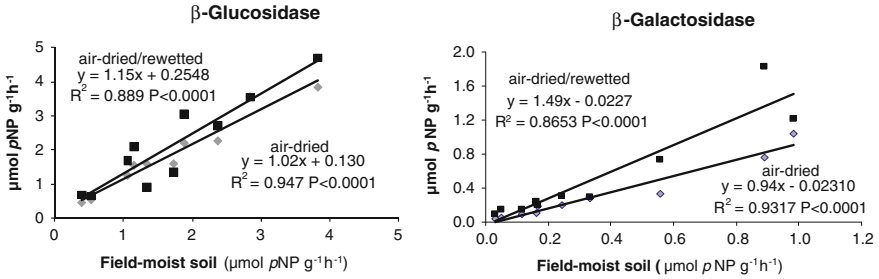

Fig. 4 *β*-Glucosidase and *β*-galactosidase determined under field-moist conditions and air-dried, and air-dried/rewetted

4 Discussion

4.1 Effects of Air-Drying on MBC, BSR, ERP and Enzyme Activities of Field-Moist Soils

The MBC contents of air-dried soil samples were lower than those of field-moist soil. The results are in consonance with those obtained by Van Gestel et al. (1993), Magid et al. (1999), Zornoza et al. (2007) and Gordon et al. (2008), and indicate that the proportion of microbial biomass that survived the drying process was too low to reach the values attained by that of field-moist soils when both air-dried and fresh samples were incubated at 60% WHC. The rewetting of a dry soil event creates extreme stress, increases soil water potential and requires microbial cells to increase their water potential by rapidly releasing solutes to again achieve a state of equilibrium. In extreme cases, the immediate result is the release of all the cellular solutes, which, when the soils are typically poor in C, would be quickly assimilated by the surviving microorganisms. This would cause their proliferation and, once the substrate begins to be consumed completely, their diminution (Gordon et al. 2008). Thus, rewetting dry soil may alter the location of microorganisms in relation to their substrates and cause anomalously large fluctuations in the microbial biomass C. OR and LG were the soils in which MBC was most affected by air-drying. The pre-treatment caused a 39% and 35% decrease in this endpoint, respectively. Soils of the Mediterranean ecosystem typically experience rapid rewetting and drying cycles and a high moisture deficit most of the year, which means that their microbial communities are pre-adapted to moisture stress (Sparling et al. 1987). However, the particularly severe heat-drought combination that occurred in 2006 in the geographical regions to which OR and LG soils belong (Table 1) must have made their microbial population more fragile as it is known that the duration of drought and the amount of moisture added on rewetting affect the soil's microbial community (Clein and Schimel 1994).

The BSR values of air-dried soils were clearly higher than those of fresh soils. The highest increases due to the pre-treatment were found in the LG and OR soils, which

were the soils in which air-drying had led to the greatest losses in microbial biomass content. These results indicate that increases in the BSR of fresh soils due to air-drying were mainly due to the mineralization of organic matter from microorganisms that were killed by the pre-treatment and not recovered during the incubation process at WHC. The mineralization of the protected organic matter that was released by the drying and rewetting process (Fierer and Schimel 2003) may also have contributed to the increase in BSR by air-drying. Wu and Brookes (2005) also found that, after air-drying; the mineralization of the organic matter in a grassland soil after it had been rewetted was proportional to the loss of its microbial biomass due to the pre-treatment.

The CO_2 released during the first day of incubation at 60% WHC was also higher in air-dried than in field-moist samples. This phenomenon (respiration pulse) is commonly cited in the literature (Bottner 1985; Kieft et al. 1987; Franzluebbers et al. 2000; Fierer and Schimel, 2003; Wu and Brookes 2005) and has been attributed to the rapid mineralization of highly enriched intracellular compounds released by the microbial biomass in response to the rapid increase in soil water potential (Fierer and Schimel 2003). In our case, the mineralization of the organic matter of the microbial biomass that was killed by air-drying probably also contributed to the pulse. The clearly higher value of the respiration pulse in PN soil compared with that of the other soils suggests that the mineralization of the protected organic matter released by the drying and rewetting process also affected the pulse. PN was the soil with the highest organic C content and, taking into account its characteristics, it also had the highest proportion of organic matter susceptible to be released by the aforementioned process. This suggestion is reinforced by the small difference in the MBC content of PN soil after air-drying compared with that of the fresh soil. Hence, our results are in accordance with the hypothesis of Scheu and Parkinson (1994) that both biomass C and non-biomass soil organic matter contribute to the CO_2 pulse.

Air-drying did not modify or increase the β-glucosidase activity of field-moist soils. This result was in consonance with the findings of Bandick and Dick (1999), Jiménez et al. (2007), who showed that the enzyme was partly associated with humus colloids. Trasar-Cepeda et al. (2008) hypothesized that environmental stress enhanced the capacity of soil organic matter to retain β-glucosidase activity by physical and chemical bonds. The β-galactosidase activity of field-moist samples was either not modified or decreased by air-drying, which means that this enzyme was probably less protected by humified organic matter than β-glucosidase. Indeed VC, IG, PN and SG, the soils in which air-drying caused a significant decrease in β-galactosidase activity, were also among those that suffered the highest CO_2 pulse and, hence, among those with the highest content of decomposable organic matter.

4.2 Effects of Air-Drying Plus Rewetting on the Enzyme Activities of Field-Moist Soils

Our results showed that air-drying plus rewetting generally increased the assayed enzyme activities of field-moist soil samples. As cited in Lee et al. (2007), this was

probably due to disruption of soil that exposes stabilized enzymes to substrate (Tabatabai and Bremner 1970) or to induction of the enzymes during the wetting cycle because of substrate exposure from lysed cells (Kuprevich and Shcherbakova 1971; Pesaro et al. 2004). Nevertheless, other authors did not observe higher values of β-glucosidase in air-dried plus rewetted soils (Hinojosa et al. 2004; Zornoza et al. 2006; Lee et al. 2007).

4.3 Comparison of the Effects of Air-Drying With the Effects of Air-Drying Plus Rewetting on the Eoc Values of Soils

The EOC content of the air-dried soils corresponds to the extractable organic matter that was already present in the field-moist samples and also to the microbial biomass that was killed by air-drying. However, the EOC content of air-dried plus rewetted samples corresponds to: (1) the extractable organic matter that was already present in the field-moist samples; (2) the microbial biomass that was killed by air-drying and was not recovered during the incubation process at WHC; and (3) the part of the organic matter that was solubilised by drying and rewetting as a result of the release of protected organic matter due to disruption of microaggregates during rewetting due to 'slacking' (soil structural level) and/or by increasing the solubility of humic substances (molecular level) (Magid et al. 1999; Denef et al. 2001; Wu and Brookes 2005). The higher EOC content in air-dried soil compared with air-dried plus rewetted samples indicates that the value of the organic matter corresponding to the killed microbial biomass that was recovered during incubation was higher than that of the organic matter released by drying and rewetting. This hypothesis is reinforced by the fact that OR was the only air-dried soil whose EOC value was not diminished by rewetting. It was also the field-moist soil with the greatest reduction in MBC and the greatest increase in BSR due to air-drying. Consequently, it was the soil in which least MBC was recovered by rewetting.

5 Conclusions

1. Air-drying field-moist soils caused a decrease in microbial biomass. The mineralization of the killed biomass was the main cause of the increase in basal respiration that was observed in air-dried samples compared with fresh samples.
2. The differences in the effects of pre-treatments on the two enzyme activities and on the respiration pulse of the soils allowed us to hypothesize that β-glucosidase was more protected by humic colloids than β-galactosidase.
3. With respect to the validity of the pre-treatments in the evaluations of the quality of the soils
   (a) air-drying was found to be an accurate pre-treatment for microbial biomass and β-galactosidase activity determinations because, although it decreased

their corresponding values, it showed the same relative differences between-soils as those present in fresh samples.

(b) air-drying could also be considered an accurate pre-treatment for β-glucosidase activity determinations, as the values of this enzyme activity in air-dried samples were representative and showed the same ranking of values as those present in fresh samples; however, it caused a decrease in the between-soils discrimination capacity.

(c) air-dying modified the ranking of BSR and the CO_2 pulse that were present in field-moist samples.

(d) air-drying plus rewetting overestimated and modified the ranking of β-glucosidase and β-galactosidase activities of field-moist samples.

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References

Bandick A, Dick RP (1999) Field management effects on soil enzyme activities. Soil Biol Biochem 31:1471–1479

Bastida F, Zsolnay A, Hernández MT, García C (2008) Past, present and future of soil quality indices: a biological perspective. Geoderma 147:159–171

Bottner P (1985) Response of microbial biomass to alternate moist and dry conditions in a soil incubated with ^{14}C and ^{15}N-labelled plant material. Soil Biol Biochem 17:329–337

Carter MB, Gregorich EG, Anderson DW, Doran JW, Janzen HH (1997) Concepts of soil quality and their significance. In: Gregorich EG, Carter MR (eds) Soil quality for crop production and ecosystem health. Developments in soil science, vol 25. Elsevier, Amsterdam, The Netherlands, pp 1–17

Clein JS, Schimel JP (1994) Reduction in microbial activity in birch litter due to drying and rewetting events. Soil Biol Biochem 26:403–406

Denef K, Six J, Bossuyt H, Frey SD, Elliott ET, Merckx R, Paustian K (2001) Influence of dry-wet cycles on the interrelationship between aggregate, particulate organic matter, and microbial community dynamics. Soil Biol Biochem 33:1599–1611

Dick WA, Tabatabai MA (1993) Significance and potential uses of soil enzymes. In: Metting FB (ed) Soil microbial ecology: application in agricultural and environmental management. Marcel Dekker, New York, USA, pp 95–125

Eivazi F, Tabatabai MA (1990) Factors affecting glucosidase and galactosidase activities in soils. Soil Biol Biochem 22:891–897

Fierer N, Schimel JP (2003) A proposed mechanism for the pulse in carbon dioxide production commonly observed following the rapid rewetting of a dry soil. Soil Sci Soc Am J 67:798–805

Franzluebbers AJ, Haney RI, Honeycutt CW, Schomberg HH, Hons FM (2000) Flush of carbon dioxide following rewetting of dried soil relates to active organic pools. Soil Sci Soc Am J 64:613–623

Gordon H, Haygarth PM, Bardgett RD (2008) Drying and rewetting effects on soil microbial community composition and nutrient leaching. Soil Biol Biochem 40:302–311

Hernández T, García C (2003) Estimación de la respiración microbiana del suelo. In: García C, Gil F, Hernández T, Trasar C (eds) Técnicas de Análisis de Parámetros Bioquímicos en

Suelos Medida de Actividades Enzimáticas y Biomasa Microbiana. Mundi-Prensa Editorial, Madrid, Spain, pp 311–346
Hinojosa MB, Carreira JA, García-Ruíz R, Dick RP (2004) Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal-contaminated and reclaimed soils. Soil Biol Biochem 36:1559–1568
Jiménez P, Ortiz O, Tarrasón D, Ginovart M, Bonmatí M (2007) Effect of differently post- treated dewatered sewage sludge on β-glucosidase activity, microbial biomass carbon, basal respiration and carbohydrates contents of soils from limestone quarries. Biol Fertil Soils 44:393–398
Joergensen RG, Brookes PC (1990) Ninhydrin-reactive nitrogen measurements of microbial biomass in 0.5 K_2SO_4 soil extracts. Soil Biol Biochem 22:1023–1027
Kieft TL, Soroker E, Firestone MK (1987) Microbial biomass response to a rapid increase in water potential when dry soil is wetted. Soil Biol Biochem 19:119–126
Kuprevich VT, Shcherbakova TA (1971) Soil enzymes. USDA, Washington, DC, USA
Lee YB, Lorenz N, Dick LK, Dick RP (2007) Cold storage and pretreatment incubation effects on soil microbial properties. Soil Sci Soc Am J 71:1299–1305
Magid J, Kjaergaard C, Gorissen A, Kuikman PJ (1999) Drying of rewetting of a loamy sand soil did not increase the turnover of native organic matter, but retarded the decomposition of added ^{14}C-labelled plant material. Soil Biol Biochem 31:595–602
Pesaro M, Nicollier G, Zeyer J, Widmer F (2004) Impact of soil drying-rewetting stress on microbial communities and activities and on degradation of two crop protection products. Appl Environ Microbiol 70:2577–2587
Ros M, Hernández MT, García C (2003) Bioremediation of soil degraded by sewage sludge: effects on soil properties and erosion losses. Environ Manage 31:741–747
SAS Institute (1990) SAS procedure guide, Version 6, 3rd edition. SAS Institute, Cary, NC, USA
Scheu S, Parkinson D (1994) Changes in bacterial and fungal biomass C, bacterial and fungal biovolume and ergosterol content after drying, remoistening and incubation of different layers of cool temperate forest soils. Soil Biol Biochem 26:1515–1525
Shan-Min S, Brookes PC, Jenkinson DS (1987) Soil respiration and the measurement of microbial biomass C by the fumigation technique in fresh and in air-dried soil. Soil Biol Biochem 19:153–158
Smith LJ, Papendick RI (1993) Soil organic matter dynamics and crop residue management. In: Metting FB (ed) Soil microbial ecology. Marcel Dekker, New York, USA, pp 65–95
Sparling GP, Speir TW, Whale KN (1986) Changes in microbial biomass C, ATP content, and soil phospho-monoesterase and phospho-diesterase activity following air-drying of soils. Soil Biol Biochem 18:363–370
Sparling GP, Milne JG, Vincent KW (1987) Effect of moisture regime on the microbial contribution to Olsen P values. NZ J Agric Res 30:79–84
Tabatabai MA (1982) Soil enzymes. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analisis. Part 2. Chemical and microbiological properties. Soil Science Society of America, Madison, WI, USA, pp 903–947
Tabatabai MA, Bremner JM (1970) Factors affecting soil arylsulfatase activity. Soil Sci Soc Am Proc 34:427–429
Tate RL III (2000) Soil microbiology. John Wiley & Sons, New York, USA
Trasar-Cepeda C, Leirós C, Gil-Sotres F, Seoane S (1998) Towards a biochemical quality index for soils: an expression relating several biological and biochemical properties. Biol Fertil Soils 26:100–106
Trasar-Cepeda C, Leirós MC, García-Fernández F, Gil Sotres F (2000) Biochemical properties of soils of Galicia (N.W. Spain): their use as indicators of soil quality. In: García C, Gil F, Hernández T (eds) Research and perspectives of soil enzymology in spain. CSIC, Madrid, pp 175–206
Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Hydrolytic enzyme activities in agricultural and forest soils. some implications for their use as indicators of soil quality. Soil Biol Biochem 40:2146–2155

Van Gestel M, Merckx R, Vlassak K (1993) Microbial biomass responses to soil drying and rewetting: The fate of fast- and slow- growing microorganisms in soils from different climates. Soil Biol Biochem 25:109–123

Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass. Soil Biol Biochem 19:703–707

Vuorinen AH (1993) Requirement of *p*-nitrophenol standard for each soil. Soil Biol Biochem 25:295–296

Wu J, Brookes PC (2005) The proportional mineralization of microbial biomass and organic matter caused by air-drying and rewetting of a grassland soil. Soil Biol Biochem 37:507–515

Yeomans JC, Bremner JM (1989) A rapid and precise method for routine determination of organic carbon in soil. Commun Soil Sci Plan 19:1467–1476

Zornoza R, Guerrero C, Mataix-Solera J, Arcenegui V, García-Orenes F, Mataix-Beneyto J (2006) Assessing air-drying and rewetting pre-treatment effect on some soil enzyme activities under Mediterranean conditions. Soil Biol Biochem 38:2125–2134

Zornoza R, Guerrero C, Mataix-Solera J, Arcenegui V, García-Orenes F, Mataix-Beneyto J (2007) Assessing the effects o air-drying and rewetting pre-treatment on soil microbial biomass, basal respiration, metabolic quotient and soluble carbon under mediterranean conditions. Eur J Soil Biol 43:120–129

Temporal Variability and the Effect of Fertilization on Biochemical Properties of a Grassland Soil from Galicia (NW Spain)

Jorge Paz-Ferreiro, Carmen Trasar-Cepeda, M. Carmen Leirós, Socorro Seoane and Fernando Gil-Sotres

Abstract The aim of this study was to analyze the effects of soil management, particularly inorganic fertilizer, and to monitor the temporal variability of soil biochemical properties in two grassland plots under contrasting management and located next to each other, trying to link climate and soil biochemical properties. We recorded the following biochemical properties: labile carbon, microbial biomass-C, soil basal respiration, net nitrogen mineralization, and catalase, dehydrogenase, CM-cellulase, β-glucosidase, invertase, casein-protease, BAA-protease, urease, phosphodiesterase, phosphomonoesterase and arylsulphatase activities. Soil microbial activity was higher in the unfertilized plot than in the fertilized plot. Seasonal patterns were identified for some biochemical properties, especially when they were expressed on an organic carbon basis. Additionally, an equation to assess soil quality showed that the unfertilized plot had better quality that the fertilized plot. Our data show that temporal variability should be taken into account when using soil biochemical properties as indicators of soil quality.

1 Introduction

There is a growing interest in the identification and definition of soil quality indicators (Visser and Parkinson 1992). In particular, there is an interest in quantifying the loss of soil quality generated by grassland management as a result

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of the large areas of land covered by this type of vegetation, which constitutes a quarter of all agricultural land.

Some authors, e.g., Visser and Parkinson (1992), consider that the most suitable biological and biochemical properties for evaluating soil quality are those related to the cycling of biogenic elements and to the transformation of organic matter in the soil. According to Nannipieri et al. (1995), the majority of biochemical soil properties used as soil quality indicators are biochemical properties that reflect either the activity of microbial processes (general biochemical parameters), or that of hydrolytic soil enzymes (specific biochemical parameters).

On the other hand, inorganic and organic amendments have been widely used to increase the availability of plant nutrients and thus, enhance soil productivity. The use of amendments affects soil microbial communities, which in turn affect soil biochemical properties. Productivity of unfertilized grassland is limited in some seasons by N deficiency. Despite this, there is currently great interest in encouraging self-regulating agricultural systems, rather than systems that rely on fertilizer input. Mineral fertilizers increase plant biomass, which leads to increased root exudation and, consequently, the input of debris into the soil. This input is assumed to increase soil biological activity (Dick 1992). However, the resulting increases in nutrients in soils may cause a negative feedback, suppressing some of the enzymes involved in nutrient cycling (Olander and Vitousek 2000). On the whole, and in spite of previous publications about the effect of management on soil grassland quality (Paz-Ferreiro et al. 2007, 2009) the effects of soil management, and in particular inorganic fertilizers and tillage, on soil quality are not yet well understood.

Additionally, the extent to which biochemical properties change during different seasons is of interest for several reasons. It is generally considered that climate is one of the main factors that affect soil biochemical properties (Wardle 1998). Climate influences soil temperature and moisture content, which affect the dynamics of soil microbial communities (Schimel and Clein 1996) thereby altering soil biochemical properties (McGill et al. 1986). Marked temporal effects on biochemical properties have been recorded in some annual studies, although to date most studies have considered the variation in general biochemical parameters (see, for example, Patra et al. 1990, for soil microbial biomass) and not in specific biochemical parameters (although there are some studies, see e.g., Ross et al. 1984). The results of some studies that have examined the seasonal effects on soil microbial population and activity have been contradictory. For example, Kaiser and Heinemeyer (1993) and Joergensen et al. (1994) observed a greater microbial biomass in summer than in winter, and suggested that this is a direct consequence of higher temperatures. Bååth and Söderström (1982) showed that soil microbial biomass was greatest in the spring and autumn and lowest in summer and winter. On the other hand, other studies (Holmes and Zac 1994; Ross et al. 1995) reported no differences in the size of the biomass in relation to season. Contradictory results have also been found for other biochemical properties, such as soil respiration, see, for example, the results reported by Ross et al. (1984, 1995) and Maraun and Scheu (1996). Fewer studies have considered the seasonality of soil enzymes, but

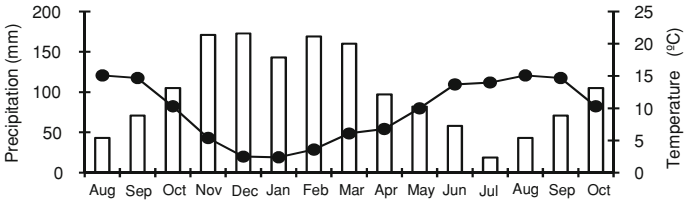

Fig. 1 Monthly temperature (*continuous line*) and precipitation (*vertical bars*) in Rodeiro between August 2003 and October 2004

some authors (Dormaar et al. 1984) have observed lower levels of soil enzymatic activity in the cold season.

Those studies published to date on the seasonality of soil biochemical properties have considered only a few properties and therefore do not reflect the soil functioning as a whole or the complexity of the processes that take place in the soil. Moreover, most studies of biochemical properties in grassland soils have not considered the temporal variation in the properties (Paz-Ferreiro et al. 2007, 2009).

The aim of the present study were (1) to monitor the seasonal patterns of several soil biochemical properties in temperate grasslands, (2) to investigate the long-term effects of fertilization on soil biochemical properties in two native grassland plots subjected to different management, (3) to see how an equation used to assess soil quality and developed by Paz-Ferreiro et al. (2007) responds to seasonal variation on soil biochemical properties. This information may allow changes in management practices that would enable preservation of soil quality in grassland ecosystems.

2 Materials and Methods

The study site is located in Rodeiro (Galicia, NW Spain), at a latitude of 7°58′ 17″ W and a longitude of 42° 41′ 27″ N. The altitude of the study site is 620 m.a.s.l. The climate in the area is Atlantic, with a mean annual rainfall ca. 1,200 mm. Rainfall is maximal between November and March, when two-thirds of the yearly precipitation occurs. The mean annual temperature is 10.2°C. Figure 1 shows the mean monthly precipitation and temperature for the period of study.

Two 0.5 ha plots managed in different ways (fertilized compared with unfertilized) were selected for analysis. The soils in both plots were Umbrisols (ISSS Working Group R.B., 1998), developed over schists. The unfertilized grassland was dominated by low-fertility plant species, including *Agrostis capillaris* L., *Holcus lanatus* L., *Anthoxanthum odoratum* L., *Lolium perenne* L. and *Poa annua* L., with a scarce presence of legumes, mainly *Trifolium repens* L. The vegetation in the fertilized grassland was dominated by *Lolium perenne* L. and *Trifolium repens* L. Additionally, the unfertilized grassland has never been tilled, while the fertilized

grassland has been tilled when grass was seeded (every 2–4 years). Both, fertilized and unfertilized plots, were rotationally grazed by cattle. The stocking rate is 2 cows ha^{-1} and 8 cows ha^{-1} in the unfertilized and fertilized grasslands, respectively. The fertilized soil received an input of 170 kg N ha^{-1} $year^{-1}$ and 30 kg P ha^{-1} $year^{-1}$ for the last 40 years. During the period of time considered in this work, all the fertilizer was added to the land on the 10th May of 2004.

Soils were collected, with a shovel, during 2003–2004 on the following dates: 23 September, 31 October, 25 November, 29 December of 2003 and 27 January, 27 February, 7 April, 28 April, 28 May, 24 June, 22 July, 2 September, 1 October and 28 October of 2004. In each of the two plots, a representative sample was obtained from the top 10 cm of the upper horizon at 10–15 points distributed uniformly over the whole area of the plot. Approximately five kilograms of soil were sampled in each plot. Samples were pooled in the field to obtain composite samples representative of each site, which were transported in isothermal bags to the laboratory and then sieved (< 4 mm). Moisture content was determined gravimetrically. A sub-sample of the soil was air-dried to ascertain general soil properties, and the remaining soil was stored at 4°C pending biochemical analyses. All biochemical analyses were carried out within two weeks of sampling.

Total C (dichromate oxidation) and N (Kjeldahl digestion) contents and pH in water (1:2.5 soil:water ratio) and in KCl (same ratio as for pH in water) were determined following the methods described by Guitián and Carballas (1976).

General biochemical parameters (soil microbial biomass C, soil basal respiration, net N mineralization, qCO_2 and catalase and dehydrogenase activities) and specific biochemical parameters (CM-cellulase, β-glucosidase, invertase, casein-protease, BAA-protease, urease, phosphodiesterase, phosphomonoesterase and arylsulphatase activities) were measured.

Soil microbial biomass C (Biomass-C) was determined by the chloroform fumigation-extraction method (Vance et al. 1987). The difference in C content of the fumigated and unfumigated extracts was converted to microbial biomass C (expressed in mg kg^{-1} of dry soil) by applying a factor (K_c) of 0.45. The C extracted with 0.5 M K_2SO_4 from the unfumigated samples was used as a measure of the labile pool of C.

Soil basal respiration was determined by static incubation (Guitián and Carballas 1976). The CO_2 produced during a 10-day period by 25 g soil samples incubated at field moisture content at 25°C was collected in 10 ml of a 1 M NaOH solution, which was then titrated against HCl with an automatic titrator. The metabolic coefficient, qCO_2 (μg CO_2–C released mg^{-1} biomass-C h^{-1}), was calculated as the ratio between basal respiration and microbial biomass C.

To determine net N mineralization, 10 g soil samples were extracted for 30 min with 50 ml of 2 M KCl before and after incubation for 10 days at 25 °C at field moisture content. Total inorganic N was determined in the extracts by Kjeldahl distillation (Bremner 1965). Net nitrogen mineralization (mg kg^{-1} 10 d^{-1}) was calculated as the difference between the values obtained before and after incubation.

The activities of oxidorreductases (dehydrogenase and catalase) and hydrolytic enzymes of the carbon (CM-cellulase, β-glucosidase and invertase), nitrogen

Table 1 Substrates and references for all enzymes analyzed in this study

Enzyme	Substrate	Reference
Catalase	H_2O_2 8.8 mM	Trasar-Cepeda et al. (1999)
Dehydrogenase	Iodonitrotetrazolium violet 8.87 mM	Camiña et al. (1998)
CM-cellulase	Carboxymethyl-cellulose 0.7%	Schinner and von Mersi (1990)
β-glucosidase	*p*-nitrophenyl-β-D-glucopyranoside 25 mM	Eivazi and Tabatabai (1988)
Invertase	Sucrose 35.6 mM	Schinner and von Mersi (1990)
Casein-protease	Casein 1%	Ladd and Butler (1972)
BAA-protease	N-α-benzoyl-L-argininamide 30 mM	Nannipieri et al. (1980)
Urease	Urea 1,065.6 mM	Nannipieri et al. (1980)
Phosphodiesterase	bis-*p*-nitrophenyl phosphate 10 mM	Bowman and Tabatabai (1978)
Phosphomonoesterase	*p*-nitrophenyl phosphate 16 mM	Saá et al. (1993)
Arylsulphatase	*p*-nitrophenyl sulphate 5 mM	Tabatabai and Bremner (1970)

(casein-protease, BAA-protease and urease), phosphorus (phosphodiesterase and phosphomonoesterase) and sulphur (arylsulphatase) cycles were determined. The references for all the methods used to determine enzymatic activities, and the substrates used for the determinations are shown in Table 1. All enzyme activities were determined in triplicate.

All statistical analyses were performed with SPSS, version 15.0. Differences in mean values were tested by analysis of variance (ANOVA).

3 Results

Statistical analysis showed that humidity and pH values were not significantly different between the fertilized and the unfertilized soil. Both soils were moderately acid; the mean pH in water value was 5.37 ± 0.16 for the unfertilized soil and 5.28 ± 0.21 for the fertilized soil. Carbon and nitrogen contents were significantly higher in the unfertilized than in the fertilized soil (Table 2). The C/N ratio was similar in both soils (mean 12 ± 2). This suggests that grassland management tends to affect C and N levels to the same degree as showed in previous works for other grassland soils located in the same area (Paz-Ferreiro et al. 2009).

The variation in soil biochemical properties during the period of analysis are shown in Figs. 2 and 3. In general, the values of the biochemical properties varied widely, and no apparent short-term effect of the single addition of fertilizer on the 10th of May was found; there were no sharp increases or decreases or at least not more different than in other months in soil biochemical properties. The fertilized plot showed in general lower activities of soil biochemical properties than the unfertilized plot which can be attributed to differences in management in both fields during the last 30 years.

Table 2 General properties for the fertilized and the unfertilized grassland plots

	Fertilized	Unfertilized
Soil humidity (%)	55 ± 20[a]	66 ± 12[a]
pH H_2O	5.28 ± 0.21[a]	5.37 ± 0.16[a]
pH KCl	4.14 ± 0.11[a]	4.28 ± 0.11[a]
Ct (%)	7.75 ± 0.81[a]	10.00 ± 0.76[b]
Nt (%)	0.669 ± 0.042[a]	0.871 ± 0.042[b]
C/N	12 ± 2[a]	12 ± 2[a]

Different letters indicate statistically significant differences between plots ($P \leq 0.05$)

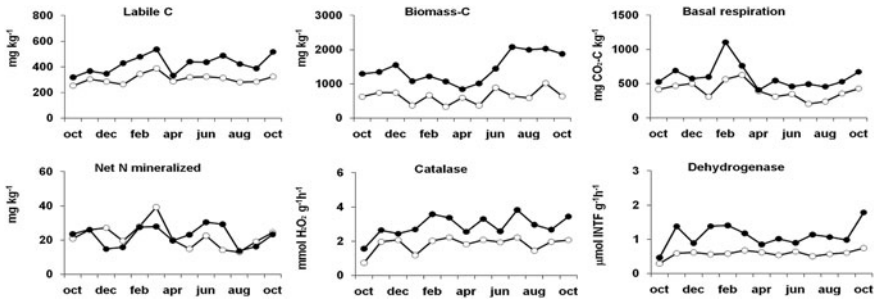

Fig. 2 Annual variations in general soil biochemical parameters. The filled circles represent the unfertilized grassland, and the open circles represent the fertilized grassland. From Paz-Ferreiro et al. (in press), with permission from Springer-Verlag

As regards the seasonal variability, the properties that displayed the greatest differences between maximum and minimum values were soil basal respiration (in the case of the fertilized grassland, the value in the month when respiration was maximal was 3.38 times the value in the month were it was minimal, and in the unfertilized plot the maximum value was 3.61 times higher than the minimum value), urease (the maximum values were 4.74 and 4.65 times the minimum values, in the corresponding respective plots) and β-glucosidase (the maximum values were 2.85 and 3.93 times the minimum values, in the corresponding respective plots). Moreover, the seasonal variability depended on the property under study. For example, the maximum values of net nitrogen mineralization occurred in spring, whereas the maximum value of BAA-protease activity was observed in summer. On the other hand, the values of other properties were higher in winter than in summer (labile carbon and arylsulphatase activity, for example). It is worth mentioning that the monthly variability found on soil biochemical properties was very high (soil biochemical properties can double or reduce to half from one month to the following month), and it seems that no clear, common for all biochemical properties, seasonal patterns are apparent.

To assess how management and seasonality affected soil biochemical properties, we conducted a two-way ANOVA using management (two soils, fertilized vs. unfertilized) and season (soils sampled in spring, summer, autumn or winter) as variables and considering both, absolute values and values relative to total carbon

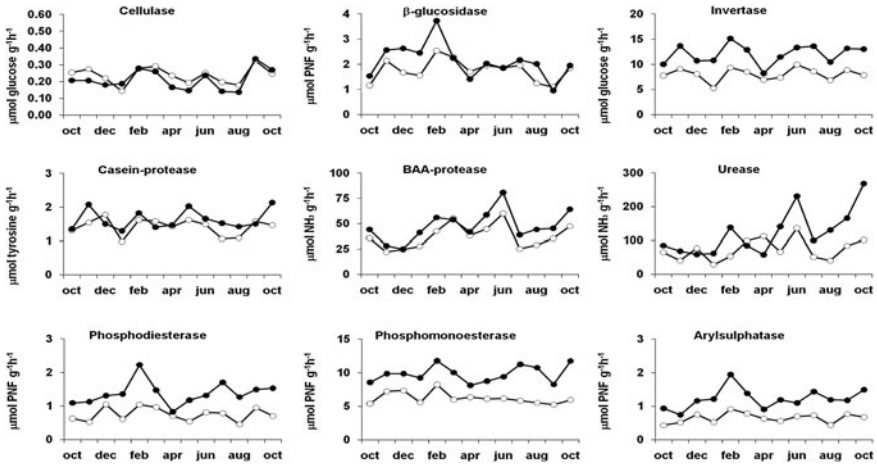

Fig. 3 Annual variations in soil specific biochemical parameters. The filled circles represent the unfertilized grassland, and the open circles represent the fertilized grassland. From Paz-Ferreiro et al. (in press), with permission from Springer-Verlag

content. Soil management influenced all soil biochemical properties with the exceptions of net N mineralization, cellulase, β-glucosidase, casein-protease and BAA-protease. This means, that most of the general biochemical parameters have been affected by management, while most enzymes belonging to the cycles of C and N did not. In the case of season only microbial biomass was affected, showing statistically significant higher values in summer and in autumn compared with spring. When the values of the biochemical properties were expressed divided by the carbon content of the samples, we found similar results for management (it affected most of the biochemical properties, except those enzymes involved in the carbon and nitrogen cycles), while the season affected many biochemical properties, both general parameters and enzymatic activities (especially those hydrolytic activities involved in the biogeochemical cycles of P and S).

4 Discussion

The lower organic matter content in the fertilized grassland is consistent with previous studies (Paz-Ferreiro et al. 2009) and can be attributed, among other reasons, to the oxidation of soil organic matter and the mixture of topsoil horizons, brought about by ploughing in the fertilized plot, which is carried out every 2–4 years and to lower amounts of root exudates typical from managed agricultural systems (Shay and Hale 1973). In this sense, it should be noted that the long-term use of inorganic fertilizers causes decreases in the amount of root mass (Ennik et al. 1980). However, the C/N ratios were similar in both grasslands, which suggest that the loss of carbon and nitrogen occur at the same rate in grassland soils, regardless of soil management.

As regards the mean values of all biochemical properties, it is important to note that they were lower in the fertilized than in the unfertilized plot (data not shown). Differences were statistically significant for most properties (see Table 3) and are consistent with previous findings (Paz-Ferreiro et al. 2009). Our data show that the general biochemical parameters are lower in the fertilized plot, reflecting lower microbial populations in the fertilized than in the unfertilized plot. It seems that the number and activity of soil microorganisms has been modified (decreased) in the fertilized soil. This decline could be attributed to a reduction in microhabitats generated by the loss of organic matter. However, some enzymatic activities (from the carbon and nitrogen cycles) do not seem to be affected by soil management, probably meaning that they have become stabilized in the soil matrix. It seems that for urease activity, the decline in activity seen in the fertilized plot (Fig. 3) could just be due to a reduction in organic matter content in the fertilized plot, as the activity of urease per amount of organic carbon did not show any difference between the two plots studied. Most of the other biochemical properties showed an influence of management, both, when considering their absolute values or when considering their value per unit of organic carbon.

Biochemical properties showed lower levels of activity per unit of organic carbon in the fertilized than in the unfertilized soil (data not shown). In the case of cellulase per unit of carbon, the activity was higher in the fertilized than in the unfertilized soil, which suggests that, in this case, organic matter is lost at a greater rate than this enzymatic activity and, although part of the soil organic carbon is lost, there is a pool of organic matter in which the cellulase has stabilized.

The results also indicate that the metabolic activities of the microbial populations were higher in fertilized (2.59 ± 1.49 μg CO_2–C released mg^{-1} biomass-C h^{-1}) than in unfertilized grassland (1.61 ± 0.72 μg CO_2–C released mg^{-1} biomass-C h^{-1}), although the values are not statistically different ($P = 0.054$). The difference found in qCO_2 values could be due to food webs which have low C assimilation efficiencies and faster turnover rate being favoured in the fertilized soil.

Seasonal variations in microbial and enzyme activities reflect the combined effects of temperature, moisture, substrate availability and other environmental factors. The temporal variability in soil enzyme activities was very high, as found in other studies in which soil biochemical properties were monitored over a similar period of time (Dormaar et al. 1984; Rastin et al. 1990). It is interesting to note that the range of values found for the biochemical properties in the unfertilized grassland is always higher than for the fertilized grassland. It is also remarkable that soil biochemical properties vary greatly from one month to another. Properties which showed higher values in summer were biomass-C, BAA-protease and urease. Casein-protease and net N mineralization showed higher values on months with mild temperatures (spring and autumn). In the case of labile C, respiration, dehydrogenase, phosphodiesterase, phosphomonoesterase and arylsulphatase higher values were shown on winter. Moreover, these properties showed a high correlation with soil humidity (see Table 3). Some other properties (catalase, β-glucosidase and invertase activities) did show seasonal variation, although they

Table 3 ANOVA results showing the effect of management and season on the different soil biochemical properties

	Absolute values						Values per unit of C					
	Management			Season			Management			Season		
	df	F	P	df	F	P	df	F	P	df	F	P
Labile C	1	24.699	**0.000**	3	0.605	0.620	1	4.854	**0.041**	3	5.766	**0.006**
Biomass-C	1	70.893	**0.000**	3	7.059	**0.002**	1	42.066	**0.000**	3	6.019	**0.005**
Basal respiration	1	12.925	**0.002**	3	2.884	0.064	1	5.311	**0.033**	3	6.404	**0.004**
Catalase	1	23.204	**0.000**	3	0.785	0.518	1	13.448	**0.002**	3	4.970	**0.011**
Dehydrogenase	1	23.698	**0.000**	3	0.179	0.909	1	16.566	**0.001**	3	0.866	0.477
Net N mineralization	1	0.518	0.481	3	2.385	0.103	1	5.551	**0.030**	3	0.814	0.503
Cellulase	1	1.546	0.230	3	2.456	0.096	1	19.278	**0.000**	3	1.364	0.286
β-glucosidase	1	3.263	0.088	3	2.761	0.072	1	0.370	0.551	3	8.957	**0.001**
Invertase	1	33.332	**0.000**	3	0.686	0.572	1	13.436	**0.002**	3	1.660	0.211
Casein-protease	1	3.107	0.095	3	1.006	0.413	1	2.130	0.162	3	0.927	0.448
BAA-protease	1	3.359	0.083	3	0.979	0.425	1	0.035	0.853	3	1.415	0.271
Urease	1	4.876	**0.040**	3	0.898	0.461	1	1.720	0.206	3	0.900	0.460
Phosphodiesterase	1	32.105	**0.000**	3	1.492	0.250	1	24.392	**0.000**	3	4.503	**0.016**
Phosphomonoesterase	1	69.844	**0.000**	3	1.210	0.335	1	19.724	**0.000**	3	4.814	**0.012**
Arylsulphatase	1	35.466	**0.000**	3	1.131	0.363	1	29.678	**0.000**	3	4.202	**0.020**

The interaction management x season is not shown as it was not statistically significant for any of the parameters measured

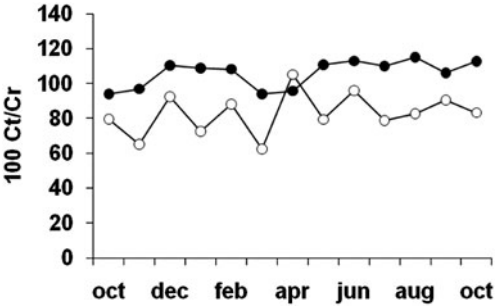

Fig. 4 Annual variations in 100 Ct/Cr. The full circle represents the unfertilized grassland, and the open circle represents the fertilized grassland

did not exhibit a marked seasonal pattern. It is important to note that these seasonal differences were only statistically significant for microbial biomass C. As other studies have shown, soil biochemical properties are generally higher in areas where temperature is higher, as it implies larger microbial populations (Insam 1990) due to larger substrate availability (Li and Sarah 2003). On the other hand, wet soils also lead to an increase of soil biochemical properties (Cooper 1972) due to the positive effects of soil humidity on the stabilization of soil enzymes. In fact, the high enzyme activity in the winter could lead to increased enzyme release into the soil because of faster turnover when more water is available (Krämer and Green 2000). It seems that, in our soils, there are two groups of properties, some biochemical properties seem to be favoured by an increase in soil temperature, while others seem favoured by the high soil humidity on winter months. This could be due to the different origins of soil enzymatic activities, as it has been shown in other studies (Li and Sarah 2003). In the case of enzymatic activities that did not show any clear seasonal pattern, it can be assumed that root exudates (Campbell et al. 1999) or the input of vegetation debris (Franzluebbers et al. 1994) affect the values to a greater extent than soil temperature or moisture. As an example, in the present study, invertase activity did not show any clear seasonal pattern and previous studies state that there is an important effect of root exudates on this enzymatic activity (Ross 1976). Another possible explanation that may account for the lack of any clear seasonal pattern is that most of the activity proceeds from enzymes stabilized in humic or clayey colloids.

Biochemical properties showed the same seasonal patterns when they were expressed as activity per unit of total carbon, with the exception of β-glucosidase, which is this last case showed statistically significant higher values in winter than in autumn and in summer. β-glucosidase activity can detect changes in soil management, has been reported to be stable with seasons (Bandick and Dick 1999; Ndiaye et al. 2000) and it has been found in other studies to be stabilized in the soil clay fraction (Knight and Dick 2004). Our data contradict the findings of these authors, which suggest that β-glucosidase activity in temperate grasslands is not as stable as reported in other ecosystems.

As regards the P and S cycle activities, the results of other studies showed that P cycle enzymes (Ross et al. 1995) and also arylsulphatase (Cooper 1972; Li and Sarah 2003) are inhibited under dry soil conditions. Nevertheless, other authors

(Klose and Tabatabai 1999) found no seasonal variation in the case of arylsulphatase. We have found that these enzymatic activities in our study remain constant for most of the year, with a peak of activity on February.

Biochemical soil properties have been widely used as indicators of soil quality because of their essential role in soil biology, ease of measurement, and sensitivity to environmental change compared to most physical and chemical properties. However, the use of these parameters as indicators of soil quality, both individually and combined, has been criticized due to the lack of reference values and the seasonal and regional variations of these properties. To address these limitations, Paz-Ferreiro et al. (2007) proposed a quality index for temperate grasslands that is independent of among-site variations in soil conditions. This expression has been used to assess soil quality in intensively managed grasslands (Paz-Ferreiro et al. 2009), but to date has not been tested for seasonal variations. These authors showed that in native grasslands there is equilibrium between soil organic carbon content and biological activity. This equilibrium was expressed by the following equation:

$$\begin{aligned}\text{Total carbon} &= 0.764 + (2.304\ 10^{-3}\ \text{biomass}-\text{C}) + (0.936\ \text{catalase activity}) \\ &+ (0.017\ \text{urease activity}) + (0.206\ \text{phosphomonoesterase activity})\end{aligned}$$

This equation explained 96% of the total variance in the estimation of total C. In this equation, total C is expressed as percentage, microbial biomass C as mg kg^{-1}, urease and phosphomonoesterase activities as μmol of released product $g^{-1}\ h^{-1}$ and catalase activity as mmol H_2O_2 consumed $g^{-1}\ h^{-1}$. Soil quality was estimated comparing the total carbon content measured by dichromate oxidation (Cr), with the total carbon content estimated from this equation. Soils with biochemical equilibrium should have a 100 Ct/Cr ratio equal to 100 and it has been found to range from 85 to 115 in native grasslands (Paz-Ferreiro et al. 2007) Fig. 4.

In this case, the unfertilized soil showed a 100 Ct/Cr value of 106 ± 8, with a minimum of 94 and a maximum of 115, while the fertilized grassland exhibited a value of 83 ± 12, ranging from 63 to 105. Both values were statistically different ($P < 0.01$). The lower values of 100 Ct/Cr in the fertilized grassland can be explained as this soil is losing soil quality, probably related to the slowing down of the biogeochemical cycling of elements. The index 100 Ct/Cr followed the order spring > winter > autumn > summer for the fertilized soil, with values ranging from 86 ± 4 in spring to 79 ± 14 in summer. No statistically significant influence of season was found in the values of 100 Ct/Cr. Our results also show that, in spite of the high temporal variability of soil biochemical properties, which makes difficult to find suitable indicators to evaluate soil quality, the equation found by Paz-Ferreiro et al. (2007) is not very dependent on the temporal dynamics of soil biochemical properties. We have found a coefficient of variation of only 8% for the unfertilized grassland soil and the 100 Ct/Cr values for this soil where always between 85 and 115%, as expected for unmanaged high quality grasslands. On the contrary, in the fertilized grassland we have found that 100 Ct/Cr was in 8 occasions between the threshold values of 85–115% and in five cases below this

value. Our data show that the above equation is quite robust to seasonal changes, particularly in unfertilized grassland, although more research would be needed to know why the range of values was larger in the fertilized grassland.

5 Conclusions

The present study shows that soil biochemical properties undergo a marked seasonal variation, the extent of which differs depending on the property considered. In most cases this seasonal variation is driven by climatic parameters (temperature, precipitation), although divergences were observed for some soil biochemical properties. Temporal variability should be considered when interpreting biochemical activities, as good indicators of soil quality in grassland soils should be sensitive to soil management rather than to climatic parameters.

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References

Bååth E, Söderstroöm B (1982) Seasonal and spatial variation in fungal biomass in a forest soil. Soil Biol Biochem 14:353–358

Bandick AK, Dick RP (1999) Field management effects on soil enzyme activities. Soil Biol Biochem 31:1471–1479

Bowman MG, Tabatabai MA (1978) Phosphodiesterase activity of soils. Soil Sci Soc Am J 42:284–290

Bremner JM (1965) Inorganic forms of nitrogen. In: Black CA, Evans DD, White JL, Ensminger LE, Clark FE (eds) Methods of soil analysis, Part 2. Chemical and microbiological properties. American society of agronomy-soil science society of America. Madison, WI, USA, pp 1179–1237

Camiña F, Trasar-Cepeda C, Gil-Sotres F, Leirós MC (1998) Measurement of dehydrogenase activity in acid soils rich in organic matter. Soil Biol Biochem 30:1005–1011

Campbell CA, Lafond GP, Biederbeck VO, Wen G, Schoenau J, Hahn D (1999) Seasonal trends in soil biochemical attributes: Effects of crop management on a Black Chernozem. Can J Soil Sci 79:85–97

Cooper PJM (1972) Arylsulfatase activity in northern Nigeria soils. Soil Biol Biochem 4:333–337

Dick RP (1992) A review: long-term effects of agricultural systems on soil biochemical and microbial parameters. Agric Ecosyst Environ 40:25–36

Dormaar JF, Johnston A, Smoliak S (1984) Seasonal changes in carbon content, dehydrogenase, phosphatase and urease activity in mixed prairie and fescue grassland Ah horizons. J Range Manag 37:31–35

Eivazi F, Tabatabai MA (1988) Glucosidases and galactosidases in soils. Soil Biol Biochem 20:601–606

Ennik GC, Gillet M, Sibma L (1980) Effect of high nitrogen supply on sward deterioration and root mass. In: Prins WH, Arnold GH (eds) The role of N in intensive grassland production. Pudoc Wageningen, The Netherlands, pp 67–76

Franzluebbers AJ, Hons FM, Zuberer DA (1994) Seasonal changes in soil microbial biomass and mineralizable C and N in wheat management systems. Soil Biol Biochem 26:1469–1475

Guitián F, Carballas T (1976) Técnicas de Análisis de Suelos. Pico Sacro Editorial, Santiago de Compostela, Spain
Holmes WE, Zac DR (1994) Soil microbial biomass dynamics and net nitrogen mineralization in Northern hardwood ecosystems. Soil Sci Soc Am J 58:238–243
Insam H (1990) Are the soil microbial biomass and basal respiration governed by the climatic regime? Soil Biol Biochem 22:525–532
ISSS Working Group RB (1998) World reference base for soil resources: introduction. International soil reference and information centre and food and agriculture organization of the United Nations. International Society of Soil Science Acco, Leuven, p 165
Joergensen RG, Meyer B, Mueller T (1994) Time course of the soil microbial biomass under wheat: A one year field study. Soil Biol Biochem 26:987–994
Kaiser EA, Heinemeyer O (1993) Seasonal variations of soil microbial biomass carbon within the plough layer. Soil Biol Biochem 25:1649–1655
Klose S, Tabatabai MA (1999) Arylsulfatase activity of microbial biomass in soils. Soil Sci Soc Am J 63:569–574
Knight TR, Dick RP (2004) Differentiating microbial and stabilized β-glucosidase activity relative to soil quality. Soil Biol Biochem 36:2089–2096
Krämer S, Green DM (2000) Acid and alkaline phosphatase dynamics and their relationship to soil microclimate in a semiarid woodland. Soil Biol Biochem 32:179–188
Ladd JN, Butler HA (1972) Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivates as substrates. Soil Biol Biochem 4:19–30
Li X, Sarah P (2003) Enzyme activities along a climate transect in the Judean Desert. Catena 53:349–363
Maraun M, Scheu S (1996) Seasonal changes in microbial biomass and activity in leaf litter layers of beech (*Fagus sylvatica*) forests on a basalt-limestone gradient. Pedobiologia 40:21–31
McGill WB, Cannon KR, Robertson JA, Cook FD (1986) Dynamics of soil microbial biomass and water-soluble organic C in *Breton* L. after 50 years of cropping to two rotations. Can J Soil Sci 66:1–19
Nannipieri P, Ceccanti B, Cervelli S, Matarese E (1980) Extraction of phosphatase, urease, proteases and organic carbon from soil. Soil Sci Soc Am J 44:1011–1016
Nannipieri P, Landi L, Badalucco L (1995) La capacità metabólica e la qualità del suolo. Agronomia 29:312–316
Ndiaye EL, Sandeno JM, McGrath D, Dick RP (2000) Integrative biological indicators for detecting change in soil quality. Am J Alternative Agric 15:26–36
Olander LP, Vitousek PM (2000) Regulation of soil phosphatase and chitinase activity by N and P availability. Biogeochemistry 49:175–190
Patra DD, Brookes PC, Coleman K, Jenkinson DS (1990) Seasonal changes of soil microbial biomass in an arable and a grassland soil which have been under uniform management for many years. Soil Biol Biochem 22:739–742
Paz-Ferreiro J, Trasar-Cepeda C, Leirós MC, Seoane S, Gil-Sotres F (2007) Biochemical properties of acid soils under native grassland in a temperate humid zone. NZ J Agric Res 50:537–548
Paz-Ferreiro J, Trasar-Cepeda C, Leirós MC, Seoane S, Gil-Sotres F (2009) Biochemical properties in managed grassland soils in a temperate humid zone: modifications of soil quality as a consequence of intensive grassland use. Biol Fertil Soils 45:711–722
Rastin N, Schlechte G, Hüttermann A, Rosenplänter K (1990) Seasonal fluctuations of some biological and biochemical factors and their dependence on certain soil factors on the upper and lower slope of a spruce forest. Soil Biol Biochem 22:1049–1061
Ross DJ (1976) Invertase and amylase activities in ryegrass and white clover plants and their relationships with activities in soils under pasture. Soil Biol Biochem 8:351–358
Ross DJ, Speir TW, Cowling JC, Whale KN (1984) Temporal fluctuations in biochemical properties of soils under pasture. Part II. Nitrogen mineralization and enzyme activities. Aust J Soil Res 22:319–330

Ross DJ, Speir TW, Kettles HA, Mackay AD (1995) Soil microbial biomass C and N mineralization and enzyme activities in a hill pasture: influence of season and slow-release P and S fertilizer. Soil Biol Biochem 27:1431–1443

Saá A, Trasar-Cepeda MC, Gil-Sotres F, Carballas T (1993) Changes in soil phosphorus and acid phosphatase activity immediately following forest fires. Soil Biol Biochem 25:1223–1230

Schimel JP, Clein JS (1996) Microbial response to freeze-thaw cycles in tundra and taiga soils. Soil Biol Biochem 28:1061–1066

Schinner F, von Mersi W (1990) Xylanase-, CM-cellulase- and invertase activity in soil: An improved method. Soil Biol Biochem 22:511–515

Shay FJ, Hale PG (1973) Effect of low levels of calcium on exudation of sugar acid derivatives from intact peanut roots under axenic conditions. Plant Physiol 51:1061–1063

Tabatabai MA, Bremner JM (1970) Arylsulfatase activity of soils. Soil Sci Soc Am Proc 34:225–229

Trasar-Cepeda C, Camiña F, Leirós MC, Gil-Sotres F (1999) An improved method to measure catalase activity in soils. Soil Biol Biochem 31:483–485

Vance ED, Brookes PC, Jenkinson DJ (1987) An extraction method for measuring soil microbial biomass carbon. Soil Biol Biochem 19:703–707

Visser S, Parkinson D (1992) Soil biological criteria as indicator of soil quality: Soil microorganisms. Am J Alternative Agric 7:33–37

Wardle DA (1998) Controls of temporal variability of the soil microbial biomass: A global-scale synthesis. Soil Biol Biochem 30:1627–1637

Short-Term Effect of Fire Severity in Chemical and Biochemical Soil Properties in a Sandy Soil

María Belén Turrión, Francisco Lafuente and Rafael Mulas

Abstract The objectives of the study were to determine how fire severity influences some chemical and biochemical soil properties and to evaluate which properties are more sensitive to this factor. Two forest burned areas and their corresponding unburned areas in Valladolid Province (Spain) were selected. The vegetation in both areas was mixed forest plantation of *Pinus pinea* L. and *Pinus pinaster* Aiton, with *Albic Arenosols*. The fires occurred in June 2004 and the samples at 0–2 cm were taken five months later. Sampling was stratified on the basis of fire severity, defining three levels (high, moderate and low) based on pine canopy consumption, organic litter layer quantity and aspect, and ash quantity and color. In soil samples, pH, total soil organic C (SOC), Walkley–Black C (C_{W-B}), total nitrogen (N), available P (P_{Olsen}), microbial activity (C_{min}), microbial biomass C and P (MBC and MBP), and acid and alkaline phosphatase activity were determined. SOC and total N concentrations increased in burned soils from external inputs. Fire increased P_{Olsen} concentrations from their incorporation into the soil as ash. Our results showed a significant increase in C_{min}, MBC and MBP in burned plots, indicating that at least a part of the microbial community is favored by nutrient availability and pH increases. These fires caused a short-term fertilizing effect in the plots studied due to the very low soil fertility and the low temperature reached.

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1 Introduction

Wildfire disturbance has received a great deal of attention because of its critical role in forest ecosystems of the Mediterranean Basin (Kara and Bolat 2009). In modern forestry, wildfires are considered undesirable, because fire destroys vegetation cover and reduces soil fertility due to erosion and nutrient losses (Fernández et al. 2007). Fires affect physical, chemical and biochemical soil properties, primarily by transferring heat into soil. High soil temperatures kill soil microbes, kill or damage plant roots and seeds, destroy soil organic matter (SOM), and alter soil nutrient and water status. The degree of soil heating during fire depends on a variety of variables, including fuel characteristics, fire intensity and residence time, soil properties, such as moisture content, soil textures and organic matter content, and litter layer properties, such as moisture content, depth. Many of the fire-induced changes in soil are linked to SOM changes (Certini 2005).

There is some confusion in the literature over the concepts of fire intensity and fire severity (Pausas et al. 2003). Fire intensity is the rate of energy or heat release per unit length of fire front, kW/m; it is related to flame length. The relative amount of damage attributed to an area of vegetation is known as fire severity, which is most frequently described in discrete classes ranging from low to high or extreme (Hammill, Bradstock and van Wagtendonk 2001; Pausas et al. 2003; Chafer 2008). Fire severity is thus the effect of fire on an ecosystem, as well as on the amount and location of organic matter consumed during a fire (Ryan and Noste 1985). Fire intensity and fire severity may or may not be related (Pausas et al. 2003). Knowledge on fire severity and intensity can be used by land managers to better predict the susceptibility of burned areas to post-fire soil erosion and its implications affecting water quality, forest regeneration, etc. (Chafer 2008; Pausas et al. 2003). Wildfires often appear as a chaotic mosaic of low affected areas alternating with severely affected areas (Rab 1996).

The human impact has resulted in the depletion of SOM of most semi-arid soils. Quantity and quality of SOM is vital not only for plant growth but also for the development of native microbial populations. Microorganisms have a fundamental role in the biogeochemical cycles and in the formation of soil structure, some works have indicated that a high level of microbial activity is needed to maintain adequate soil quality (Bastida et al. 2007; Llorente and Turrión 2010). It has been shown that different soil management regimes affect the structure and activity of the soil microbial community, via changes in the quantity and quality of the plant remains that enter the soil (Christensen 1996). However, the use of a single parameter to study the effect of soil disturbance on microbial populations and their activity is not valid because the spatial heterogeneity of soil, as well as the complex dynamics of the soil ecosystem, can affect different parameters in differing ways (García et al. 2005; Gil-Sotres et al. 2005). Many authors have proposed the combined use of a number of parameters as early indicators of stress or soil restoration (Dick et al. 1996; Gil-Sotres et al. 2005). Among the parameters related to biochemical and microbiological soil state, the indicators of soil

microbial activity have a great usefulness; the main indicators of such action are enzymatic activities, respiration, different C fractions in the soil, those related to the size of microbial populations (microbial biomass C, N and P), and those related to the availability of labile sources of C. These parameters can provide information on the microbiological quality of a soil (Bastida et al. 2008).

Soil studies in semi-arid areas have focused on the effects of a single fire, dealing with short-term nutrient dynamics in mineral soils (Carreira et al. 1997; Romanyà et al. 2001; Turrión et al. 2010), soil erosional responses, especially after the occurrence of rainfall events (Andreu et al. 2001; Pardini et al. 2004). Few studies have focused on the short-term effects of fire on semi-arid forest systems from the microbiological and biochemical point of view (Dumontet et al. 1996; Hernández et al. 1997; Hamman et al. 2007), and they have showed a depletion of soil microbial populations. An understanding of wildfire impact is needed to effectively manage forest ecosystems, including post-fire management decisions regarding seeding options, erosion control, and other managements. In this study, fire severity was classified according to the degree of canopy consumption after a crown fire. We hypothesized that soil properties could be related to fire severity (as defined above) and that the microbial activity will be depleted.

The objectives of the study were to evaluate the short-term effect of wildfires on some physical, chemical and microbiological characteristics of a forest soil under semi-arid climate, and to relate fire severity with these effects.

2 Materials and Methods

2.1 Study Site and Soil Sampling

The study sites were located near the city of *Valladolid* in Central-Northern Spain. Two burned forest areas (*Monte Arenas* and *Monte Llanillos*) and their corresponding adjacent unburned areas (Control) located in the southeastern section of Valladolid Province (Spain) at the villages of *Portillo*, *Aldeamayor de San Martín* and *La Parrilla* were selected. The altitudes were 766 m.a.s.l. in *Monte Arenas* and 842 m.a.s.l. in *Monte Llanillos*.

The soils were Albic Arenosols, characterized by high contents of sands, low C and N contents, low fertility, and absence of carbonates. Soil horizon properties of a typical profile of the Area are shown in the Table 1.

The vegetation in both plots was mixed forest plantation of *Pinus pinea* L. and *Pinus pinaster* Aiton. The climate of this region was semi-arid, characterized by dry warm summers. Based on climatological data from the past 30 years, mean annual temperature was 12.3°C and mean annual rainfall was 444 mm. The mean annual temperatures of the summer and autumn months were 21°C and 12.4°C, respectively. The mean summer precipitation was 63 mm and the mean autumn precipitation was 131 mm.

Table 1 Characteristics of the studied soils

Hor	Depth (cm)	pH H_2O	E.C.[a] (μS/cm)	SOC[b] (g kg^{-1})	Total N (g kg^{-1})	C/N	P_{Olsen} (mg kg^{-1})	CEC[c] ($cmol_c$ kg^{-1})	Sand		Silt (%)	Clay (%)
									Coarse (%)	Fine (%)		
A1	0–10	6.0	34.4	6.4	0.44	14.5	2.37	4.75	68.9	24.2	2.5	4.4
A2	10–20	6.5	27.3	2.15	0.42	5.1	1.45	3.75	31.0	60.2	3.7	5.1
C	20–55	6.9	22.4	1.60	0.39	4.2	1.05	4.00	58.4	33.6	4.5	4.5

[a] Electrical Conductivity
[b] Soil Organic C
[c] Cation Exchange Capacity
Coarse Sand (0.50–2 mm); Fine Sand (0.05–0.50 mm); Silt (0.05–0.002 mm); Clay (<0.002 mm)

Table 2 Fire severity classes judged from pine canopy damage; description and post-fire mortality of the adult pines (Pausas et al. 2003)

Fire severity classes	Description	Post-fire mortality
LOW	Light fire; canopy trees retain >20% of green leaves (top of the canopy) Trees remain mainly green after the fire.	No
MODERATE	Most leaves (>80%) of canopy trees are scorched (dead) but not consumed. Green leaves may occur on the top (<5%). Trees are mainly brown (retained scorched leaves) after the fire.	Yes
HIGH	Severe fire; canopy trees with >80% of the leaves consumed and the rest (if any) scorched (top). No green leaves left.	Yes

A canopy fire occurred in these areas on June 19th, 2004. Both fires were caused by lightning storms, affecting 554.5 ha of forest in *Monte Arenas* and 155.4 ha of forest in *Monte Llanillos*. It lasted for less than a day. Measurements of fire intensity were not available because of the accidental nature of the fire. Areas little affected by fire alternating with areas seriously impacted could be observed, indicating different fire severity levels.

In the present study, we classified fire severity following the indications of Pausas et al. (2003), who provided a threefold classification of fire severity according to pine canopy damage: low, moderate and high (Table 2).

Three fire severity classes were differentiated in the *Monte Arenas* burned area (low, moderate and high). In the Monte Llanillos burned area only two fire severity classes were found (low and moderate). Only in the high fire severity class, the organic litter layer was consumed.

Soil samples were taken on November 2004, at 0–2 cm depth, after elimination of ashes and litter remains in disturbed soil samples. The precipitation between the dates of fire and the sampling was 99.0 mm. Fifteen sampling points were randomly selected along each experimental area in the burned area and fifteen in the adjacent unburned one. Every sample was made up of 10 sub-samples taken randomly around each sampling point to obtain a composite sample. In Table 3 it can be seen the data of composite samples analyzed.

Table 3 Physical and chemical properties of burned and unburned soils

	Fire severity	n[1]	WHC[2]	Porosity (%)	pH	P_{Olsen}	SOC	N (g kg^{-1} soil)	C^{3}_{W-B}	C_{labile} (mg kg^{-1} soil)	SOC/N	C_{W-B}/SOC	C_{labile}/SOC (%)
Monte Arenas	Control	15	8.8^{a}	46.4^{a}	6.58^{b}	3.1^{d}	17.4^{b}	0.65^{b}	1.0^{b}	78.6^{b}	26.8^{a}	0.64^{a}	4.5^{a}
	L[4]	5	9.5^{a}	48.2^{a}	7.82^{a}	19.0^{c}	16.3^{b}	0.54^{b}	1.1^{b}	84.8^{b}	29.4^{a}	0.66^{a}	5.0^{a}
	M[5]	5	11.2^{a}	46.1^{a}	7.66^{a}	25.0^{b}	22.3^{b}	0.78^{b}	1.4^{b}	126.5^{ab}	26.6^{a}	0.64^{a}	5.7^{a}
	H[6]	5	10.5^{a}	40.7^{a}	7.94^{a}	41.1^{a}	35.8^{a}	1.94^{a}	2.2^{a}	187.0^{a}	18.9^{b}	0.62^{a}	5.2^{a}
Monte Llanillos	Control	15	8.0^{a}	49.5^{a}	6.15^{b}	4.6^{c}	18.7^{a}	0.56^{a}	0.9^{b}	63.7^{b}	29.7^{a}	0.52^{a}	3.5^{a}
	L	7	9.3^{a}	50.9^{a}	7.96^{a}	18.9^{b}	15.4^{a}	0.55^{a}	0.8^{b}	103.4^{a}	31.0^{a}	0.54^{a}	5.3^{a}
	M	8	10.8^{a}	47.7^{a}	7.75^{a}	28.2^{a}	20.1^{a}	0.67^{a}	1.2^{a}	110.8^{a}	29.5^{a}	0.57^{a}	7.8^{a}

[1] Number of composite soil samples
[2] Water holding capacity
[3] Easily oxidizable C determined by the Walkley and Black procedure
[4] Low fire severity
[5] Moderate fire severity
[6] High fire severity
Values followed by the same letter are not significantly different ($P < 0.05$) within the same zone

2.2 Physical and Chemical Soil Characteristics

Water holding capacity (WHC) was determined gravimetrically. Soil porosity was calculated with bulk density (Blake 1965a) and the soil particle density was determined by the pycnometer method (Blake 1965b). Soil pH was determined in a 1:2.5 (w/v) soil:water suspension, easily oxidizable carbon (C_{W-B}) by the Walkley–Black method, and available P (P_{Olsen}) by the Olsen method. Labile organic C (C_{labile}) was extracted by 0.5 M K_2SO_4. The C content in the extracts was estimated using a SKALAR FormacsHT Analyzer. Soil organic carbon (SOC) and total nitrogen were determined with a LECO CHN-2000 analyzer.

2.3 Microbiological Soil Characteristics

Microbial biomass C (MBC) and microbial biomass P (MBP) were determined by the chloroform fumigation-extraction method, using 0.5 M K_2SO_4 as the extractant for MBC (Vance et al. 1987) and 0.5 M $NaHCO_3$ for MBP (Brookes et al. 1982). C contents in the fumigated and non-fumigated extracts were determined and P contents were determined by colorimetry (Murphy and Riley 1962). Carbon concentration in the fumigated sample extracted with 0.5 M K_2SO_4 was considered as labile C (C_{labile}; Haynes 1999).

In order to measure the soil potential microbial respiration (C_{min}), fifty grams of moist soil sample (at 55% of water holding capacity) were placed in 500 ml stoppered glass jars and incubated at 28°C. The CO_2 evolved was collected, after 3 days of incubation, in 10 ml 0.5 M NaOH and determined by back-titration with 0.5 M HCl, after carbonate precipitation with $BaCl_2$ (Alef 1995).

The activity of microorganisms was expressed as metabolic quotient (qCO_2: mg CO_2–C mg^{-1} MBC d^{-1}) which represents the microbial respiration per unit of biomass, and was calculated as reported by Anderson and Domsch (1993). The microbial quotient (MBC/SOC) represented the percentage of microbial biomass carbon with respect to the total organic carbon of soil (Anderson and Domsch 1993). The rate of organic carbon mineralization (C_{min}/SOC) represented the coefficient of endogenous mineralisation (Fierro et al. 2007) and it was expressed as mg CO_2–C g^{-1} SOC h^{-1}. As the available P concentrations in soil were low (Table 2), we considered interesting to know the enzymatic activities related to the P cycle, and acid and alkaline phosphatase activities were measured (Tabatabai and Bremner 1969).

2.4 Statistical Analyses

Normality and homocedasticity of the residuals were tested using the Kolmogorov–Smirnov and Levene tests, respectively. Since most of the variables did not satisfy these assumptions, and the transform of the variables using logarithm and square root

did not resemble a normal distribution, the non-parametric Kruskall-Wallis test was applied. When the Kruskall-Wallis null hypothesis was rejected, post-hoc pair wise comparisons were performed to investigate differences between pairs of means. All statistical analyses were performed using the Statistica 7.0 software package.

3 Results

3.1 Physical and Chemical Soil Characteristics

Physical and chemical characteristics of the soils studied are presented in Table 3. The WHC and porosity values were low and not significant differences were observed due to fire effect. The soil of unburned sites had low organic matter content, with organic C and total N concentrations of 18 and 0.6 g kg^{-1} of soil, respectively. The mean value of P_{Olsen} in unburned soils was also low (3.8 mg P kg^{-1}). The low content of organic matter and mineral nutrients was also reflected in the C_{W-B} and labile C amounts.

The fires did not cause enough soil heating to produce significant changes to the soil physical properties studied. The pH in burned soils was significantly higher than in the control; however, no significant differences were observed in pH among fire severity levels. Olsen P concentrations increased significantly with the increase in fire severity. The concentrations of SOC, N, C_{W-B} and C_{labile} were significantly higher in high fire severity burned soils than in unburned soils. In high severity burned soil, SOC concentration was twice that of the control. The ratios of these parameters (N, C_{W-B} and C_{labile}) referred to SOC were less sensitive to the fire effect than the individual properties.

3.2 Microbiological Soil Characteristics

Microbiological characteristics of the studied soils are presented in Table 4. C_{min}, MBC and MBP, were significantly lower in unburned than in burned soils. Acid phosphatase showed significantly higher values in control than in burned soils. The mean MBC was 108 mg C kg^{-1} in the unburned soils and 206 mg C kg^{-1} in the burned soils. Only MBP showed significant differences among the fire severity levels in both studied areas. The higher the fire severity, the higher the BMP concentrations were obtained. The alkaline phosphatase concentrations were not significantly different either between burned and unburned soils, or among fire severity levels.

Values of the MBC/SOC and C_{min}/SOC corresponding to unburned soils were low; the highest values corresponded to low fire severity soils, while severely burned and control soils presented similar values for these ratios. Microbial biomass was enriched in P, MBC to MBP ratios being two times lower in soils

Table 4 Microbiological properties of burned and unburned soils

	Fire severity	MBC[1]	MBP[2]	C_{min}[3]	Phos.[4] acid	Phos.[4] alk.	MBC/SOC[1] (%)	MBC/MBP[1]	C_{min}/SOC	qCO_2[5]	Phos. acid/MBC[4]	Phos. acid/MBP	Phos. ac/ Phos. alk
Monte Arenas	Control	118.6^{b}	23.5^{d}	36.02^{b}	0.861^{a}	0.490^{a}	0.66^{c}	5.1^{b}	2.18^{ab}	0.015^{a}	7.28^{a}	36.86^{a}	6.34^{a}
	L[6]	229.8^{a}	30.9^{c}	49.76^{ab}	0.214^{b}	0.415^{a}	1.40^{a}	7.4^{a}	3.02^{a}	0.009^{a}	0.94^{b}	6.97^{b}	0.52^{b}
	M[7]	229.6^{a}	73.0^{b}	61.56^{a}	0.215^{b}	0.339^{a}	1.07^{b}	3.1^{c}	2.92^{a}	0.010^{a}	0.94^{b}	2.95^{b}	0.63^{b}
	H[8]	227.6^{a}	107^{a}	69.98^{a}	0.104^{b}	0.382^{a}	0.64^{c}	2.1^{c}	1.96^{b}	0.014^{a}	0.45^{b}	0.98^{b}	0.27^{b}
Monte Llanillos	Control	97.0^{b}	20.9^{c}	30.32^{b}	0.749^{a}	0.267^{a}	0.64^{b}	4.6^{a}	1.76^{b}	0.011^{a}	7.56^{a}	35.37^{a}	2.74^{a}
	L	171.4^{a}	46.7^{b}	65.96^{a}	0.418^{b}	0.553^{a}	1.22^{a}	3.7^{ab}	3.92^{a}	0.014^{a}	2.45^{b}	8.96^{b}	0.37^{b}
	M	172.8^{a}	55.0^{a}	60.21^{a}	0.170^{b}	0.458^{a}	0.88^{b}	3.1^{b}	3.19^{a}	0.016^{a}	0.99^{b}	3.09^{b}	0.76^{b}

[1] Microbial biomass C in mg kg^{-1} soil; MBC/SOC and MBC/MBP in %.
[2] Microbial biomass P in mg kg^{-1} soil
[3] C mineralizable in mg CO_2-C kg^{-1} soil d^{-1} ; C_{min}/SOC in mg CO_2-C g^{-1} SOC d^{-1}
[4] Phosphatase acid or alkaline in µmol *p*NP g^{-1} soil h^{-1}; Phos. acid/MBC in µmol *p*NP g^{-1} MBC h^{-1}; Phos. acid/MBP in µmol *p*NP g^{-1} MBP h^{-1}
[5] Metabolic quotient in mg CO_2-C mg^{-1} MBC h^{-1}
[6] Low fire severity
[7] Moderate fire severity
[8] High fire severity
Values followed by the same letter are not significantly different ($P < 0.05$) within the same zone

affected by high severity fire than in control soils. A significant decrease in acid phosphatase to MBC and to MBP ratios was observed after fire occurrence. These ratios were not significantly different among fire severity levels The metabolic quotient qCO_2 did not show significant differences either between burned and unburned soils, or among fire severity classes. There were significant differences in the acid phosphatase to alkaline phosphatase ratio between unburned and burned soils, but not among fire severity levels.

4 Discussion

The WHC and porosity values of the control are commonly found in sandy soils (Marshal and Holmes 1988), and fire did not affect them.

The effect of fire on SOM amount is highly variable, and depends on several factors, including fire type (canopy or aboveground *versus* underground fires), intensity, slope. These effects may range from almost the total destruction of the SOM to increases that may reach 30% in the surface layers as a consequence of external inputs, mainly from dry leaves and incompletely burned plant materials (González-Pérez et al. 2004). Generally, low severity fires increase total N and SOC concentrations (Fierro et al. 2007; Kara and Bolat 2009; Gray and Dighton 2009) and a decrease of SOC and N is observed after highly severe wildfire (González-Pérez et al. 2004). Our results showed no significant differences in total N and SOC between unburned and low or moderate affected soils and only the areas affected with high severity fire showed significantly higher concentrations than the unburned soils. This fact could indicate that the wildfires studied did not reach high temperatures, although the classification based on the visual inspection of the remnant vegetation allowed differentiating among low, moderately and highly impacted areas. Besides the external inputs of SOM from plant necromass, it is also necessary to consider that litter turns, after fire, into particulate (fine-earth sized particles), which mix with the whole soil material in the organic horizon. This, in turn, causes a net increase in the C content, friable charred organic matter and particulate charcoal (González-Pérez et al. 2004).

Klopatek et al. (1991) stated that one of the greatest initial effects of fire is the reduction of the SOC/N ratio due to the increased mineralization process; however, our results do not support such a statement for low and moderate severity fire level.

Significant increases in easily oxidisable and labile C due to fire effect were observed. Pardini et al. (2004) found that fire tends to affect short-term organic carbon content by converting inaccessible organic forms into usable labile forms via combustion of the litter layer. Fierro et al. (2007) pointed out that fire can supply easily decomposable organic matter in the short time both due to incorporation of partially burned aboveground biomass and to drying and killing of belowground and microbial biomass, being less probable the second if only low soil temperature values have been reached during the fire.

Significant higher pH were found in the burned samples than in the control probably due to the so-called "ash bed effect" of fire on acidic soils (Certini 2005). The soils studied had low available P, and the P added in ash due to the fire increased the availability of P for plants and microorganisms. Phosphorus availability was probably increased not only due to incorporation of nutrients to the soil as ash, but also to increased P mineralization due to higher soil pH (Romanyà et al. 1994). Several authors have reported an increase of soil mineral nutrients following fires (DeBano 1991; Klopatek et al. 1991). Such an increase was explained by an enrichment of soil by ash, which would represent a reservoir for mineral N, P, Ca, K, and Mg (Dumontet et al. 1996). Saá et al. (1993) reported a meaningful 5-fold increase in inorganic soluble P in the 0–5 cm layer of a humic cambisol under *Pinus pinaster* and *Ulex europaeus* one month after wildfire, as compared with the unburned sites. In our study, the P_{Olsen} and pH surface layer increases could be explained by an enrichment of soil by ash and an absence of lixiviation due to the semi-arid climate, characterized by relatively low annual precipitation (less than 450 mm), summer drought and mesic temperature. After fire events, mineral nutrients may temporarily remain in the ash layer, but eventually they may be solubilised, brought downward through the soil profile, and utilized by the growing plants and soil microorganisms (Belillas and Rodá 1993). Although leaching of mineral nutrients could be expected from the pedological characteristics of the soils studied, such leaching was limited in time and intensity by the semi-arid climate and by the low rainfall on the area from fire event to sampling date.

The control MBC and MBP values found in the present study are very low as compared to the values reported by other authors for forest soils. They are even lower than the values found by Dumontet et al. (1996), who obtained a mean of 176 mg C kg^{-1} soil for MBC in unburned soil from a dunal Mediterranean environment.

Biological and biochemical parameters are sensitive to the slight modifications that soil can undergo in the presence of any degrading agent (Nannipieri et al. 1990). Microbial biomass C represents the living component of the organic matter of soil, excluding animals and plant roots. Although microbial biomass usually makes up less than 5% of SOM (Dalal 1998), it carries out many critical functions in the soil ecosystems, among which the following could be pointed out: microbial biomass is both a source and sink for nutrients, it participates in the C, N, P and S transformations, it plays an active role in the degradation of xenobiotic organic compounds and in the immobilization of heavy metals, it participates in the formation of soil structure, etc. (Nannipieri et al. 2002). The MBC/SOC has been proposed as a more sensitive index of soil changes than total organic C (Bastida et al. 2008), since the microbial biomass of a soil responds more quickly to changes than organic matter does. In the soils studied, MBC only represented around 0.65% of SOC for unburned soils and around 1% for burned soils. Wildfire is reported to have variable effects of forest soil microbial biomass. The hypothesis that fire depleted the microbial activity was not supported by the data. The N and P_{Olsen} enrichment and the increase in soil pH observed in the surface layer may be

responsible for the stimulation of microbial biomass growth, resulting in an increase in MBC and MBP concentrations and C mineralization (C_{min}). The effects of fire on soil microbial biomass depend on the intensity and duration of the fire; such effects can range from complete sterilization to little or no effect (Kara and Bolat 2009) and can also be increased, so Fierro et al. (2007) have indicated that the few data concerning Mediterranean-type ecosystems provide evidence of an increase of soil microbial activity and thus mineralization after fire. Microorganisms have a fundamental role in the biogeochemical cycles and in the formation of soil structure, some works have indicated that a high level of microbial activity is needed to maintain adequate soil quality (Bastida et al. 2007; Llorente and Turrión 2010). However, it is not always in this way, a clear example of the opposite situation is the effect of wildfire, which can increase microbial activity and not improve soil quality as can be observed in the present study.

The hypothesis that soil properties could be related to fire severity was supported by the data in the case of chemical properties. The soil in the burned areas was nutrient enriched and this enrichment was higher when high fire severity was reached and no significant differences in MBC and, C_{min} were observed with the fire severity, we can conclude that the three fire severity levels studied had a similar short-term effect on microbial parameters.

The lowest values of the MBC/SOC and C_{min}/SOC corresponded to high fire severity. The increase of MBC and C_{min} due to fire, but not influence of the fire severity level, and the SOC increase with the fire severity due to a higher addition of organic materials could explain this pattern.

The qCO_2 values found in the soils studied are high in comparison with the data reported by other authors (Wardle and Ghani 1995), indicating low microbial efficiency. The qCO_2 values were not affected by the fire-produced disturbance. In the soils studied, qCO_2 was not a sensitive indicator of short-term fire effect and, as Sojka and Upchurch (1999) point out, the use of one or two biochemical properties is not sufficient to demonstrate the complexity of the functioning of this soil system. In general, the qCO_2 value is greater in a distorted ecosystem than in a stable ecosystem (Dalal 1998). However, the significance of this increase is unclear, because it may be due to a drop in efficiency of substrate utilization by the microbiota, to a microbiota response to adverse conditions, to the predominance of zymogene microorganisms over the autochthonous microorganisms, or to the alteration of the bacteria/fungi ratio as they have different carbon use strategies (Dilly and Munch 1998).

Fire often results in effects on organic matter and microbial community structure that can be detected by quantifying enzyme activity. For example, acid phosphatase activity, as an indicator of overall microbial activity, often decreases as a consequence of fire (Saá et al. 1993; Eivazi and Bryan 1996; Boerner et al. 2000). Our results corroborate this affirmation. In the unburned soils studied, acid phosphatase activity was higher than alkaline phosphatase activity; however, both concentrations were similar in burned soils.

Our results showed that acid phosphatase to MBC and to MBP ratios were indexes very sensitive to fire effect, but were not sensitive to fire severity. These ratios decreased significantly with the fire. Landi et al. (2000) indicated that the

ratio between activity parameters and microbial biomass represents a single combination of two different measurements in a single criterion that can give some indications of changes occurring in microbiological activity and ecological information on specific aspects of microbial activity. An increasing ratio may indicate either increasing enzyme production and enzyme release by microorganisms or enhanced release of enzymes immobilized on clay or humic colloids to the soil solution (Kandeler and Eder 1993). On the other hand, rising pH and P content has found to decrease acid phospatase activity (Haynes and Swift 1988).

5 Conclusions

Although the classification based on the visual inspection of the remnant vegetation after fire allowed differentiating three fire severity levels (low, moderate and high), however the results showed that in all cases the reached temperature was low.

Our results showed that low-impact burning in very poor soils can cause short-term increases in the availability of plant and microbial nutrients, and these mineral mobilizations increased microbial activity, although the soil quality was not improved.

The labile carbon fractions (C_{labile} and $C_{W\text{-}B}$) were more sensitive to fire severity than SOC. P_{Olsen} and MBP were the most sensitive of the studied parameters to the effects of fire and fire severity.

The metabolic quotient did not appear as an adequate indicator of microbial response to fire disturbance.

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References

Alef K (1995) Soil respiration. In: Alef K, Nanipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic Press, London, UK, pp 214–222

Anderson T, Domsch KH (1993) The metabolic quotient for CO_2 (qCO_2) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of the soil. Soil Biol Biochem 36:859–868

Andreu V, Imeson AC, Rubio JL (2001) Temporal changes in soil aggregates and water erosion after a wildfire in a Mediterranean pine forest. Catena 44:69–84

Bastida F, Moreno JL, Hernández T, García C (2007) The long-term effects of the management of a forest soil on its carbon content, microbial biomass and activity under a semi-arid climate. Appl Soil Ecol 37:53–62

Bastida F, Zsolnay A, Hernández T, García C (2008) Past, present and future of soil quality indices: a biological perspective. Geoderma 147:159–171

Belillas CM, Rodá F (1993) The effects of fire on water quality, dissolved nutrient losses and the export of particulate matter from dry heathland catchments. J Hydrol 150:1–17

Blake GR (1965a) Bulk density. In: Black CA (ed) Methods of soil analysis: Part I physical and mineralogical properties. American Society of Agronomy, Madison, WI, USA, pp 383–390

Blake GR (1965b) Particle density. In: Black CA (ed) Methods of soil analysis: Part I physical and mineralogical properties. American Society of Agronomy, Madison, WI, USA, pp 371–373

Boerner REJ, Sutherland EK, Morris SJ, Hutchinson TF (2000) Spatial variation in the effects of prescribed fire on N dynamics in a forested landscape. Landscape Ecol 15:425–439

Brookes PC, Powlson DS, Jenkinson DS (1982) Measurement of microbial biomass phosphorus in soil. Soil Biol Biochem 14:319–329

Carreira JA, Lajtha K, Niell FX (1997) Phosphorous transformations along a soil/vegetation series of fire-prone, dolomitic, semi-arid shrublands of southern spain. Biogeochemistry 39:87–120

Certini G (2005) Effects of fire on properties of forest soils: a review. Oecologia 143:1–10

Chafer CJ (2008) A comparison of fire severity measures: an australian example and implications for predicting major areas of soil erosion. Catena 74:235–245

Christensen BT (1996) Carbon in primary and secondary organo-mineral complexes. In: Carter MR, Stewart BA (eds) Advances in soil science structure and organic matter storage in agricultural soils. CRC Lewis Publishers, Boca Raton, FL, USA, pp 97–165

Dalal RC (1998) Soil microbial biomass–what do the numbers really mean? Aust J Exp Agric 38:649–665

DeBano LF 1991 The effect of fire on soil. In: Harvey AE, Neuenschwander LF (Eds) Management and productivity of western-montane forest soils. General technical reported INT-280. U.S. Department of Agriculture, Forest Service, Intermountain Research Station, Ogden, UT, USA, pp 32–50

Dick RP, Breakwill D, Turco R (1996) Soil enzyme activities and biodiversity measurements as integrating biological indicators. In: Doran JE, Jones AJ (eds) Methods for assessment of soil quality. Soil Science Society of America, Specifial Publication 49, Madison, WI, USA, pp 242–272

Dilly O, Munch J-C (1998) Ratios between estimates of microbial biomass content and microbial activity in soils. Biol Fertil Soils 27:374–379

Dumontet S, Dinel H, Scopa A, Mazzatura A, Saricino A (1996) Post-fire soil microbial biomass and nutrient content of a pine forest from a dunal mediterranean environment. Soil Biol Biochem 28:1467–1475

Eivazi F, Bryan MR (1996) Effects of long-term prescribed burning on the activity of selected soil enzymes in an oak-hickory forest. Can J For Res 26:1799–1804

Fernández C, Vega JA, Fonturbel T, Pérez PG, Jiménez E, Madrigal J (2007) Effects of wildfire, salvage jogging and slash treatments on soil degradation. Land Degrad Dev 18:591–607

Fierro A, Rutigliano FA, De Marco A, Castaldi S, Virzo De Santo A (2007) Post-fire stimulation of soil biogenic emission of CO_2 in a sandy soil of a mediterranean shrubland. Int J Wildland Fire 16:573–583

García C, Roldán A, Hernández T (2005) Ability of different plant species to promote microbiological processes in semiarid soil. Geoderma 124:193–202

Gil-Sotres F, Trasar-Cepeda C, Leiros MC, Seoane S (2005) Different approaches to evaluating soil quality using biochemical properties. Soil Biol Biochem 37:877–887

González-Pérez JA, González-Vila JF, Almendros G, Knicker H (2004) The effect of fire on soil organic matter–a review. Environ Int 30:855–870

Gray DM, Dighton J (2009) Nutrient utilization by pine seedlings and soil microbes in oligotrophic pine barrens forest soils subjected to prescribed fire treatment. Soil Biol Biochem 41:1957–1965

Hamman ST, Burke IC, Stromberger ME (2007) Relationships between microbial community structure and soil environmental conditions in a recently burned system. Soil Biol Biochem 39:1703–1711

Hammill KA, Bradstock R, van Wagtendonk J (2001) Remote sensing of fire severity in the blue mountains: influence of vegetation type and inferring fire severity. Int J Wildland Fire 15:213–216

Haynes RJ (1999) Labile organic matter fractions and aggregate stability under short-term grass-based leys. Soil Biol Biochem 31:1821–1830

Hernández T, García C, Reinhard I (1997) Short-term effect of wildfire on the chemical, biochemical and biological properties of mediterranean pine forest soils. Biol Fertil Soils 25:109–116
Kandeler E, Eder G (1993) Effect of cattle slurry in grassland on microbial biomass and on activities of various enzymes. Biol Fertil Soils 16:249–254
Kara O, Bolat I (2009) Short-term effects of wildfire on microbial biomass and abundance in Black pine plantation soils in Turkey. Ecol Indic 9:1151–1155
Klopatek JM, Klopatek CC, DeBano LF (1991) Fire effects on nutrient pools of Woodland floor materials and soils in a pinyin-juniper ecosystem. In: Nodvin SC, Waldrop TA (eds) Fire and the Environment: ecological and cultural perspectives USDA forest service. Knoxville, TN, USA, pp 154–160
Landi L, Renella G, Moreno JL, Falchini L, Nannipieri P (2000) Influence of cadmium on the metabolic quotient, L-:D-glutamic acid respiration and enzyme activity, microbial biomass ratio under laboratory conditions. Biol Fertil Soils 32:8–16
Llorente M, Turrión MB (2010) Microbiological parameters as indicators of soil organic carbon dynamics in relation to different land use management. Eur J For Res 129:73–81
Marshal TJ, Holmes JW (1988) Soil Physics. Cambridge University Press, Cambridge, UK
Murphy J, Riley JP (1962) A modified single solution method for the determination of phosphate in natural water. Anal Chim Acta 27:31–36
Nannipieri P, Ceccanti B, Grego S (1990) Ecological significance of biological activity in soil. In: Bollag J-M, Stotzky G (eds) Soil biochemistry, vol 6. Marcel Dekker, New York, USA, pp 293–355
Nannipieri P, Kandeler E, Ruggiero P (2002) Enzyme activities and microbiological and biochemical processes in soil. In: Burns RG, Dick RP (eds) Enzymes in the environment: activity, ecology and applications. Marcel Dekker, New York, USA, pp 1–34
Pardini G, Gispert M, Dunjó G (2004) Relative influence of wildfire on soil properties and erosion processes in different mediterranean environments in NE Spain. Sci Total Environ 328:237–246
Pausas JG, Ouadah N, Ferran A, Gimeno T, Vallejo R (2003) Fire severity and seedling establishment in pinus halepensis woodlands, eastern iberian peninsula. Plant Ecol 169:205–213
Rab MA (1996) Soil physical and hydrological properties following jogging and slash burning in the eucalyptus regnans forest in southeastern australia. For Ecol Manag 84:159–175
Romanyà J, Khanna PK, Raison RJ (1994) Effects of slash burning on soil phosphorus fractions and sorption and desorption of phosphorus. For Ecol Manag 65:89–103
Romanyà J, Casals P, Vallejo R (2001) Short-term effects of fire on soil nitrogen availability in mediterranean grasslands and shrublands growing in old fields. For Ecol Manag 147:39–53
Ryan KC, Noste NV 1985 Evaluating prescribed fires. In: Lotan JE, Kilgore BM, Fischer WC, Mutch RW (Eds) Proceedings-symposium and workshop on wilderness fire. General technical. report INT–182. USDA, Forest Service, Intermountain Forest and Range Experiment Station, Ogden, UT, USA, pp 230–238
Saá A, Trasar-Cepeda MC, Gil-Sotres F, Carballas T (1993) Changes in soil phosphorus and acid phosphatase activity immediately following forest fires. Soil Biol Biochem 25:1223–1230
Sojka RE, Upchurch DR (1999) Reservations regarding the soil quality concept. Soil Sci Soc Am J 63:1039–1054
Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307
Turrión MB, Lafuente Aroca MJ, López O, Mulas R, Ruipérez C (2010) Characterization of soil phosphorus in a fire-affected forest cambisol by chemical extractions and ^{31}P-NMR spectroscopy analysis. Sci Total Environ 408:3342–3348
Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C. Soil Biol Biochem 19:703–707
Wardle DE, Ghani A (1995) A critique of the microbial metabolic quotient (qCO_2) as a bioindicator of disturbance and ecosystem development. Soil Biol Biochem 27:1601–1610

Part II
Applied Enzymology to Recycle Organic Residues and Environmental Restoration

Use of Microbial Activity and Community Structure Shifts to Estimate the Toxicological Risk of Heavy Metal Pollution in Soils with Different Organic Matter Contents

José L. Moreno, Teresa Hernández and Carlos García

Abstract Microorganisms and extracellular enzymes, which they synthesize and segregate in the soil matrix, are vital for soil fertility and the degradation of organic matter and organic pollutants in soil. The human activities have raised the concentration of heavy metals in the environment, producing toxic effects on soil microorganisms. For these reasons, microbial activity and specific activity of enzymes have been used as sensitive environmental indicators of heavy metals pollution in soils. A paramount factor responsible for the differences in the effects of heavy metals on the microbial activity of soil is its organic matter content. The uncontrolled addition of sewage sludge to soil in order to increase its organic C content may result in a higher concentration of pollutants. The aim of this chapter is to review some results obtained in the study of the toxic effects of heavy metals (Cd, Ni, Zn and Cu) on microbial community structure and activity in soil. Additionally, the importance of soil organic matter in modulating the effect of heavy metals on microbial parameters is reviewed. The results are discussed with regard to recent results obtained by other authors using various methods and approaches to examine this problem.

1 Introduction

Heavy metals are present in background levels as native elements in some soils as a result of the weathering process that acts upon the soil's parent material. However the human activities have raised the concentration heavy metals in the

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environment, producing toxic effects on microorganisms in the soil. The main difference between heavy metal and organic pollution of soils is than once the metals enter the soil, they remain there for extremely long periods of time, having a half-life, which, depending upon the metal, can last several thousands of years. As reported by Adriano (2001), the man-made sources of heavy metals in soils are: commercial fertilizers, liming materials, sewage sludge, animal waste, pesticides, irrigation waters, coal combustion residues, metal-smelting industries and mine waste, auto emissions, and others in the form of municipal solid waste or compost. These sources of heavy metals can impact agro-ecosystems, natural ecosystems, as well as urban and rural areas. Recently, important sources of heavy metal soil pollution are the uncontrolled use of composts obtained from municipal solid wastes, sewage sludge and other biowastes (Moreno et al. 1996; Smith 2009). Cadmium, zinc, nickel, copper, chrome, lead and arsenium are, perhaps, the most studied heavy metals when the toxicological risk of heavy metal pollution on microorganisms was estimated. This is due to the fact that these heavy metals are the most frequently found in polluted soils due to organic waste amendment, deposition of atmospheric pollution, and irrigation with sewage water.

Soil is a paramount, non renewable, resource which is essential for global environment and human activities. Soil performs different functions that affect other compartments of the environment, principally the hydrosphere and the atmosphere. Moreover, these and other functions permit the use of soil in human activities, principally, agriculture, livestock and infrastructural support. As a result, the evaluation and monitorization of the effects of different stresses or pressures on soil functionality and health have been important concerns in recent years. One of the most studied pressures or stressors on soil quality has been heavy metal contamination, specifically its effects on soil microbial communities. Microorganisms and the extracellular enzymes, which they synthesize and segregate in the soil matrix, are vital for soil fertility and the degradation of organic matter and organic pollutants. Soil microbes react quickly to stressful factors and they are evenly distributed in soil. For this reasons, the microbial activity and specific activity of enzymes can be used as a sensitive environmental indicator of heavy metal pollution in soils (Van Beelen and Doelman 1997; Brookes 1995). The early review of Baath (1989) about the effects of heavy metals on microbial processes and populations in non-cultivated temperate forest soil also note this fact. McGrath (1992) analyzed the effect of heavy metal on microbial activity of agricultural soil when sewage sludge was applied as an organic amendment. Giller et al. (1998) focused their review on a discussion of the problems and pitfalls of research on this topic to explain the disparities between the results and conclusions from different types of scientific studies.

Threshold levels of heavy metal in soil have been proposed by different European countries to establish the ecotoxicological risks of soil contamination. The limits proposed in the early regulations were focused on protecting against the negative effects of heavy metals added to soil via the sewage sludge amendment

on crops, on animal grazing on this soil and to protect human health from metal exposure (EEC 1986). In the last regulations, threshold limits are based on microbial toxicity tests (Van Beelen and Doelman 1997). However, as a result of new evidence about the effects of heavy metals on microbial activities and community structure, these limits are currently under review in Europe with heavier restrictions under consideration (ComEC 2006).

Thus, the aim of this chapter is to discuss the results obtained in the study of the toxic effects of heavy metals on the activity and structure of soil microbial communities. These results are discussed with regard to recent results obtained by other authors using various methods and approaches to examine this problem. To estimate these toxic effects on soil microbial population, researchers frequently use tools to measure the microbial population size (microbial biomass C), microbial activity (C mineralization, ATP content, and soil dehydrogenase), hydrolase enzyme activities (urease, phosphatase, β-glucosidase activity, etc.), and microbial diversity (DNA profiling by DGGE and PLFA biomarkers) and the Community Level Physiological Profile (BIOLOG system). Additionally, the importance of soil organic matter for modulating the effect of heavy metals on microbial parameters is reviewed.

2 Effect of Heavy Metals on Microbial Biomass and Activity in Soils with Different Organic Matter Content

A paramount factor responsible for the differences in the effects of heavy metal on soil microbial activity is the organic matter content (Niklinska et al. 1998). As previously mentioned, the uncontrolled addition of sewage sludge to soil in order to increase the organic C content may result in higher heavy metal concentration in soil. This practice is increasingly important in southern European countries which must recycle both sewage water as an extra resource to irrigate crops, after its depuration in treatment plants, and sewage sludge, produced as a result of this process, as an organic soil amendment. Thus, the organic C and other nutrients (N, P and K) present in sewage sludge or compost may be useful to soil microorganisms and plants. This environmentally friendly practice, when it is applied in a controlled form, contributes to decreased use of chemical fertilizers in agriculture and increases the quality and health of degraded soil in semiarid areas (García et al. 2000), which preserves it from the effects of erosion and desertification. Although a heavy metal pollution risk exists when these organic materials are applied to the soil, the heavy metal availability and tolerance of the soil microorganisms may change with the amount of organic matter added (Xie et al. 2009). Thus, when soil is amended with these organic materials, it is important evaluate the effect of heavy metals.

2.1 Effect of Heavy Metals on Microbial Biomass and C Mineralization

One of the experiments about this topic (Moreno et al. 1999) consisted of the incubation of a semiarid soil amended at two different doses (20 t ha^{-1} and 80 t ha^{-1}) of two sewage sludge composts with different concentrations of Cd (2 and 815 mg kg^{-1} dry matter respectively) by adding of Cd salt solutions. Both sewage sludge composts were also contaminated with Zn, Ni and Cu solutions to achieve concentrations of 776, 275 and 105 mg kg^{-1} dry matter respectively of these elements. The Cd concentration of heavily polluted compost surpassed the maximum limit that the EU permits for sewage sludge utilization in agriculture (EEC 1986), while concentrations of the rest of the heavy metals were lower than the EU limits. The concentrations of DTPA-extractable Cd fraction in soils amended with composts at low and high doses were 2.8 and 7.8 mg kg^{-1} respectively. Both composts increased the total organic C (TOC) content, humic substances, and water-soluble C contents of the semiarid soil. These beneficial effects were still noticeable after 120 days of incubation. However, the presence of high Cd levels in the organic amendment inhibited mineralization of the water soluble C. An increase of the available Cd content in the soil amended with the Cd contaminated composts was evident. The high Cd concentration of compost decreased the favourable effect of the organic matter added on microbial biomass and dehydrogenase activity, but increased the metabolic quotient (qCO_2). In this respect, qCO_2 was a very sensitive indicator of the microbial stress in the soil amended with the Cd contaminated compost.

After publication of this study, other authors (Chander et al. 2001) reported that the content of microbial biomass C, ergosterol and ATP did not necessarily decrease with increasing heavy metal content, when the long-term effects of different sources of heavy metal on soil microbial properties were studied. However, increased heavy metal concentrations from different sources of contamination decreased biomass C/soil C ratio and caused a shift in the microbial community structure towards fungi, as demonstrated by the increase of the ergosterol/biomass C ratio. A low biomass C/soil C ratio indicates reduced substrate utilisation efficiencies (Anderson and Domsch 1990). This fact means that more substrate is diverted towards catabolic processes at the expense of anabolic processes, leading to reduced microbial biomass in the long-term (Chander and Brookes 1991). After heavy metal pollution of soil, this shift towards catabolic processes was generally observed and this fact is frequently reflected by increases of qCO_2 (Giller et al. 1998).

The effect of heavy metal in different polluted soils of a landfill area for urban wastes in India was studied by Bhattacharya et al. (2008). These authors found that landfill soils showed significantly higher microbial biomass C than the unpolluted background soil, possibly due to the higher organic matter content of former soils than the latter soil. They explained that the variation of microbial biomass C in the landfill soils was related to the interplay of physicochemical properties as well as

bioavailable metals in these soils. They used the ratio of microbial biomass C to organic C as an indicator of perturbation due to heavy metal. The ratio values of the landfill soils were much lower than the normal range of this ratio (1–4%). Brookes (1995) reported than the ratio values of metal polluted soil generally were 0.4–1%. Bhattacharya et al. (2008) also measured higher basal respiration and SIR values in landfill soils than the background soil. Xie et al. (2009) observed a decrease of Cd and Cu bioavailability and dehydrogenase activity promotion with a long-term compost application. Gibbs et al. (2006) studied the effects of heavy metals (Zn, Cu or Cd) contained in sewage sludge on soil fertility and microbial activity. In this study, metal-rich sludge cakes were applied annually for four years to nine sites. Positive and significant relationships between soil organic C concentration, biomass C and respiration rates were observed. The sludge cake application generally increased soil microbial biomass C and soil respiration rates, although the most probable numbers of clover *Rhizobium* were not affected. In conclusion, these authors observed no evidence that metal applications were damaging to soil microbial activity in the short term after the cessation of sludge cake addition.

Similarly, microbial communities of forest soils, pasture soils or other non-agricultural soils may have different sensitivities to heavy metal exposures, depending on their organic matter content. Moreno et al. (2009) studied the influence of organic matter content of two soil (from a forest and an abandoned agricultural plot) on the toxic effects of different doses of Cd and Zn added to both soils, singly or in combination, on biomass C, C mineralization rate (soil basal respiration) and other parameters related to microbial activity (dehydrogenase activity and ATP content). Cd and Zn were applied in three different doses (3, 6.5 and 12.5 mg Cd kg^{-1} soil; 300, 650 and 1300 mg Zn kg^{-1} soil) which correspond to average values of the new proposed reference levels for these heavy metals in different national regulations of European countries (Aguilar et al. 1995). In general, a significantly higher decrease of the above parameters was noted with the Cd and Zn contamination in the abandoned soil with a lower organic matter content than the forest soil that was rich in organic matter (OM). The different sensitivities of microbial biomass and activity to heavy metal contamination in the two soils may be due to their different total organic C content and OM quality, which can both buffer the negative effects of heavy metals.

2.2 Heavy Metal Effect on Soil Enzyme Activities

The essential catalysts of organic matter decomposition are extracellular enzymes that degrade macromolecules to soluble substrates for microbial assimilation, thus, extracellular enzymes allow microbes to access the otherwise biologically unavailable C and other nutrients in soil organic matter (SOM). Some of these extracellular enzymes catalyse the hydrolysis of polysaccharides as cellulose or lignin, the most abundant components of plant litter, to oligosaccharides. Then, low molecular-weight sugars are used as substrate by other hydrolases such

as β-glucosidase. Proteases, ureases and phosphatases are other important hydrolytic enzymes that are involved as bio-catalysts in the mineralization processes of organic N and P. Moreover, the activities of enzymes have been related to soil microbial community structure (Waldrop et al. 2000). Soil enzymes studies have been used to know the functional diversity of soil microbial communities (Caldwell 2005). In other previous studies, soil biochemical parameters, as enzymatic activities, were measured to determine the toxic effect of Cd-polluted sewage sludge compost on soil microbial activity (Moreno et al. 1999). The exact effect of Cd on enzymatic activities depended on the enzyme studied. Thus, dehydrogenase activity was negatively affected by heavily polluted sewage sludge compost added to soil, suggesting a negative effect of Cd on the synthesis of this endocellular enzyme, which is similar to its effect on the microbial biomass. However, protease-BAA (protease that hydrolyse N-α-benzoil-L-argininamide) and β-glucosidase activities seemed to be unaffected, while urease and alkaline phosphatase activities were stimulated by Cd-polluted compost added to soil. In general, the organic matter added with the sludge compost had a positive effect on the enzymatic activities, which, in some cases, counteracted the negative effects that a high Cd concentration might have had on them.

In the above-mentioned incubation experiment that analysed two semiarid soils with varying organic matter content and artificially polluted with three Cd and Zn doses (Moreno et al. 2009), different effects on urease, β-glucosidase, protease-BAA and alkaline phosphatase activity were observed in the forest soil in comparison to the shrubland soil. The effect of heavy metal contamination on the enzymatic activities was higher in the scrubland soil than the forest soil. The higher TOC content of forest soil and consequently its higher content of available substrates enabled microbial community to grow and resist heavy metal contamination to a higher degree. In the shrubland soil, the most sensitive enzymatic activities were urease and β-glucosidase. The combination of Cd and Zn addition at the highest dose had the most significant effect in decreasing enzymatic activities in both soils. A contrary effect was observed by Chaperon and Sauve (2008) when they assessed the toxicity of Cd, Cu and Pb, alone and in combination, by measuring the dehydrogenase and urease activity of a forest soil. Significant antagonist effects were observed for the majority of the combinations for both the dehydrogenase and the urease assays. Thus, a stimulation of enzymatic activities was observed in the mixtures in comparison to the enzymatic activities obtained with the individual metal.

3 Methods to Analyze Changes on Microbial Diversity in Metal Polluted Soils with Different Organic Matter Content

In addition to microbial activity, the composition of microbial communities determines the biological quality of soil (Martinez-Inigo et al. 2009). The resilience or recovery capacity of soil microbial communities to contamination is

related to high degree of functional redundancies of microbes (Girvan et al. 2005). Heavy metal pollution tends to lead to a decrease in microbial diversity and shifts in the dominance of certain strains and new pollutant-resistant strains occur in contaminated soil (Singh et al. 2003; Renella et al. 2004). This occurs, in terms of species richness, due to the extinction of species which lack sufficient tolerance to the stress imposed, and can potentially lead to the enrichment of particular species which survive well in the face of the stress (Giller et al. 1998). As non-cultivation-based methods have become available, researchers have begun examining the impact of metal exposures on the entire indigenous community (Baath et al. 1998; Kozdroj and van Elsas 2001). DNA, phospholipids fatty acid (PLFA), and Biolog analysis are three commonly used methods for the evaluation of the variation of microbial diversity in polluted soil.

3.1 DNA Diversity

DNA extraction from soil, prior DNA amplification by polymerase chain reaction (PCR), and terminal restriction fragment length polymorphism (TRFLP) or denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) are techniques specifically used to analyze the products (18S or 16S rRNA genes) that characterise microbial communities. These are wide-spread methods to assess the impact of heavy metal contamination on soil microbial diversity. Exposures to elevated concentrations of heavy metals can change the structure of soil microbial communities, as detected by DGGE analysis (Ros et al. 2009; Anderson et al. 2009a and 2009b; Martinez-Inigo et al. 2009; Renella et al. 2004). In the study of Ros et al. (2009), an experiment was conducted to investigate the effect of two different concentrations of Cd, 6.5 and 12.5 mg kg^{-1} on microbial community activity, abundance, and structure in semiarid soil after a 60-day lab incubation. Changes in the microbial community structure were observed by PCR-DGGE of group-specific 16S rRNA genes (acidobacteria, α-proteobacteria, and β-proteobacteria) and 18S rRNA genes (fungi). High level contamination of soil significantly reduced the microbial diversity of different bacterial groups, suggesting that this concentration had a negative effect on bacterial diversity compared to the low level contamination, which produced an increased bacterial diversity index. Meanwhile, fungi diversity index decreased as soil Cd concentration increased, suggesting that both concentrations have a negative effect on fungal diversity, and they were less adapted to or more inhibited by Cd contamination than bacterial groups. However, the fungal abundances were less influenced by increased Cd pollution and more easily thrive in this Cd-polluted soil. This data suggested that although fungus diversity diminished, the abundance of the fungal population adapted to high-level Cd concentration could increase.

In another study by Anderson et al. (2009b), three soils located near a copper smelting plant, previously contaminated with background, low and high levels of aerially deposited metals were amended with metal-salts to determine the potential

of metal contamination to shape the structural and functional diversity of microbial communities. Amendment of these soils with metal-salts resulted in shifts in microbial community structure. Microbial communities from each site did not converge on a structurally or functionally similar community following metal-salt amendment, indicating that other factors may be equally important in shaping the microbial communities in soil. Among these factors, soil physicochemical parameters like organic matter and pH, which can both influence the bioavailability and toxicity of metals in soils, may be critical. Martinez-Iñigo et al. (2009) studied the bacterial community PCR-DGGE profiles of two soils with different physicochemical characteristics and treated them with two doses of Pb, Cr, Zn and Cd and cultivated with the metal-tolerant plant *Silene vulgaris* (Moench) Garcke. They observed that after growing the metal-tolerant plant, new bands appeared in the PCR-DGGE profiles of the rhizosphere bacterial community, and this fact seemed to be useful biological indicator for monitoring the reclamation process.

Anderson et al. (2008) reported the effect of sewage addition, including sludge rich in the metals cadmium, copper and zinc, on soil fungal community composition using both an rDNA and rRNA-DGGE approach. RNA was used alongside DNA, as it is thought that metabolically active species will transcribe more rRNA than inactive species (Prosser 2002). Thus, RNA- and DNA-based approaches can detect different members of the fungal community from the same sample (Anderson and Parkin 2007). Within the rRNA gene cluster of fungi, the target regions most commonly used in ecological studies of fungal communities are the genes encoding 18S rRNA and 25/28S rRNA, and the internal transcribed spacer (ITS) region that incorporates the 5.8S rRNA gene. The highly variable nature of rapidly evolving rDNA spacer regions has made the ITS the most popular choice for species level identification of fungal taxa in environmental DNA pools (Anderson and Cairney 2004). Anderson et al. (2008) observed that sewage sludge addition altered fungal ITS-DGGE banding patterns; however, no additional effects of an increase in soil heavy metal concentrations were observed in the soil. Their data demonstrates that although an increase in soil organic matter content alters fungal community diversity and composition, increasing soil concentrations of Cd, Cu and Zn up to current UK legislative limits had little additional effect regardless of whether rRNA or rDNA was analysed. This suggested that current UK limits for these three heavy metals are within a concentration range that the dominant soil fungi at this field site can tolerate.

Lazzaro et al. (2006) compared indicators of Cd bioavailability and ecotoxicity in forest soils with contrasting physicochemical characteristics. They investigated microbial activity parameters and changes in bacterial community structure using terminal restriction fragment length polymorphism (T-RFLP) with the addition of different Cd doses to the forest soils in a microcosm experiment. These authors found that soil with a pH higher than 6.7 and clay content higher than 50% showed inhibited basal respiration, but no marked shift in bacterial community structure with Cd additions at bioavailable concentrations exceeding United Nations/European Economic Commission guidelines for Cd concentration in the soil solution (0.8 μg dissolved Cd L^{-1}; De Vries et al. 2001). However, soils with a pH

lower than 5.8 and clay content lower than 50% showed additional changes in bacterial community structure.

Nakatsu et al. (2005) conducted a microcosm experiment on soil contaminated with Pb, Cr and aromatic hydrocarbons to determine the response of microbial communities to the addition of glucose or xilene, either alone or simultaneously, with Cr or Pb concentrations. The aim of this study was to segregate the effects of the two metals, as well as to examine the impact of aromatic hydrocarbons on microbial community structure and activity. Organic substrates were added as a driving force for change in the microbial community. Glucose represented an energy source used by a broad variety of bacteria, whereas fewer soil species were expected to use xylene, which mimics aromatic compounds present in these soils. The addition of organic carbon alone had the most significant impact on community composition and led to the proliferation of a few dominant phylotypes, as detected by PCR-DGGE of 16 rRNA genes. The effect of heavy metal addition on soil microbial communities differed between the two carbon sources. For glucose, either Pb or Cr produced significant changes and replacement with new phylotypes. In contrast, many phylotypes selected by xylene treatment were retained when either metal was added. *Actinobacteria* and gene copies of biphenyl dioxigenase and phenol hydroxilase were very prevalent in microcosms with xilene and Cr(VI). Much lower metal concentrations were needed to inhibit the catabolism of xilene than of glucose.

Renella et al. (2005) reported shifts on bacterial diversity, as determined by PCR-DGGE in soil amended with bimetallic sludges. The most important changes were observed in the soils amended with high-level Ni–Cd sludge. In another experiment, where the long-term effects of high Cd concentration on microbial activity and bacterial community structure of soils was assessed (Renella et al. 2004), slight changes to the bacterial community structure in maize cropped soil containing high Cd concentrations as compared to the control were reported. These authors concluded that high Cd concentrations induced mainly physiological adaptations rather than selection for metal-resistant bacterial species.

3.2 PLFA Biomarkers

Phospholipid fatty acids (PLFA) are essential components of every living cell and are useful biomarkers that offer sensitive and reproducible measurement for characterizing the numerically dominant portion of soil microbial communities without cultivating organisms (Kaur et al. 2005). They are considered to be representative of the viable microbial community; they are not found in storage products or in dead cells because, upon cell death, the phosphate group is quickly hydrolyzed (Zelles 1999). PLFA profiles give estimates of both microbial community composition and biomass size. Unlike nucleic acid profiling, which is high specie specific, PLFAs largely profile functional groups. PLFA analysis has been used to detect various environmental stresses, and as result, this method has

been frequently used to characterise microbial communities from heavy metal-contaminated soils (Shi et al. 2002; Rajapaksha et al. 2004; Hinojosa et al. 2005; Shentu et al. 2008). Farrell et al. (2010), using PLFA profiling of metal-contaminated soil from a mine spoil, which was amended with compost, reported that this remediation strategy enhanced microbial diversity in the subsoil. However, these authors did not observe an increase in microbial activity in the subsoil. It was concluded that although compost can successfully immobilize heavy metals and promote ecosystem diversity and function, surface incorporation had little remedial effect below the surface layer over the course of the short term trial. Shentu et al. (2008) studied microbial community diversity in soil affected by different Cd exposure levels. Cd-contaminated soil showed decreases in PLFA biomarkers for Gram-negative bacteria and actinomycetes while the PLFA biomarker for Gram-positive and fungi increased with higher levels of Cd contamination. Akerblom et al. (2007) also observed an increase of Gram-positive bacteria and a decrease of Gram-negative bacteria that could be attributed to heavy metal stress in soil incubated with several heavy metals for 64 days. However, these authors reported that PLFA biomarkers for actinomycetes increased with heavy metal contamination of soil. The fungi/bacteria ratio is found to increase with higher levels of Cd contamination; this finding supported the suggestion that fungi were more resistant to heavy metals than bacteria (Rajapaksha et al. 2004; Baath et al. 2005; Akerblom et al. 2007; Shentu et al. 2008). Hinojosa et al. (2005) found that the monounsatured to satured PLFA ratio was higher in non-polluted soil than in polluted soil. High values of this ratio indicate high substrate availability but the toxic effects of the metals inhibit their utilization (Zelles et al. 1992 and 1995, Bossio and Scow 1998; Hinojosa et al. 2005). In other soil incubation studies, an increase in the *trans*/*cis* ratio of 16:1ω7 with different heavy metals has been documented, and therefore, this ratio can be used as a metal stress bioindicator (Kaur et al. 2005).

3.3 Biolog Profiles

The Biolog approach gives information about substrate utilization and the functional diversity (Zak et al. 1994) of soil bacteria. The Biolog ECO plates system uses 31 carbon substrates, six of which are not present in the GN plates; these substrates are predominantly amino acids, carbohydrates and carboxylic acids. The assay is based on measuring the oxidative catabolism of the substrates to generate patterns of potential sole carbon source utilization. This simple technique uses an automated measuring apparatus and provides a more meaningful assay of community structure than isolate-based methods. The advantage of the Biolog approach is its easy applicability, which makes this method feasible for use in large-scale field studies. However, there are several aspects and drawbacks that must be taken into account. Standardization of inoculum density is important, since inoculum cell density and rate of colour development are strongly related

(Palojärvi et al. 1997). Additionally, this method reflects the metabolic capabilities of only a subset of the whole community since the method is selective for organisms actively metabolizing under the given conditions on the microtiter plates. These conditions usually deviate largely from those in the environment.

Several studies have indicated that Biolog was a useful method for assessing the effect of heavy metals on the functional diversity of soil microbial communities. Anderson et al. (2009a) observed evidence of functional redundancy within microbial communities native to the smelter-impacted sites based on overlapping carbon substrate utilization patterns. A decrease of the functional diversity of soil bacterial communities has been the most common effect of short-term heavy metal pollution as reported by several authors (Stefanowicz et al. 2008). Microbial communities of polluted soils are influenced not only by heavy metal, but also by other environmental factors such as soil pH, temperature, moisture and organic matter quality. Therefore, it may be difficult to attribute the observed effects to heavy metal (Gong et al. 2002). In order to overcome this problem, the measurement of pollution-induced community tolerance (PICT) by the Biolog test has been recently used by several authors (Stefanowicz et al. 2009; Niklinska et al. 2005, Van Beelen et al. 2004). The increased community tolerance to the soil pollution indicates a damaging effect of the pollutant. In tolerant communities, the biodiversity may be decreased and the tolerant species may not always be able to perform the same ecological functions as the sensitive ones (Giller et al. 1998). Stefanowicz et al. (2009) found pollution-induced tolerance of bacterial communities in polluted meadow soils as an effect of long-term heavy metal pollution with the use of the Biolog ECO plates system. Similar findings were previously reported by Niklinska et al. (2005) based on Biolog ECO plate assays. PICT of microorganisms to Cu at the organic layer of Cu-polluted forest soils was found for five substrates. At the organic layer of Zn-polluted forest soils significant PICT to Zn was found for 10 out of 31 substrates. Moreover, the Cu-polluted samples exhibited lower resistance to Zn and the Zn-polluted samples to Cu compared with the unpolluted samples, which indicate that at the polluted sites, microbial resistance to additional stress caused by another metal was decreased Fig. 1.

3.4 Assessing Toxicological Risk of Heavy Metals to Soil Microorganisms by Estimation of Their Ecological Doses

It is important to ascertain the maximum amounts of heavy metals that can be supported by a soil without any effect on its quality. In order to easily quantify the influence of pollutants on microbe-mediated processes in soil, Babich et al. (1983) developed the concept of an "ecological dose 50%" (ED_{50}), defined as the concentration of a toxicant that inhibits a microbe-mediated ecological process by 50%. However, a 50% reduction in a basic ecological process may be too extreme for the continued function of a soil. Moreover, in ecotoxicology, it was recognized than only a few species and/or microbial functions of whole present in a polluted

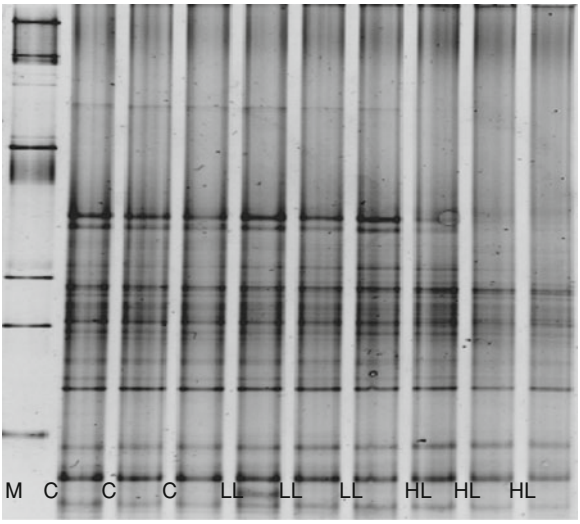

Fig. 1 DGGE banding pattern of acidobacteria 16S rRNA genes in soil under different Cd concentration. *M*: marker; *C*: control; *LL*: low level of Cd; *HL*: high level of Cd (Ros et al. 2009)

soil can be tested in the laboratory. Thus, lower percentage values of inhibition (5, 10 or 25%) equivalent to ED_5, ED_{10} or ED_{25} need to be established for regulatory purposes. The ED_{50} can also be useful in determining which factors affect the toxicity of the heavy metal. These factors can include time (Doelman and Haanstra 1986) and soil abiotic properties (Doelman and Haanstra 1984) affecting the speciation and mobility of heavy metals in soil and controlling their availability and residence time. In addition, the ED_{50} can be used to establish which microbiological and biochemical properties of soil are the most sensitive to heavy metal contamination (Speir et al. 1995). Different mathematical models have been used to calculate the ED_{50} values. Speir et al. (1995, 1999) employed a Michaelis–Menten kinetic approach to model inhibition of soil biological properties by Cr (VI) and As (V). The algebraic expressions of kinetic models were:

$$v = c/(1 + bi) \qquad \text{(Model 1)}$$

$$v = c(1 + ai)/(1 + bi) \text{ (Model 2).}$$

The constants a, b and c were always positive with $b > a$. The constant c represents the uninhibited value of the tested parameter, and the constants a and b depends on the curve slope. Model 1 describes the full inhibition of v (tested parameter) by i, the concentration of inhibitor (Cd concentration) and Model 2 describes the partial inhibition. The mathematical equation for the sigmoidal dose–response model was:

$$y = a/\{1 + \exp[b(x - c)]\} \text{ (Model 3),}$$

where y is the tested parameter, x is the natural logarithm of Cd concentration, a is the uninhibited value of y, b is a slope parameter indicating the inhibition rate, and

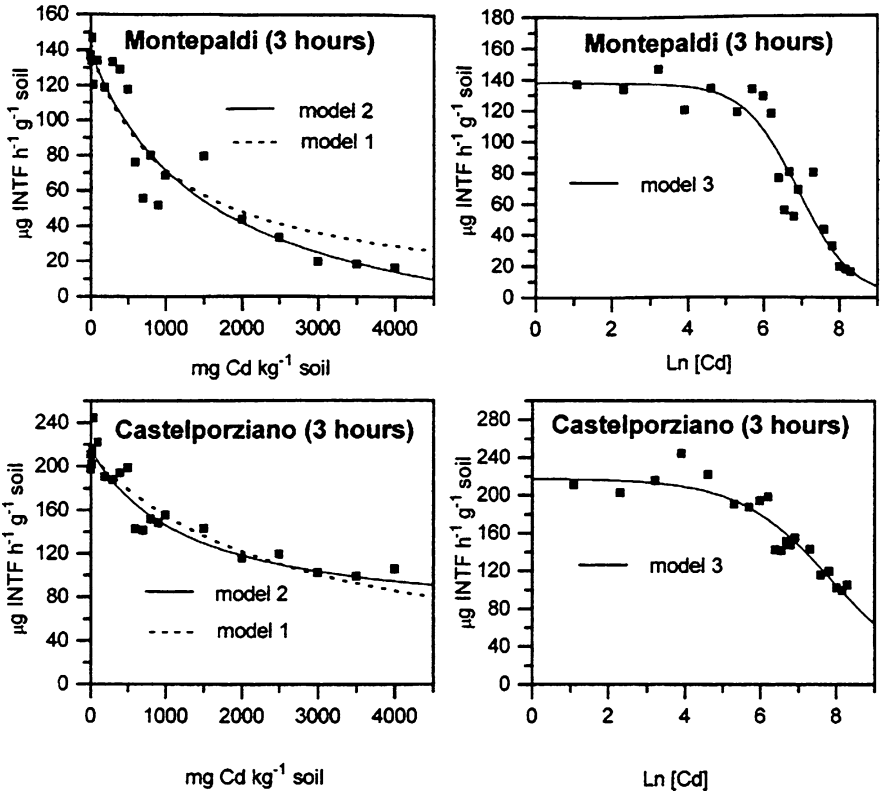

Fig. 2 Experimental data and calculated plots of the relationships between soil dehydrogenase activity and Cd concentration according to the three models used to calculate ecological doses (Moreno et al. 2001)

c is the natural logarithm of ED_{50}. A graphic representation of these functions is shown in Fig. 2.

It was reported that Model 1 was the most successful one in calculating the ED_{50} values for the inhibition of the soil ATP content, urease and dehydrogenase activities when increasing Cd doses on two contaminated soils with different physicochemical characteristics (Moreno et al. 2001). In this paper, it was shown that different ED_{50} values were estimated in function of the soil characteristics and the duration of Cd exposures. ED_{50} values of Cd estimated in the clayey soil were higher than those in the sandy soil (Fig. 2). An increase of ED_{50} values with the duration of Cd exposure was also reported.

Urban wastes, such as sewage sludge, are increasingly used as soil organic amendments, especially to soils containing little organic matter, to maintain or improve soil quality, and this practice can affect the mobility of heavy metal in the polluted soil. The effects of soil pollution by Cd and Ni on the activities of urease, phosphatase, β-glucosidase and protease-BAA, which are important in the cycling

of soil C, N, and P, have been investigated, and the consequences on this effect of the addition of sewage sludge to soil have been determined (Moreno et al. 2003). Two soils (amended and unamended with sewage sludge) were spiked with a wide range of Cd and Ni concentrations. The ED_{50} values and two other ecological values (ED_{10} and ED_5) over three different incubation periods were also determined. Amendment with sewage sludge decreased the negative effects of Cd and Ni on urease and phosphatase activities. There was no effect of exposure time on the toxicity of Cd or Ni to the enzymatic activities. Recently, Speir et al. (2007) established a field trial to assess the impacts on some biological properties of the application of heavy metal-spiked sewage sludge, with the aim of determining toxicity threshold concentrations of heavy metals in soil. A sigmoidal dose–response model was used to calculate ED_{50} and ED_{20} using normalized data by expressing the activities as percentages of those measured in a control plot. The model parameters had very broad 95% confidence intervals and/or the fits to the model had small R^2 values, and thus only ED_{20} values could be calculated. It was concluded that Cu and Zn at the highest concentrations used in this experiment were possibly having adverse effects on some biological properties of the soil.

4 Conclusions

Heavy metals represent and stressing factor for soil microbial communities and the determination of maximum levels in the heavy metal concentrations which no produce effect in the soil functionality is a common aim in numerous studies. Numerous studies have demonstrated the importance of soil organic matter (SOM) on the response of the microbial communities to heavy metal concentration. Different approaches and measurement of soil parameters, such as enzymes activities, microbial respiration and biomass C have been used in order to understand the ecotoxicological risk of heavy metal to soil microbial communities. The data obtained in these early studies together with other obtained with molecular techniques (DNA and PLFA profiling) and BIOLOG assay, used to determine shifts in the soil microbial communities structure and the functional diversity respectively, are helping to establish more accurately the consequences of heavy metal pollution in soil microbial ecology. Accordingly, new criteria and threshold values for heavy metal concentration are being proposed in order to avoid irreversible effect on soil quality and preserve crops, animals and human beings from these kinds of toxic pollutants.

References

Adriano DC (2001) Trace elements in Terrestrial environments. Biogeochemistry bioavailability and risk of metals, Springer, New York

Aguilar, J., Dorronsoro, C., Gómez-Ariz, J.L., Galán, E. Los criterios y estándares para declarar un suelo contaminado en Andalucía y la metodología y técnicas de toma de muestra y análisis

para su investigación. In: Investigación y Desarrollo Medioambiental en Andalucía (1995-1998). Universidad de Sevilla, Seville, Spain, pp. 61-64

Akerblom S, Bååth E, Bringmark L, Bringmark E (2007) Experimentally induced effects of heavy metal on microbial activity and community structure of forest mor layers. Biol Fertil Soils 44:79–91

Anderson IC, Cairney JWG (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ Microbiol 6:769–779

Anderson T-H, Domsch KH (1990) Application of eco-physiological quotients (qCO_2 and qD) on microbial biomasses from soils of different cropping histories. Soil Biol Biochem 22:251–255

Anderson IC, Parkin PI (2007) Detection of active soil fungi by RT-PCR amplification of precursors rRNA molecules. J Microbiol Meth 68:248–253

Anderson IC, Parkin PI, Campbell CD (2008) DNA- and RNA-derived assessments of fungal community composition in soil amended with sewage sludge rich in cadmium, copper and zinc. Soil Biol Biochem 40:2358–2365

Anderson JAH, Hooper MJ, Zak JC, Cox SB (2009a) Characterization of the structural and functional diversity of indigenous soil microbial communities in smelter-impacted and nonimpacted soils. Environ Toxicol Chem 28:534–541

Anderson JAH, Hooper MJ, Zak JC, Cox SB (2009b) Molecular and Functional Assessment of Bacterial Community Convergence in Metal-Amended Soils. Microb Ecol 58:10–22

Bååth E (1989) Effects of heavy metals in soil on microbial process and population (a review). Water Air Soil Pollut 47:335–379

Bååth E, Frostegard A, Díaz-Ravina M, Tunlid A (1998) Microbial community-based measurements to estimate heavy metal effects in soil: The use of phospholipid fatty acid patterns and bacterial community tolerance. Ambio 27:58–61

Bååth E, Diaz-Ravina M, Bakken LR (2005) Microbial biomass, community structure and metal tolerance of a naturally Pb-enriched forest soil. Microb Ecol 50:496–505

Babich H, Bewley RJF, Stotzky G (1983) Application of the "Ecological Dose" concept to the impact of heavy metal on some microbe-mediated ecologic processes in soil. Arch Environ Contam Toxicol 12:421–426

Bhattacharyya P, Mitra A, Chakrabarti K, Chattopadhyay DJ, Chakraborty A, Kim K (2008) Effect of heavy metals on microbial biomass and activities in century old landfill soil. Environ Monit Assess 136:299–306

Bossio DA, Scow KM (1998) Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. Microb Ecol 35:265–278

Brookes PC (1995) The use of microbial parameters in monitoring soil pollution by heavy metals. Biol Fertil Soil 19:269–279

Caldwell BA (2005) Enzyme activities as a component of soil biodiversity. A review Pedobiologia 49:637–644

Chander K, Brookes PC (1991) Microbial biomass dynamics during the decomposition of glucose and maize in metal contaminated and non-contaminated soils. Soil Biol Biochem 23:1169–1177

Chander K, Dyckmans J, Joergensen RG, Meyer B, Raubuch M (2001) Different sources of heavy metals and their long-term effects on soil microbial properties. Biol Fertil Soils 34:241–247

Chaperon S, Sauve S (2008) Toxicity interactions of cadmium, copper, and lead on soil urease and dehydrogenase activity in relation to chemical speciation. Ecotoxicol Environ Saf 70:1–9

ComEC (Commission of the European Communities) (2006) Thematic strategy for soil protection. COM(2006) 231 final, 22 September. Brussels

De Vries W, Shuetze G, Farret R, Ashmore M, Groenenberg BJ, Romkens P (2001) Proposed harmonised critical limits and transfer functions for the calculation of critical loads for lead and cadmium. Final report of working phase. DLO Winand Staring Centre, Wageningen, The Netherlands 56

Doelman P, Haanstra L (1984) Short-term and long-term effects of cadmium, chromium, copper, nickel, lead and zinc on soil microbial respiration in relation to abiotic soil factors. Plant Soil 79:317–327

Doelman P, Haanstra L (1986) Short-and long-term effects of heavy metals on urease activity in soils. Biol Fertil Soil 2:213–218

EEC (1986) Council directive on the protection of the environment, and in particular of soil, when sewage sludge is used in agriculture. Official J Eur Community, Directive 86/278/ECC, No L181/6-12

Farrell M, Griffith GW, Hobbs PJ, Perkins WT, Jones DL (2010) Microbial diversity and activity are increased by compost amendment of metal-contaminated soil. FEMS Microbiol Ecol 71:94–105

García C, Hernández T, Pascual J, Moreno JL, Ros M (2000) Microbial activity in soils of SE Spain exponed to degradation and desertification processes-strategies for their rehabilitation. In: García C, Hernández MT (eds) Research and perspectives of soil enzymology in spain. CSIC, Spain, pp 93–143

Gibbs PA, Chambers BJ, Chaudri AM, McGrath SP, Carlton-Smith CH, Bacon JR, Campbell CD, Aitken MN (2006) Initial results from a long-term, multi-site field study of the effects on soil fertility and microbial activity of sludge cakes containing heavy metals. Soil Use Manag 22:11–21

Giller KE, Witter E, McGrath SP (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol Biochem 30:1389–1414

Girvan MS, Campbell CD, Killham K, Prosser JI, Glover LA (2005) Bacterial diversity promotes community stability and functional resilience after perturbation. Environ Microbiol 7:301–313

Gong P, Siciliano SD, Srivastava S, Greer CW, Sunahara G (2002) Assessment of pollution-induced community tolerance to heavy metals in soil using ammonia-oxidizing bacteria and biolog assay. Human Ecol Risk Assess 8:1067–1081

Hinojosa MB, Carreira JA, Garcia-Ruiz R, Dick RP (2005) Microbial response to heavy metal-polluted soils: Community analysis from phospholipid-linked fatty acids and ester-linked fatty acids extracts. J Environ Qual 34:1789–1800

Kaur A, Chaudhary A, Kaur A, Choudhary R, Kaushik R (2005) Phospholipid fatty acid-a bioindicator of environment monitoring and assessment in soil ecosystem. Curr Sci 89:1103–1112

Kozdroj J, van Elsas JD (2001) Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. J Microbiol Meth 43:197–212

Lazzaro A, Hartmann M, Blaser P, Widmer F, Schulin R, Frey B (2006) Bacterial community structure and activity in different Cd-treated forest soils. FEMS Microbiol Ecol 58:278–292

Martinez-Inigo MJ, Perez-Sanz A, Ortiz I, Alonso J, Alarcon R, Garcia P, Lobo MC (2009) Bulk soil and rhizosphere bacterial community PCR-DGGE profiles and beta-galactosidase activity as indicators of biological quality in soils contaminated by heavy metals and cultivated with *Silene vulgaris* (Moench) Garcke. Chemosphere 75:1376–1381

McGrath SP (1992) Effects of heavy metals from sewage sludge on soil microbes in agricultural ecosystems. In: Ross SM (ed) Toxic metals in soil-plant systems. Wiley, New York, pp 247–274

Moreno JL, Garcia C, Hernandez T, Pascual JA (1996) Transference of heavy metals from a calcareous soil amended with sewage-sludge compost to barley plants. Bioresour Technol 55:251–258

Moreno JL, Hernández T, García C (1999) Effects of a cadmium-contaminated sewage sludge compost on dynamics of organic matter and microbial activity in an arid soil. Biol Fertil Soil 28:230–237

Moreno JL, Garcia C, Landi L, Falchini L, Pietramellara G, Nannipieri P (2001) The ecological dose value (ED_{50}) for assessing Cd toxicity on ATP content and dehydrogenase and urease activities of soil. Soil Biol Biochem 33:483–489

Moreno JL, Garcia C, Hernández T (2003) Toxic effect of cadmium and nickel on soil enzymes and influence of adding sewage sludge. Eur J Soil Sci 54:64–71

Moreno JL, Bastida F, Ros M, Hernandez T, Garcia C (2009) Soil organic carbon buffers heavy metal contamination on semiarid soils: Effects of different metal threshold levels on soil microbial activity. Eur J Soil Biol 45:220–228

Nakatsu CH, Carmosini N, Baldwin B, Beasley F, Kourtev P, Konopka A (2005) Soil microbial community responses to additions of organic carbon substrates and heavy metals (Pb and Cr). Appl Environ Microbiol 71:7679–7689

Niklinska M, Laskowski R, Maryanski M (1998) Effect of heavy metals and storage time on two types of forest litter: Basal respiration rate and exchangeable metals. Ecotoxicol Environ Saf 41:8–18

Niklinska M, Chodak M, Laskowski R (2005) Characterization of the forest humus microbial community in a heavy metal polluted area. Soil Biol Biochem 37:2185–2194

Palojärvi A, Sharma S, Rangger A, von Lützow M, Insam H (1997) Comparison of biolog and phospholipid fatty acid patterns to detect changes in microbial community. In: Insam H, Rangger A (eds) Microbial Communities. Functional versus structural approaches, Springer-Verlag, Berlin, Heidelberg, pp 37–48

Prosser JI (2002) Molecular and functional diversity in soil microorganisms. Plant Soil 244:9–17

Rajapaksha R, Tobor-Kaplon MA, Baath E (2004) Metal toxicity affects fungal and bacterial activities in soil differently. Appl Environ Microbiol 70:2966–2973

Renella G, Mench M, van der Lelie D, Pietramellara G, Ascher J, Ceccherini MT, Landi L, Nannipieri P (2004) Hydrolase activity, microbial biomass and community structure in long-term Cd-contaminated soils. Soil Biol Biochem 36:443–451

Renella G, Mench M, Gelsomino A, Landi L, Nannipieri P (2005) Functional activity and microbial community structure in soils amended with bimetallic sludges. Soil Biol Biochem 37:1498–1506

Ros M, Pascual JA, Moreno JL, Hernández MT, García C (2009) Evaluation of microbial communty, abundante and structure in a semiarid soil under cadmium pollution at laboratory level. Water Air Soil Pollut 203:229–242

Shentu JL, He ZL, Yang XE, Li TQ (2008) Microbial activity and community diversity in a variable charge soil as affected by cadmium exposure levels and time. J Zhejiang Univ-Sci B 9:250–260

Shi W, Becker J, Bischoff M, Turco RF, Konopka AE (2002) Association of microbial community composition and activity with lead, chromium, and hydrocarbon contamination. Appl Environ Microbiol 68:3859–3866

Singh BK, Walker A, Morgan JAW, Wright DJ (2003) Effects of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. Appl Environ Microbiol 69:5198–5206

Smith SR (2009) A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge. Environ Int 35:142–156

Speir TW, Kettles HA, Parshotam A, Searle PL, Vlaar LNC (1995) A simple kinetic approach to derive the ecological dose value, ED_{50}, for the assessment of Cr(VI) toxicity to soil biological properties. Soil Biol Biochem 27:801–810

Speir TW, Kettles HA, Percival HJ, Parshotam A (1999) Is soil acidification the cause of biochemical responses when soils are amended with heavy metal salts? Soil Biol Biochem 31:1953–1961

Speir TW, van Schaik AP, Hunter LC, Ryburn JL, Percival HJ (2007) Attempts to derive EC_{50} values for heavy metals from land-applied Cu-Ni-, and Zn-spiked sewage sludge. Soil Biol Biochem 39:539–549

Stefanowicz AM, Niklinska M, Laskowski R (2008) Metals affect soil bacterial and fungal functional diversity differently materials and methods. Environ Toxicol Chem 27:591–598

Stefanowicz AM, Niklinska M, Laskowski R (2009) Pollution-induced tolerance of soil bacterial communities in meadow and forest ecosystems polluted with heavy metals. Eur J Soil Biol 45:363–369

Van Beelen P, Doelman P (1997) Significance and application of microbial toxicity tests in assessing ecotoxicology risk of contaminants in soil and sediments. Chemosphere 34:455–499

Van Beelen P, Wouterse M, Posthuma L, Rutgers M (2004) Location-specific ecotoxicological risk assessment of metal-polluted soils. Environ Toxicol Chem 23:2769–2779

Waldrop MP, Balser TC, Firestone MK (2000) Linking microbial community composition to function in a tropical soil. Soil Biol Biochem 32:1837–1846

Xie WJ, Zhou JM, Wang HY, Chen XQ, Lu ZH, Yu JB, Chen XB (2009) Short-term effects of copper, cadmium and cypermethrin on dehydrogenase activity and microbial functional diversity in soils after long-term mineral or organic fertilization. Agric Ecosyst Environ 129:450–456

Zak JC, Willig MR, Moorhead DL, Wildman HG (1994) Functional diversity of microbial communities: A quantitative approach. Soil Biol Biochem 26:1101–1108

Zelles L (1999) Fatty acid patterns of phopholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. Biol Fertil Soil 29:111–129

Zelles L, BAI QY, Beck T, Beese F (1992) Signature fatty acids in phopholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biol Biochem 24:317–323

Progress in Microbial Activity and Chemical Properties of a Trace Element Polluted Soil Under Assisted Natural Remediation

Alfredo Pérez-de-Mora, Pilar Burgos, Francisco Cabrera and Engracia Madejón

Abstract In this work, we studied the temporal dynamics of several microbiological properties in a trace element polluted soil under the influence of various amendments and/or a plant cover during a 30 month-period. The experiment was carried out in containers filled with ca. 150 kg of contaminated soil. Seven treatments were established: four organic (leonardite LEO, litter LIT, municipal waste compost MWC and biosolid compost BC) and one inorganic (sugarbeet lime SL), where the grass *Agrostis stolonifera* L. was sown, and two control treatments (with plant CTRP or without plant CTR). Soil was sampled four times during the experimental period. The microbiological properties studied were: microbial biomass C, microbial biomass C/total organic C, dehydrogenase, aryl-sulphatase, β-glucosidase, acid-phosphatase and protease enzyme activities. Dynamics of microbiological properties differed between treatments being results not only affected by soil pH or trace element concentrations, but also by changes derived from the different treatments in organic matter quality and quantity, as well as nutrient content in soil. While microbial biomass C, dehydrogenase, aryl-sulphatase and protease activities were highly correlated with soil pH and soluble trace element contents, changes in β-glucosidase activity were mainly influenced by water soluble C concentrations. It was also observed that enzymatic activities generally decreased over time after no more amendment additions occurred. Nonetheless, during the experiment microbial biomass and activities were generally higher in all treatments compared to the untreated control and thus remediation practices had a positive and significant effect on trace element stabilization and microbial activity in the contaminated soil.

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1 Introduction

Mining activities can release significant amounts of trace elements such as As, Cd, Cu, Pb and Zn into the environment negatively affecting organisms and ecological processes within atmospheric, aquatic and terrestrial systems (Iskandar and Adriano 1997). In terrestrial systems, trace elements tend to accumulate in the surface of soils due to binding with its components (clay minerals, iron/manganese oxides, organic matter, etc.) in a variety of ways. Owing to its small size and crucial role in nutrient cycling, the soil microbiota is the first group of organisms that undergoes direct and indirect impact due to accumulation of trace elements in soil (Giller et al. 1998). Although high concentrations of trace elements may promote the development of tolerant microbial populations (Ellis et al. 2003), trace element polluted soils generally show less microbial biomass and altered microbial activity patterns compared to non-affected soils (Kandeler et al. 2000). Among the different methodologies to assess microbial activity in soil, enzymatic assays, either based on colorimetric or fluorescence reactions, have been widely used due to: (1) their specificity, (2) their simplicity and (3) the integrative nature of enzymatic activities, from the micro-up to the macroscale (Nannipieri et al. 2002). Soil enzymatic assays may be therefore used to evaluate not only anthropogenic disturbances such as those related to contaminant accumulation, but also restoration and remediation practices in affected systems. Since trace elements cannot be degraded, remediation of soils polluted with trace elements is based either on the extraction or the stabilization of the contaminants. Among stabilization techniques, assisted natural remediation has been proposed as a potential low-cost and environmentally friendly alternative to treat extensive areas moderately contaminated (Madejón et al. 2006). This technique is based on the use of amendments to accelerate those processes (sorption, precipitation and complexation reactions) that take place naturally in soils and reduce the mobility and bioavailability of toxic elements (Bolan and Duraisamy 2003). In addition to the incorporation of amendments, the development of a plant cover may prevent wind-blow of contaminated particles and reduce water pollution (Tordoff et al. 2000). There is, however, concern regarding the longevity of utilizing amendments to assist natural remediation. Reacidification of soil may reverse the action of amendments that make soils more alkaline. Mineralization of organic matter present in biosolids may also release trace elements in potentially bioavailable forms. Traditionally, repeated applications of amendments have been recommended to maintain trace element immobile, but more work is required to refine these procedures and understand the effects of such practices on soil dynamics in the mid-and long-term.

Stabilization techniques aim at immobilizing trace elements in the soil to reduce their availability to biological targets, run-off transport and leaching. However, reliance on abiotic properties is insufficient to assess the efficiency of remediation practices from an environmental perspective. Alternative parameters are required which can be used as bioindicators to monitor changes in soil ecological processes such as those related to anthropogenic and natural disturbances,

Table 1 Mean values ± standard deviation of some chemical characteristics of the soil

pH		3.32 ± 0.76
TOC[a]	g kg^{-1}	5.40 ± 0.07
Tot-As	mg kg^{-1}	120 ± 3
Tot-Cd	mg kg^{-1}	2.43 ± 0.04
Tot-Cu	mg kg^{-1}	78.3 ± 1.4
Tot-Mn	mg kg^{-1}	645 ± 25
Tot-Pb	mg kg^{-1}	201 ± 6
Tot-Zn	mg kg^{-1}	226 ± 3

[a] TOC total organic carbon

remediation practices or land management. Enzymatic activities are particularly attractive for this purpose due to their crucial role in soil organic matter transformations and their direct link to the soil microbiota.

The aim of this study was to evaluate the mid-term effects of various amendments and/or a plant cover on trace element stabilization and the different processes related to the cycling of nutrients (C, N, P and S) in a soil moderately contaminated with As, Cd, Cu, Pb and Zn. A 30 month experiment was conducted in containers to simulate potential field remediation practices under more controlled conditions. In addition to trace element availability, general soil physical and chemical characteristics as well as various microbiological and biochemical properties were investigated.

2 Materials and Methods

2.1 Soil Characteristics

Soil was sampled in an area affected by the Aznalcóllar mine accident named "El Vicario", where the only remediation work carried out by the authorities was the removal of the sludge layer together with the first 15 cm of topsoil. The soil was clayey loamy classified as Typic Xerofluvent (Soil Survey Staff 1996). Some relevant characteristics are presented in Table 1.

2.2 Experimental Design

The experiment was carried out in 28 containers (70 cm long × 60 cm wide × 40 cm deep) that were placed outdoors in the experimental farm "La Hampa" (IRNAS-CSIC) in Coria del Río (Southern Spain) (485 mm mean rainfall, average for 1971-2008; Mean annual daily temperature is around 17°C, with maximum and minimum temperatures in July of 33.5°C and in January of 5.2°C). The containers

were filled with the upper 20 cm of the soil (1.32 g cm^{-3} bulk density). Containers were arranged according to a complete randomized block design with seven treatments (four organic, one inorganic and two controls) and four replicates per treatment. The organic treatments were: leonardite (LEO), a low rank coal between peat and sub-bituminous, rich in humic acids from a coal mine (DAYMSA), litter (LIT) collected from a deciduous forest (*Castanea sativa* Miller.) in the Sierra of Aracena (Huelva, Southern Spain), municipal waste compost (MWC) from a city refuse treatment plant (Villarrasa, Southern Spain), and biosolid compost (BC) constituted from wastewater sludge from a water treatment plant and green waste from parks and gardens (EGMASA, Sevilla, Southern Spain). The inorganic treatment was sugar beet lime (SL), a residual material from the sugar manufacturing process with 70–80% of $CaCO_3$ (dry basis) (AZUCARERA EBRO, San José de la Rinconada, Southern Spain). Two control treatments without amendments were also established: control with plant (CTRP) and control without plant (CTR). These amendments were chosen because they constitute low-cost, representative materials for land treating extensive areas. The characteristics of the amendments are described in Pérez de Mora et al. (2005). Trace element contents of all amendments were below the limits established by the European Union (CEC 1986) for sewage sludge. The annual loads of trace elements of the products used were also in accordance with the same directive. The amendments were applied on a fresh basis (20–25% moisture content) and mixed with the topsoil (10 cm) in the containers. Within the 30 months of the study two doses of each amendment were applied: the first one at the beginning of the experiment (70–75 Mg dw ha^{-1} for leonardite and composts and 50–60 Mg dw ha^{-1} for sugarbeet lime and litter) and again after 12 months (February 2003) (35.0–37.5 Mg dw ha^{-1} for leonardite and composts and 25–30 Mg dw ha^{-1} for sugarbeet lime and litter). The grass *Agrostis stolonifera* L. was sown (167 kg ha^{-1}) in the containers and grown for 5 months (March–July) for 3 consecutive seasons (2002–2004). *A. stolonifera* L. was selected because it is known to show metal tolerance. The containers were routinely watered when necessary to maintain plant growth.

2.3 Soil Sampling and Chemical Analysis

Soil was sampled on four occasions: 1, 6, 18 and 30 months after the beginning of the experiment. In each case, 10 soil cores (2 cm diameter, 10 cm depth) regularly distributed were taken from each container to make a composite sample. Subsamples for chemical analysis were previously air-dried, crushed and sieved (2 mm). Soil and amendment aliquots for trace element determinations were additionally ground to 60 μm. Subsamples for microbial biomass and enzymatic activities were sieved (2 mm) and stored at 4°C until analysis (within two weeks after the sampling). Soil pH values were measured in a 1:2.5 sample:1 M KCl extract after shaking for one hour The 0.01 M $CaCl_2$-extractable trace element

concentrations in soils were determined in 1:10 soil sample (< 2 mm):0.01 M $CaCl_2$ extracts (Ure et al. 1993) via ICP-OES (Inductively coupled plasma-optical emission spectrometry). Total organic carbon (TOC) in soil was analysed by dichromate oxidation and titration with ferrous ammonium sulphate (Walkley and Black 1934).

2.4 Microbial Biomass and Enzyme Activities

Microbial biomass carbon (MBC) content was determined by the chloroform fumigation-extraction method modified by Gregorich et al. (1990). The concentration of C in the extract was measured as described by Jenkinson and Powlson (1976) using dichromate digestion. An extraction efficiency coefficient of 0.38 was used to convert the difference in soluble C between the fumigated and the unfumigated soil to MBC (Vance et al. 1987). Dehydrogenase activity (DH) was determined by the method of Trevors (1984), using INT (2 (*p*-iodophenyl)-3-(*p*-nitrophenol) 5-phenyl tetrazolium chloride) as the electron acceptor (García et al. 1993). Arylsulphatase activity (Aryl) was determined as proposed by Tabatabai and Bremmer (1970) after soil incubation with *p*-nitrophenyl sulphate and measurement of *p*-nitrophenol absorbance at 400 nm. *β*-glucosidase activity (*β*-gluc) was measured as indicated by Tabatabai (1982) after soil incubation with p-nitrophenyl glucoside and measurement of *p*-nitrophenol absorbance at 400 nm. Acid phosphatase activity (Phosph) was measured after soil incubation with *p*-nitrophenyl phosphate disodium in a 0.5 M maleate buffer (pH 6.5) and measurement of PNP absorbance at 398 nm (Nannipieri et al. 1980). Protease activity (Prot) was calculated after incubation of soil with casein and measurement of the absorbance of the extracted tyrosine at 700 nm (Ladd and Butler 1972).

DH was expressed in mg INTF kg^{-1} dw h^{-1}. Aryl, *β*-gluc and Phosph activities were expressed in mg PNP kg^{-1} dw h^{-1}. Prot was expressed in mg Tyrosine kg^{-1} dw 2 h^{-1}.

2.5 Statistical Analysis

All statistical analyses were carried out with the program SPPS 15.0 for Windows. A normality test was carried out for all variables prior to analysis of the variance. If necessary, non-normal distributed data was transformed accordingly. Differences between treatments within each sampling event for each variable were tested using ANOVA. Post-hoc analysis was performed using Tukey's test for equal variances and Games-Howell's test for unequal variances. To test for time-related differences within the same treatment microbiological data was also analysed by ANOVA for repeated measures using time as factor. Validity of the repeated factor for ANOVA was tested using Mauchly's sphericity test. The Greenhouse-Geisser correction was used for violations of sphericity. Significant differences for all

variables between the different sampling events within the same treatment were established by Students *t*-test pairwise comparisons using the Bonferroni correction. A *t*-test for unequal variances was computed when these were heterogenous. A correlation matrix between all chemical and biochemical parameters was calculated. The significance level reported ($\alpha = 0.01$ and $\alpha = 0.05$) is based on Pearson's coefficients.

3 Results and Discussion

In this study we focused on time-related patterns of various intra-and extracellular enzymatic activities in a soil moderately contaminated with trace elements in which different amendments were applied. Since solubility of As and Pb was very low, metal-enzyme interactions were only reported for Cd, Cu and Zn. Detailed information on biotic and abiotic properties within each sampling can be found elsewhere (Pérez de Mora et al. 2005; Pérez-de-Mora et al. 2006a, b).

Results from microbial biomass C (MBC) estimations showed various interesting trends. Firstly, the incorporation of amendments into the soil resulted in higher MBC concentrations in all samplings compared to both controls (Fig. 1a). In the second sampling and subsequently higher microbial biomass yields were recorded in the control with plants than in the control without plants (Fig. 1a). Finally, in most treatments, except LEO and BC, microbial biomass was highest in the second sampling and subsequently decreases (Fig. 1a). The incorporation of amendments and the presence of a rhizosphere in the soil had therefore a stimulatory effect on the soil microbial community. This could be related to the alkalinising effect of the amendments and the incorporation of nutrients and readily degradable organic substrates as suggested by the positive correlations between MBC and soil pH and nutrient-related properties (N-Kjeldahl, available-P and water soluble C) (Table 2) (Sakamoto and Oba 1991). The growth of a vegetation cover between the first and second sampling could partly explain why for most treatments MBC was highest in the second sampling. Plants excrete 10–20% of their photosynthetates as root exudates, which can be used up by numerous microorganisms as substrates for growth (Salt et al. 1998). In subsequent samplings, however, microbial biomass generally decreased although higher plant yields were achieved (Pérez-de-Mora et al. 2006c). More complex mechanisms may also be involved which may account for this trend such as competition between plants and soil microorganisms for nutrients and/or different degradability of organic matter incorporated with the amendments.

Since microbial biomass is generally higher in soils with higher organic matter content and amendments may also incorporate microorganisms into the soil, it may be useful to normalize shifts in soil microbial biomass C by using the ratio microbial biomass C/total organic C (MBC/TOC) (Insam and Merschack 1997). A high ratio can be attributed to a highly active microbial population or to easily degradable organic sources, at least for that population (Spargling 1992). Common

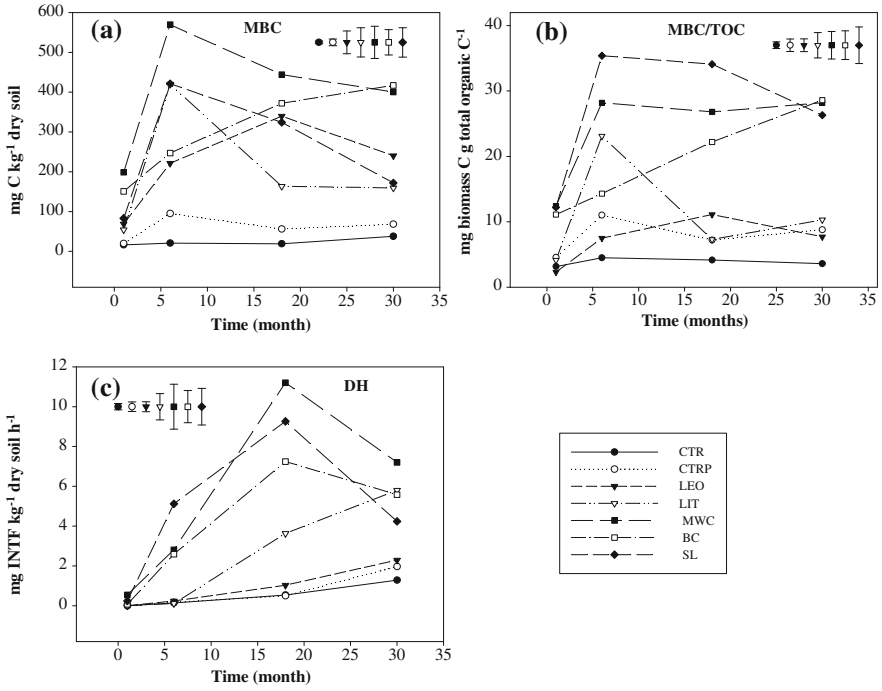

Fig. 1 Temporal dynamics of **a** microbial biomass C, **b** microbial biomass C/total organic C and **c** dehydrogenase activity. Symbols represent mean values. Error bars represent the maximum standard deviations within one treatment during the whole experiment

values ranged from 10 to 40 mg MBC g^{-1} TOC (Gigliotti and Farini 2002). The ratio showed a very similar pattern for all treatments to that of MBC (Fig. 1b). In the case of the compost and sugarbeet lime amended soils the ratio was significantly higher in all samplings compared to both controls. Nonetheless, in soils amended with litter and leonardite differences with the controls were not significant. On occasions similar values were observed in the control with plant and the amended soils. These results suggest that microorganisms may be incorporated with the amendments (hence higher MBC yields), but also that litter and leonardite are not as good as composts or lime to stimulate microbial growth in degraded soils. The fact that plant litter needs first to be cut down into smaller fractions for more effective microbial attack and the recalcitrant nature of organic C from the leonardite may explain these results.

Enzymatic patterns were enzyme-and treatment-dependent showing the extreme complexity of ecological reactions occurring in the soil. Dehydrogenase activity has been used to assess heavy metal toxicity in soils (Rossel et al. 1997) and microbial activity in semiarid Mediterranean areas (García et al. 1997). During the experiment higher DH was recorded in all treated soils compared to controls, except LEO (Fig. 1c). This enzyme was positively correlated with MBC, but there

Table 2 Correlation coefficients between chemical and biochemical properties

	MBC/TOC	MBC	DH	Aryl	β−gluc	Phosph	Prot
pH	0.565[a]	0.450[a]	0.616[a]	0.379[a]	−0.007	−0.322[a]	0.318[a]
Sol-Cd	−0.415[a]	−0.411[a]	−0.561[a]	−0.343[a]	−0.159	−0.023	−0.482[a]
So-Cu	−0.368[a]	−0.428[a]	−0.403[a]	−0.254[a]	−0.308[a]	−0.213[b]	−0.449[a]
Sol-Zn	−0.502[a]	−0.471[a]	−0.616[a]	−0.360[a]	−0.246[a]	−0.080	−0.530[a]
TOC	−0.221[b]	−0.202[b]	−0.223[b]	−0.117	−0.105	0.034	−0.011
N-Kjeld	0.312[a]	0.574[a]	0.465[a]	0.632[a]	0.384[a]	0.208[b]	0.266[a]
Avail-P	0.304[a]	0.250[a]	0.223[b]	0.059	−0.181	−0.287[a]	−0.054
WC	0.527[a]	0.665[a]	0.356[a]	0.687[a]	0.544[a]	0.388[a]	0.330[a]
MBC/TOC	1	0.852[a]	0.598[a]	0.510[a]	0.192[b]	−0.088	0.266[a]
MBC		1	0.498[a]	0.568[a]	0.384[a]	0.156	0.310[a]
DH			1	0.585[a]	0.227[b]	−0.093	0.485[a]
Aryl				1	0.383[a]	0.143	0.224[b]
β-glu					1	0.724[a]	0.393[a]
Phosph						1	0.331[a]
Prot							1

[a] Correlation is significant at the 0.01 level
[b] Correlation is significant at the 0.05 level

seemed to be a delay in DH response to remediation practices compared to MBC; in most cases DH values were highest in the third sampling (Fig. 1c). Due to its intracellular nature DH is usually better correlated to microbial biomass dynamics than extracellular enzymes (Pérez-de-Mora et al. 2008). Values of DH were significantly higher in the third and fourth samplings for most of the treatments except for CRT. These results may be therefore influenced by the higher sensitivity of DH to soil acidity compared to extracellular enzymes as the positive correlation between this activity and soil pH suggests (Table 2). In fact, soil pH in all treatments increased as time progressed including control soils (Pérez-de-Mora et al. 2006d).

Temporal dynamics of extracellular activities were very variable. Similarities with the activity patterns of other biochemical parameters estimated such as MBC and DH were only found for Aryl (Fig. 2). Here soils amended with composts and SL showed the largest activity (Fig. 2a). As observed for DH, the highest activity values were recorded in the third sampling. In the other treatments (LIT, LEO, CTR, CTRP) little variation was found during the experiment (Fig. 2a). Temporal and treatment induced differences for Aryl may be related to changes in soil pH and trace element availability as the correlations between these parameters indicate (Table 2). In fact, this activity has been outlined as a very sensitive enzyme in tracing heavy metal effects (Hinojosa et al. 2008). Given that SO_4^{-2} availability is enhanced as soil pH increases and sulphides from the spill should act as the main source of SO_4^{-2} anions in all treatments, data suggests that Aryl was less affected by sulphate concentrations in soil than by the pH or metal concentrations.

Results from β-gluc, Phosph and Prot differed from the rest of biological parameters studied (Fig. 2b–d). This may be explained by the fact that

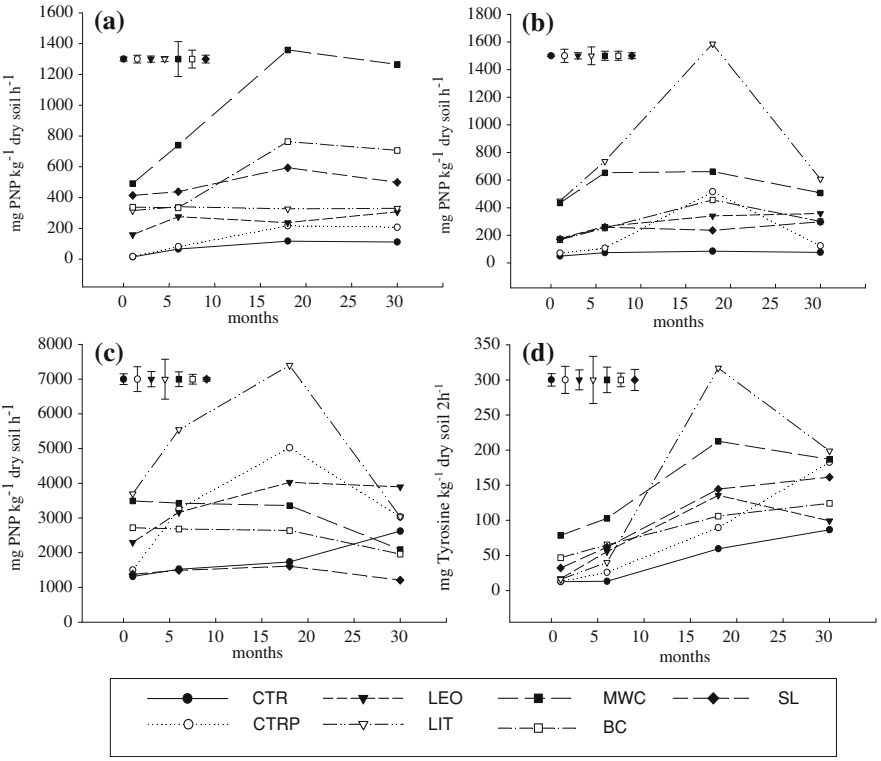

Fig. 2 Temporal dynamics of **a** aryl-sulphatase C, **b** β-glucosidase **c** acid-phosphatase and **d** protease. Symbols represent mean values. Error bars represent the maximum standard deviations within one treatment during the whole experiment

concentrations of the substrates and the final products resulting from these activities could be highly influenced by the incorporation of amendments and the development of a plant cover. In the first case, enzymatic activity would be promoted as more substrate is present. On the other hand, if the final products of enzymatic reactions accumulate and are readily available, enzymatic release and hence potential activity might decrease (feedback inhibition). In the case of β-gluc little temporal variations were recorded during the experiment, except in the LIT and CTRP treatments in which mean values for the third sampling event were significantly higher than those found in the other treatments (Fig. 2b). Both LIT and CTRP showed a significant increase in β-gluc between the second and the third samplings followed by a sharp decrease in activity towards the end of the experiment (Fig. 2b). This trend could be related to a significant improvement in vegetation cover in these two treatments between the second and the third sampling (Pérez-de-Mora et al. 2006c). In the other amended soils incorporation of C substrates through the addition of amendments and the presence of a healthy plant

cover from the beginning of the experiment could account for a more homogeneous C pool in the soil and thus a more constant pattern of β-gluc over time.

Acid-phosphatase showed a similar trend as β-gluc in the LIT and CTRP treatments (Fig. 2c). For the other soils different results were observed: in the case of LEO, Phosph increased as time progressed, whereas in CTR potential activity remained low until the third sampling and it increased sharply towards the end of the experiment. It is possible that improved soil conditions in these treatments at the end of the experiment (f.ex. less acid pH in CTR) could account for higher potential activity values (Fig. 2c). In contrast, soils amended with composts and SL showed a decrease in Phosph at the end of the experiment (Fig. 2c). This might be due to feedback inhibition of the enzyme by inorganic phosphate as suggested by the negative correlation between acid-phosphatase and available-P (Table 2). These results are also in agreement with data reported by other authors for other soils into which amendments with a high P content were incorporated (Madejón et al. 2003, Plaza et al. 2004).

Protease activity generally increased in all treatments as time progressed, except in LIT, where a similar pattern as for β-gluc and Phosph was observed (Fig. 2d). Prot was negatively correlated with soluble heavy metal concentrations and positively correlated with soil pH (Table 2). Therefore amelioration of soil properties and metal availability could partly account for this general trend. In addition, temporal variations in N availability related to amendment incorporation, plant maturity and mineralization processes could also influence Prot in the soil as the positive correlation between protease and N-Kjeldahl indicates (Table 2). Other authors have also reported positive but rather weak correlations between these two variables arguing that the complex nature of organic N (Schulten and Schnitzer 1998, Wick et al. 2002) and the substrate specificity of the various extracellular proteases (Kalisz 1988) may account for this observation.

4 Conclusions

- The incorporation of amendments and/or development of a plant cover show great potential for stabilizing trace elements in soil in the mid-term, enhancing nutrient cycling and microbial activity at the same time.
- Due to their alkaline nature, composts and sugarbeet lime performed better than other amendments and thus have more potential for field application. However, once a vegetation cover has established, amendment additions may be unnecessary or kept at a minimum.
- Remediation practices showed a positive effect on soil functionality which is enzyme and treatment dependent. Enzymatic activities such as dehydrogenase, aryl-suplhatase and protease seem to be more sensitive to changes in soil pH and trace element availability than β-glucosidase and acid-phosphatase, which seem to be more strongly influenced by substrate or enzymatic product availability.

- The utilization of microbial enzymatic assays for assessing soil remediation practices can be very helpful, but various activities are required for diagnostic monitoring of soil restoration.

References

Bolan NS, Duraisamy VP (2003) Role of inorganic and organic soil amendments on immobilisation and phytoavailability of heavy metals: a review involving specific case studies. Aust J Soil Res 41:533–555

CEC (Commission of the European Communities), 1986. Council Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Official J L181, 04/07/1986, pp. 6–12

Ellis RJ, Morgan P, Weightman AJ, Fry JC (2003) Cultivation-dependant and-independant approaches for determining bacterial diversity in heavy-metal contaminated soil. Appl Environ Microbiol 69:3223–3230

García C, Hernández T, Costa F (1997) Potential use of dehydrogenase as an index of microbial activity in degraded soils. Commun Soil Sci Plan 28:123–134

Gigliotti C, Farini F (2002) Microbial biomass response to heavy metals in the field. In: Violante A, Huang PM, Bollag JM, Gianfreda L (eds) Soil mineral-organic matter-microorganisms interactions and ecosystem health. Developments in soil science, vol 28B. Elsevier Science B.V, Amsterdam/New York, pp 195–204

Giller KE, Witter E, McGrath SP (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol Biochem 30:1389–1414

Gregorich EG, Wen G, Voroney RP, Kachanoski RG (1990) Calibration of rapid direct chloroform extraction method for measuring soil microbial biomass C. Soil Biol Biochem 22:1009–1011

Hinojosa MB, Rodríguez-Maroto JM, García-Ruíz R, Carreira JA (2008) Effects of pyrite sludge pollution on soil enzyme activities: Ecological dose-response model. Sci Total Environ 396:89–99

Insam H, Merschack P (1997) Nitrogen leaching from forest soil cores after amending organic recycling products and fertilizers. Waste Manage Res 15:277–292

Iskandar IK, Adriano DC (1997) Remediation of soil contaminated with metals. Science Reviews, Aberystswyth, UK

Jenkinson DS, Powlson DS (1976) The effect of biocidal treatment in metabolism of soil: A method for measuring microbial biomass. Soil Biol Biochem 8:209–213

Kalisz HM (1988) Microbial proteinases. Adv Biochem Eng Biotechnol 36:3–65

Kandeler E, Tscherko D, Bruce KD, Stemmer M, Hobbs PJ, Bardgett RD, Amelung W (2000) Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biol Fertil Soils 30:390–400

Ladd JN, Butler JHA (1972) Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates. Soil Biol Biochem 4:19–30

Madejón E, Burgos P, López R, Cabrera F (2003) Agricultural use of three organic residues: Effect on orange production and on properties of a soil of the "Comarca Costa de Huelva" (SW Spain). Nutr Cycl Agroecosyst 65:281–288

Madejón E, Pérez-de-Mora A, Felipe E, Burgos P, Cabrera F (2006) Soil amendments reduce trace element solubility in a contaminated soil and allow regrowth of natural vegetation. Environ Pollut 139:40–52

Nannipieri P, Ceccanti B, Cervelli S, Matarese E (1980) Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016

Nannipieri P, Kandeler E, Ruggiero P (2002) Enzyme activities and microbiological and biochemical processes in soil. In: Burns RG, Dick RP (eds) Enzymes in the environment, activity, ecology and applications. Marcel Dekker, New York, pp 1–33

Pérez de Mora A, Ortega-Calvo JJ, Cabrera F, Madejón E (2005) Changes in enzyme activities and microbial biomass after "in situ" remediation of a heavy metal-contaminated soil. Appl Soil Ecol 28:125–137

Pérez-de-Mora A, Burgos P, Madejón E, Cabrera F, Jaeckel P, Schloter M (2006a) Microbial community structure and function in a soil contaminated by heavy metals: effect of plant growth and different amendments. Soil Biol Biochem 38:327–341

Pérez-de-Mora A, Burgos P, Ortega-Calvo JJ, Cabrera F, Madejón E (2006b) Microbial function after assisted natural remediation of a trace element polluted soil. In: A. Méndez-Vilas (Ed) Modern Multidisciplinary Applied Microbiology. Understanding and Exploiting Microbes and Their Interactions. Biological, Physical, Chemical and Engineering Aspects. Wiley-VCH, pp 536–540

Pérez-de-Mora A, Burgos P, Cabrera F, Madejón E (2006c) Trace elements availability and plant growth in a mine-spill-contaminated soil under assisted natural remediation. Part I. Soils. Sci Total Environ 363:28–37

Pérez-de-Mora A, Burgos P, Cabrera F, Madejón E (2006d) Trace elements availability and plant growth in a mine-spill-contaminated soil under assisted natural remediation. Part II. Plants. Sci Total Environ 363:38–45

Pérez-de-Mora A, Madejón E, Cabrera F, Buegger F, Fuß R, Pritsch K, Schloter M (2008) Long-term impact of acid resin waste deposits on soil quality of forest areas II. Biological indicators. Sci Total Environ 406:99–107

Plaza C, Hernández D, García-Gil JC, Polo A (2004) Microbial activity in pig slurry-amended soils under semiarid conditions. Soil Biol Biochem 36:1577–1585

Rossel D, Tarradellas J, Bittom G, Morel JL (1997) Use of enzymes in soil ecotoxicology: a case for dehydrogenase and hydrolitic enzymes. In: Tarradellas J, Bitton G, Rossel D (eds) Soil ecotoxicology. Lewis Publishers, CRC Press, Boca Ratón, Florida, pp 179–206

Sakamoto K, Oba Y (1991) Relationship between the amount of organic material applied and soil biomass content. Soil Sci Plant Nutr 37:387–397

Salt DE, Smith RD, Raskin I (1998) Phytoremediation. Annu Rev Plant Physiol Plant Mol Biol 49:643–668

Schulten HR, Schnitzer M (1998) The chemistry of soil organic nitrogen: a review. Biol Fertil Soils 26:1–15

Soil Survey Staff (1996) Keys to soil taxonomy. US department of agriculture, soil conservation service, Washington DC

Spargling GP (1992) Ratio of microbial biomass carbon to soil organic carbon as sensitive indicator of changes in soil organic matter. Aust J Soil Res 39:195–207

Tabatabai MA (1982) Soil enzymes. In: Page AL, Miller EM, Keeney DR (eds) Methods of Soil Analyses, Part 2, Chemical and Microbiological Properties. American Society of Agronomy, Madison, WI, pp 903–947

Tabatabai MA, Bremmer JM (1970) Arylsulphatase activity of soils. Soil Sci Soc Am Proc 34:225–229

Tordoff GM, Baker AJM, Willis AJ (2000) Current approaches to the revegetation and reclamation of metalliferous wastes. Chemosphere 41:219–228

Trevors JT (1984) Dehydrogenase activity in soil: a comparison between the INT and the TTC assay. Soil Biol Biochem 16:673–674

Vance ED, Brookes PC, Jenkinson DS (1987) Microbial biomass measurements in forests soils: determination of K_c values and test of hypothesis to explain the failure of the chloroform fumigation-incubation method in acid soils. Soil Biol Biochem 19:381–387

Walkley A, Black IA (1934) An examination of the Degtjareff method for determining soil organic matter and a proposed determination of the chromic acid tritation method. Soil Sci 37:29–38

Wick B, Kühne RF, Vielhauer K, Vleck PLG (2002) Temporal variability of selected soil microbiological and biochemical indicators under different soil quality conditions in south-western Nigeria. Biol Fertil Soils 35:155–167

Ure AM, Quevauviller PH, Muntau H, Griepink B (1993) Speciation of heavy metals in soils and sediments. An account of the improvement and harmonisation of extraction techniques undertaken under the auspices of the BCR of the Comission of the European Communities. Int J Environ Anal Chem 51:135–151

Changes in Some Hydrolase Activities in Agricultural Soils in Response to Zinc Contamination

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Abstract Zinc is essential for many soil enzymes but in excess it can act as a typical inhibitor. This heavy metal often reaches toxic levels in soils, as consequence of human activities. The aim of the study was to determine whether enzyme activities (urease, phosphomonoesterase and β-D-glucosidase) can be used as sensitive indicators of the contamination of soil by zinc. The study consisted of a laboratory experiment in which five agricultural soils were spiked with four different doses of zinc: 300, 900, 2,700 and 8,100 mg Zn kg^{-1} soil. The spiked soils were incubated for 7 days before the enzyme activities were measured. The results showed that contamination with zinc at low doses either slightly stimulated or slightly inhibited the enzymatic activity. On the other hand, as zinc contamination increased, the activities were suppressed. The type of soil was an important factor in determining the effect of zinc contamination; the greatest inhibition of enzymatic activity was observed in the soils with the lowest contents of organic matter and lowest cation exchange capacity. The ED_{50} values were very variable for each enzymatic activity; the lowest values were obtained for urease activity and the highest for β-D-glucosidase activity. The enzymatic activities were negatively correlated with DTPA-extractable zinc. The soil enzymes were adversely affected not only by zinc contamination but also by increasing soil acidity and the activities were negatively correlated with low pH values. In conclusion, urease

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and phosphomonoesterase activities were more sensitive to zinc contamination than β-D-glucosidase activities and may be useful as indicators of soil contamination by zinc.

1 Introduction

Accumulation of heavy metals in the surface layers of soils is a serious problem, as heavy metals in excess may affect the biological activity of a soil as well as its physical and chemical properties, thereby altering the correct functioning of the ecosystem. Microorganisms are extremely sensitive to contamination, as toxic compounds interact with them in the polluted soils. Thus, both the microorganisms and the processes in which they are involved may be used as indicators of the degree of deterioration of soils contaminated by heavy metals. Many authors consider that the enzyme activities involved in the carbon, nitrogen, phosphorus and sulphur cycles may be used as indicators of the deterioration because the accumulation of heavy metals has been reported to have negative effects on soil biochemical properties (Giller et al. 1998; Gil-Sotres et al. 2005).

Zinc is one of the most important heavy metals as regards soil contamination, not directly because of its toxicity, but rather because of the high concentrations that are reached in soils. Zinc enters the ecosystems through various different ways and from many different sources (industrial emissions, inorganic fertilizers, application of sewage sludge, atmospheric deposition, etc.). Although zinc is essential for microorganisms, and many enzymes cannot function without it, this metal is particularly problematic because of the high concentrations that are often reached in soils. Numerous studies have demonstrated the negative effects of zinc on biological activity, although there is some discrepancy as regards the safe limits of the metal in soils (Giller et al. 1998). The toxicity is regulated by abiotic factors such as the soil pH and the organic matter and clay contents. These parameters largely determine the concentration of zinc in the soil solution and therefore its bioavailability, through the processes of hydrolysis, precipitation or retention on organo-mineral surfaces. The bioavailability of zinc is therefore a key factor in terms of its toxicity to soil microorganisms.

Although many enzyme activities have been used as biochemical indicators of soil quality of heavy metal-contaminated soils, no consensus has yet been reached on which is the most appropriate (Dick 1997).

In order to compare the influence of different heavy metals on the soil enzymatic activities, the dose-response curves are widely used as they allow the determination of the effective quantity of metal causing definite inhibitory effects. The dose-response relationship can be quantified in terms of an ecological dose (ED), defined as the metal concentration which decrease the specific microbe-mediated ecologic processes by some percentage (Babich and Stotzky 1985), being the ED_{50} dose, i.e. the dose causing a reduction of 50%, the most used. There are several graphical procedures or mathematical models to calculate the ecological

Table 1 Physiographic characteristics of the soils used in the study

	Catoira	Coutada	Rúa	Trasariz	Xinzo
Latitude (N)	42° 40′ 30″	42° 44′ 30″	42° 21′ 05″	42° 47′ 25″	42° 01′ 55″
Longitude (W)	8° 42′ 10″	8° 32′ 25″	7° 09′ 20″	8° 29′ 50″	7° 43′30″
Altitude (m.a.s.l.)	50	80	515	180	619
Parent material	Migmatitic granitoid	Schists and paragneisses	Adamelite granite	Metatexite and diatexite	Granite with megacrystals
Crop	Maize	Maize	Vine	Turnip	Potato

dose value. In numerous studies (Welp and Brümmer 1997; Moreno et al. 2002; Speir et al. 2007) it was stated that the form of such dose-response curves is characterized by an S-shaped pattern. Van Beelen et al. (2004) used a sigmoidal dose-response curve described by the equation:

$$Y = 100/1 + 10^{(X - \log_{10}[ED_{50}])}$$

where, Y is the enzymatic activity expressed as percentage in relation to the corresponding activities in the uncontaminated soil at a specific concentration, and X is $\log_{10}$ of zinc concentration.

In the present study, the activities of three enzymes involved in the N, P and C cycles (urease, phosphomonoesterase and β-D-glucosidase, respectively) were determined in different agricultural soils artificially contaminated with zinc, in order to evaluate their sensitivity to such contamination and their possible usefulness as bioindicators. Moreover, the relationships between the enzymatic activities and the Zn bioavailability (soluble in DTPA) were discussed and the sigmodal dose-response curve described by Van Beelen et al. (2004) was used to calculate the ED_{50} values for each of the enzymes and soils.

2 Materials and Methods

The soils used in this study, selected to provide a range of physicochemical properties, were obtained from the Ap horizons (0–15 cm) of five agricultural soils. A brief description of the physiographic characteristics of the sampling plots is provided in Table 1.

In each plot, a representative number of samples were collected and mixed in the field to provide composite soil samples. The samples were transported to the laboratory in isothermal bags, then sieved (< 4 mm) and homogenized. The samples were then stored at 4°C to prevent modification of the biochemical properties, before being artificially contaminated with zinc. The samples were stored for less than 96 h before analysis.

In order to determine the physicochemical properties of the soils, an aliquot of each sample was separated, then air-dried and sieved (< 2 mm). The soils were

analysed for pH in water, total carbon, total nitrogen, water field capacity (water retained at −33 kPa pressure) and particle size distribution according to the methods described by Guitián and Carballas (1976). Briefly, soil pH in water was measured in a 1:2.5 w:v, soil:solution ratio. Total carbon was determined by oxidation with dichromate in an acid medium while total nitrogen was analysed by Kjeldahl distillation. Soil particle size distribution was estimated by the Robinson pipette method, after destruction of the organic matter with hydrogen peroxide, and with Calgon as dispersant, and the textural class of soils was determined following FAO (1966).

The cation exchange capacity (CEC) was determined after extraction of the samples with ammonium acetate at pH 7, followed by centrifugation (Gillman et al. 1983). Amorphous Al and Fe were extracted with 0.2 M ammonium oxalate/oxalic acid buffer of pH 3.0 (McKeague and Day 1966) and were determined by flame atomic absorption spectrometry. The total zinc content was determined in finely ground samples (< 50 μm) after acid microwave digestion with HF/HNO_3 and measurement of Zn in the diluted solution by flame atomic absorption spectrometry. Bioavailable zinc was determined by extraction in DTPA solution (0.005 M diethylene triamine pentaacetic acid, 0.01 M $CaCl_2$ and 0.1 tetraethylammonium adjusted to pH 7.3) according to the procedure described by Lindsay and Norvell (1978) and analysed by flame atomic absorption spectrometry.

To study the influence of Zn on the enzymatic activities in soils, portions of the collected soil samples were treated with Zn as chloride salts at doses of 300, 900, 2,700 and 8,100 mg Zn kg^{-1} soil. The Zn-treated soils were incubated for 7 days in the dark at 25°C and at optimal conditions of moisture (at water field capacity), and thereafter analysed for the three enzymatic activities. The unspiked reference samples (control samples) were treated similarly by addition of distilled water only.

The activities of urease, phosphomonoesterase and β-D-glucosidase were determined as outlined by Trasar-Cepeda et al. (2000). For urease the substrate was 6.4% urea, the incubation period was 90 min, the buffer was 0.2 M phosphate buffer pH 8.0 and the released NH_4^+–N was determined with an ammonium selective electrode. For phosphomonoesterase and β-D-glucosidase, the substrates were 16 mM *p*-nitrophenyl phosphate and 25 mM *p*-nitrophenyl-β-D-glucopyranoside, the incubation periods were 30 and 60 min, respectively, and the samples were incubated at pH 5.0 with Modified Universal Buffer; the released *p*-nitrophenol (*p*NP) was determined colorimetrically at 400 nm and quantified by reference to calibration lines obtained using *p*-nitrophenol standards incubated with soil under the same conditions as for the samples. All determinations were performed in triplicate and the average values were expressed on an oven-dried soil basis (105°C).

The relationships between concentration-effect were fitted to the sigmoid model of Van Beelen et al. (2004), and the fitted curves were used to estimate the values of ED_{50}, ED_{20} and ED_{10}, i.e. the concentration of zinc that inhibits the enzymatic activity by 50, 20 and 10%.

Table 2 Some physicochemical characteristics (mean ± standard deviation) of the soils used in the study

	Catoira	Coutada	Rúa	Trasariz	Xinzo
pH H_2O (1:2.5 w:v)	5.41 ± 0.00	5.83 ± 0.00	5.29 ± 0.01	7.06 ± 0.03	5.55 ± 0.03
Total C (%)	4.15 ± 0.02	2.55 ± 0.05	1.17 ± 0.02	1.94 ± 0.04	2.09 ± 0.01
Total N (%)	0.276 ± 0.005	0.210 ± 0.000	0.108 ± 0.005	0.179 ± 0.003	0.129 ± 0.005
Sand (%)	74 ± 4	65 ± 3	70 ± 5	39 ± 2	82 ± 3
Silt (%)	16 ± 4	19 ± 3	13 ± 2	36 ± 4	8 ± 1
Clay (%)	10 ± 2	16 ± 3	17 ± 2	25 ± 3	10 ± 1
CEC ($cmol_{(+)}$ kg^{-1} ds)	12.30 ± 0.14	20.40 ± 0.85	7.90 ± 0.42	10.75 ± 0.42	5.90 ± 0.14
Fe_2O_3 (%)	0.25 ± 0.00	0.40 ± 0.00	0.16 ± 0.00	0.30 ± 0.00	0.11 ± 0.00
Al_2O_3 (%)	0.50 ± 0.00	0.53 ± 0.01	0.12 ± 0.01	0.34 ± 0.01	0.32 ± 0.01
Total Zn (mg kg^{-1} ds)	80 ± 1	147 ± 2	95 ± 4	225 ± 10	64 ± 2
Bioavailable Zn (mg kg^{-1} ds)	3.7 ± 0.0	1.5 ± 0.1	3.6 ± 0.1	4.0 ± 0.0	2.6 ± 0.1

Descriptive statistics, one-way analysis of variance (ANOVA), mean comparison using Duncan's test and linear correlation among parameters were performed with SPSS, version 15.0.

3 Results

3.1 Physicochemical Properties of the Soils

The physicochemical properties of the soil samples are presented in Table 2. The soils display a wide range of physical and chemical properties, which may influence zinc toxicity. The pH values were rather similar in all soils except the Trasariz soil, the higher pH of which was probably due to the recent addition of lime.

The total carbon and nitrogen contents varied widely among the different soils; the highest values corresponded to the Catoira soil, and the lowest to the Rúa soil. The values of the cation exchange capacity (CEC) also varied widely, with the highest values obtained for the Coutada soil and the lowest for the Xinzo soil. The particle size distribution analysis revealed a predominant sand fraction in all of the soils, although in the Trasariz soil the percentages of sand and loam were very similar; the texture of all of the soils was sandy-loam, except for the Trasariz soil, which had a loam texture. The contents of iron oxides was highest in the Coutada soil and lowest in the Xinzo soil; aluminium oxides were found to be more abundant in the Coutada and Catoira soils, which are those that contain more organic matter.

The total zinc content of the soils also varied widely, between 64 mg kg^{-1} (Xinzo soil) and 225 mg kg^{-1} (Trasariz soil). The bioavailable zinc ranged between 1.5 and 4.0 mg kg^{-1}, obtained for Coutada and Trasariz soils,

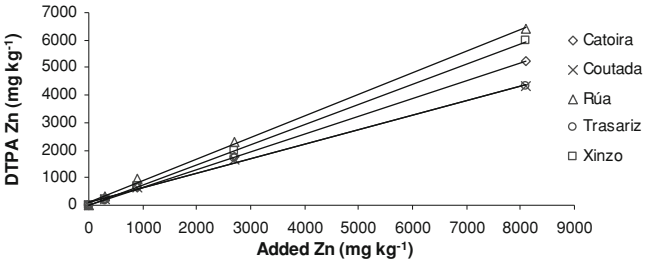

Fig. 1 Relationship between the total amounts of zinc added and zinc extracted in DTPA solution

respectively. Xinzo soil showed intermediate values between these two, and the values of Catoira and Rúa soils were similar to those of Trasariz soil.

3.2 Bioavailable Zinc

The concentration of bioavailable zinc in the different soils increased linearly with increasing doses of zinc added (Fig. 1). Most of the Zn added to soils remained in a bioavailable form, so that the percentages of Zn soluble in DTPA (bioavailable Zn) in relation to the quantities of Zn added were higher than 50%. The soils with both the lowest contents of organic matter and with the lowest cation exchange capacity displayed the highest levels of bioavailable Zn.

3.3 Soil pH

There was a substantial change in pH in the soils amended with zinc salt. The highest the dose of salt added to the soil, the highest the decrease in pH, so that in all cases the pH decreased by more than one unit in the samples contaminated with the highest dose of zinc (Table 3). The strongest decrease in pH was observed for Trasariz soil, which has an initial pH of 7.06, while for the other four soils the decreases were lower and similar for all the soils.

3.4 Hydrolase Enzymatic Activities

The values of the enzymatic activities were different in each of the soils used for the study (Table 4). The highest urease activities occurred in the Catoira and Coutada soils and the lowest in the Rúa soil, while the levels of β-D-glucosidase

Table 3 Variation in soil pH (ΔpH) after Zn addition and 7 days of incubation

Dose	Catoira	Coutada	Rúa	Trasariz	Xinzo
300	0.51	0.53	0.51	0.79	0.42
900	0.75	0.81	0.73	1.17	0.68
2,700	0.90	1.10	0.92	1.61	0.86
8,100	0.92	1.26	1.00	1.91	1.01

Table 4 Effect of increasing doses of zinc on the activities of urease (μmol NH_4^+–N g^{-1} h^{-1}), phosphomonoesterase (μmol *p*NP g^{-1} h^{-1}) and *β*-D-glucosidase (μmol *p*NP g^{-1} h^{-1}) in dry soil basis. Mean ± standard deviation values (n = 3) are shown

	Dose	Catoira	Coutada	Rúa	Trasariz	Xinzo
Urease	Control	3.40 ± 0.22	3.45 ± 0.22	1.10 ± 0.07	1.82 ± 0.04	1.72 ± 0.05
	300	3.15 ± 0.07	3.90 ± 0.20	0.68 ± 0.07	2.28 ± 0.10	1.57 ± 0.05
	900	3.11 ± 0.19	3.51 ± 0.15	0.68 ± 0.04	1.89 ± 0.13	1.14 ± 0.09
	2,700	2.18 ± 0.07	1.63 ± 0.05	0.22 ± 0.02	1.46 ± 0.06	0.49 ± 0.04
	8,100	0.60 ± 0.02	0.50 ± 0.02	0.07 ± 0.00	0.54 ± 0.05	0.16 ± 0.01
Phosphomonoesterase	Control	2.81 ± 0.07	2.19 ± 0.11	1.26 ± 0.06	2.40 ± 0.09	1.64 ± 0.04
	300	2.65 ± 0.05	1.94 ± 0.13	1.24 ± 0.06	2.02 ± 0.14	1.52 ± 0.11
	900	2.57 ± 0.10	1.79 ± 0.06	1.00 ± 0.03	1.80 ± 0.10	1.37 ± 0.07
	2,700	2.05 ± 0.08	1.40 ± 0.11	0.64 ± 0.02	1.62 ± 0.07	1.03 ± 0.04
	8,100	1.32 ± 0.10	0.90 ± 0.05	0.21 ± 0.01	1.06 ± 0.06	0.33 ± 0.01
β-D-glucosidase	Control	0.68 ± 0.01	0.63 ± 0.01	0.60 ± 0.05	0.72 ± 0.04	0.92 ± 0.07
	300	0.57 ± 0.05	0.69 ± 0.03	0.61 ± 0.05	0.67 ± 0.06	0.92 ± 0.09
	900	0.53 ± 0.02	0.53 ± 0.03	0.53 ± 0.04	0.70 ± 0.05	0.92 ± 0.09
	2,700	0.52 ± 0.05	0.53 ± 0.04	0.45 ± 0.03	0.53 ± 0.02	0.81 ± 0.06
	8,100	0.34 ± 0.02	0.30 ± 0.01	0.01 ± 0.00	0.30 ± 0.01	0.65 ± 0.04

activity were similar in all soils. Similarly to urease, Catoira soil showed the highest phosphomonoesterase activity and Rúa soil the lowest.

The addition of zinc tended to reduce all the enzymatic activities to lower values than those observed in the uncontaminated (control) soils. The urease activity decreased as the amount of zinc added increased, although in some cases at the lowest dose the activity increased. The phosphomonoesterase activity also decreased in the presence of zinc, and the reduction increased as the dose of contaminant increased. As regards the *β*-D-glucosidase activity, the values observed for the lowest doses of zinc were very similar to those in the control soil, and in all soils a clear reduction was only observed at very high levels of contaminant.

As the levels of the enzyme activities considered were very different in all of the soils, and aiming to facilitate the comparison among soils, the values of the enzyme activities in contaminated soils were expressed as the percentages of the corresponding activities in the control soils (Figs. 2, 3 and 4). Although in general the urease activity tended to decrease in the presence of the metal, especially for the highest doses of zinc, it increased by approximately 20% at the lowest dose of

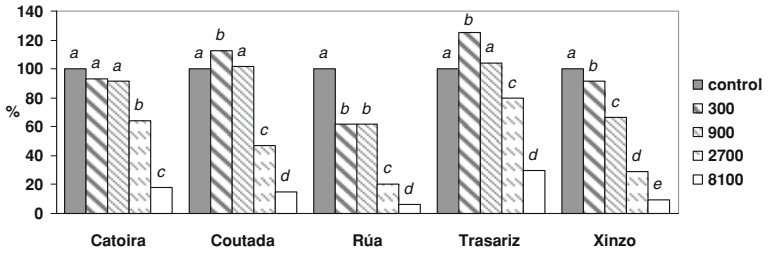

Fig. 2 Effect of increasing doses of zinc on urease activity. The same letter within the same soil indicate no significant differences at $P \leq 0.05$ (Duncan's test)

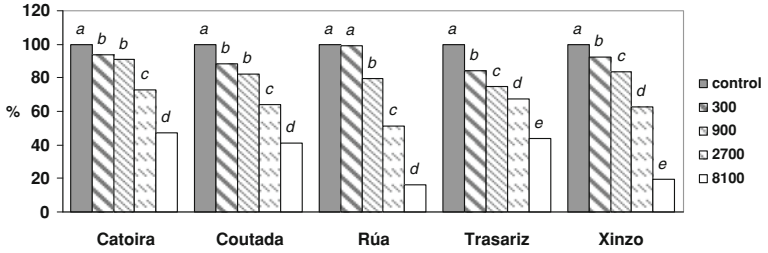

Fig. 3 Effect of increasing rates of zinc on phosphomonoesterase activity. The same letter within the same soil indicate no significant differences at $P \leq 0.05$ (Duncan's test)

contaminant in the Coutada and Trasariz soils (Fig. 2). On average for all the contaminated soils, the percentage of reduction in the urease activity observed was 3% for the lowest dose of the contaminant, 52% for the 2,700 mg kg^{-1} dose and 85% for the highest dose.

The phosphomonoesterase activity decreased significantly even at the lowest dose of contaminant, and the decrease was proportional to the rate of zinc added to the soil (Fig. 3). Thus, on average for all soils the dose of 300 mg kg^{-1} reduced the activity by a 8%, the dose of 900 mg kg^{-1} caused a 18% decrease, the dose of 2,700 mg kg^{-1} resulted in a 36% decrease and the dose of 8,100 mg kg^{-1} zinc caused a reduction of the activity of 66%.

The β-D-glucosidase activity was also inhibited in the presence of zinc (Fig. 4), although to a much lesser extent than the phosphomonoesterase and urease activities. For example, the dose of 900 mg kg^{-1} depressed the β-D-glucosidase activity by an 11% on average for all the investigated soils, and 2,700 mg kg^{-1} of Zn resulted in a reduction of the activity of 21%, but only the highest dose decreased the activity by more than 50%.

On the basis of the mean values for inhibition of enzyme activity, urease activity appeared to be the most sensitive to addition of Zn while β-D-glucosidase was the least sensitive.

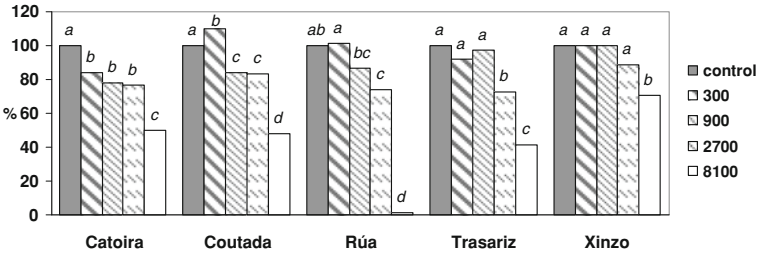

Fig. 4 Effect of increasing rates of zinc on β-D-glucosidase activity. The same letter within the same soil indicates no significant differences at $P \leq 0.05$ (Duncan's test)

Table 5 Ecological dose (ED_{50}, ED_{20}, ED_{10}) values, in mg Zn kg^{-1}, and coefficients of determination obtained for the dose-response curves

		Catoira	Coutada	Rúa	Trasariz	Xinzo
Urease	R^2	0.993	0.928	0.944	0.920	0.992
	ED_{50}	4219	3354	925	5938	1533
	ED_{20}	1491	1373	206	2989	556
	ED_{10}	720	887	65	2064	233
Phosphomonoesterase	R^2	0.996	0.994	0.994	0.953	0.999
	ED_{50}	7227	5124	2855	5916	3932
	ED_{20}	1891	1024	906	748	1245
	ED_{10}	825	395	470	236	551
β-D-glucosidase	R^2	0.891	0.898	0.991	0.973	0.985
	ED_{50}	8787	7750	4243	6418	15333
	ED_{20}	1293	2639	1818	2125	5417
	ED_{10}	269	1387	1010	986	2745

3.5 Ecological Dose (ED) Values

The values of enzymatic activities were fitted to the sigmoidal dose-response curve of Van Beelen et al. (2004), and used to calculate the ED_{50}, ED_{20} and ED_{10} values. The total amount of zinc that led to a 50% inhibition in enzyme activity (ED_{50} value) revealed a large variability in the different soils (Table 5) and for the different enzymes. The lowest ED_{50} values obtained corresponded to the urease activity and the highest to the β-D-glucosidase activity. The ED_{50} values in Rúa soil were very low in comparison with the values estimated for the other soils. The highest ED_{50} values for urease activity were obtained for Trasariz soil, while for phosphomonoesterase and β-D-glucosidase activities the highest values were obtained for Catoira and Xinzo soils, respectively. However, a 50% reduction in a basic ecological process may be too extreme for the continued functioning of a soil and so lower percentage values of inhibition (10 or 20%) equivalent to ED_{10} or ED_{20} were calculated from the dose response curves by interpolation. The ED_{10} values were between the first two doses added in this study, except for

Table 6 Correlation coefficients (r) between enzyme activities and added Zn, DTPA-extractable Zn, pH values and variation in soil pH (ΔpH)

	Added Zn	DTPA Zn	pH H_2O	ΔpH
Urease	−0.857**	−0.880**	0.580**	−0.546**
Phosphomonoesterase	−0.929**	−0.960**	0.416*	−0.717**
β-D-glucosidase	−0.906**	−0.945**	0.410*	−0.654**

** $P \leq 0.01$, * $P \leq 0.05$

β-D-glucosidase which showed higher values. Unexpectedly, no correlations between ED_{50} values and the physicochemical soil properties were observed.

3.6 Relationship between Zinc Content, pH and Enzymatic Activities

Table 6 shows the correlation coefficients obtained for the linear correlation between enzyme activities and added Zn, DTPA-extractable Zn, pH values and variation in soil pH (ΔpH).

The Zn concentration and all the enzymatic activities were negatively correlated, but there were no discernable differences in the correlation coefficients whether DTPA fraction or added zinc was considered. Significant positive correlations between enzymatic activities and the pH values and negative correlations between the enzymatic activities and the change (decrease) in pH were also observed, but this can be an indirect effect because of the decrease in pH as the dose of Zn added to the soil increases.

4 Discussion

Added Zn may be quickly adsorbed onto solid soil surfaces, but the immobilization caused by diffusion and occlusion is a much slower process. Thus, it is expected that a significant proportion of the metal remains in an active bioavailable fraction. As a result of this, the percentage of DTPA-extractable Zn was high in relation to the dose applied.

Zinc amendment resulted in considerable acidification of all studied soils, especially at the higher concentrations. Soil acidification in metal amendment studies had been previously reported by diverse authors, like Aoyama and Itaya (1995) and Speir et al. (1999). According to Percival et al. (1999), the pH reduction in the contaminated samples could be attributed to the displacement of protons from the cation-exchange complex, and hydrolysis of the zinc cations. Therefore, it could be expected that the soil organic matter would attenuate the change in soil pH values caused by addition of Zn (Speir et al. 1999). However,

and except in the case of Trasariz (which had a neutral pH, probably due to a recent addition of lime), there were not appreciable differences among the pH decreases shown by the diverse soils, indicating that either there was not such attenuation or it was similar in all soils.

Several studies have been carried out to investigate the effect of heavy metal toxicity on enzymatic activity, and considerable differences in their sensitivities have been reported (Giller et al. 1998). In this study, the addition of a Zn metal salt to agricultural soils produced a wide range of enzymatic responses, and in general urease activity appeared to be the most sensitive parameter, and β-D-glucosidase activity the least sensitive (i.e. more tolerant to the presence of Zn). Nevertheless, the effect of the addition of low doses of metal varied from slight stimulation to slight reduction. When zinc was added in larger doses, it acted causing a strong decrease of soil enzymatic activity, similarly to which has been shown by many authors (Juma and Tabatabai 1977; Eivazi and Tabatabai 1990; Hemida et al. 1997; Giller et al. 1998; Stuczynski et al. 2003; Belyaeva et al. 2005). Soils differed widely in their sensitivity to added zinc, which may be related to abiotic factors that determine the availability of the metal. In general, the highest percentages of reduction were observed in the soils with the lowest organic matter content and cation exchange capacity, which were also the soils that showed the highest proportion of added Zn remaining in bioavailable forms.

It is possible that, in addition to the toxicity of the metal, the change in pH also had some effect on the enzyme activity inhibition, as indicated by the fact that both the increase in Zn concentration and the decrease in pH affected negatively the enzymatic activities (Table 6). Similar results were reported by Speir et al. (1999), who showed that in many cases a considerable proportion of the reduction in enzyme activity occurring when soils are amended with heavy metal salts can be attributed to acidification and not to a direct metal effect.

The range of toxicity, expressed as ED_{50} values, was very variable in the different soils for each enzyme. All threshold values were within the test range, except for Catoira and Xinzo soils in which the ED_{50} values for β-D-glucosidase activity exceeded the highest concentration tested, because in these soils the maximum reduction of β-D-glucosidase activity was near (Catoira soil) or did not reach (Xinzo soil) the 50%. The variability observed in the *ED* values may be related to the different properties of the soils that determine sorption and solubility of the zinc in soils (Welp and Brümmer 1999). Thus, for urease and phosphomonoesterase activities (the two enzymes showing clear sensitivity to zinc contamination), the lowest ED_{50} values were obtained for the soils in which a high proportion of the added Zn remained as bioavailable. However, it was not possible to establish correlations between the magnitude of the ecological dose and soil properties such as pH, clay or organic matter content at the 0.05 significance level.

Although the fact that no such correlation was found might be related to the short incubation time (7 days), it is also possible that the lack of correlation was due to other factors, as some authors like Smolders et al. (2004) observed significant correlations between soil properties and EC_{50} values for potential nitrification rate in soils artificially contaminated with zinc and incubated for short

periods of time (3 days). The lack of correlation could also be related to the small number of soils studied.

5 Conclusions

In conclusion, the results indicate that addition of Zn chloride lowered the soil pH, with a reduction of about 1 unit below control values at the highest doses added.

The extent of the negative effect of soil contamination with Zn depended on the type of soil and the enzyme considered.

Among the enzymes tested, urease was the most sensitive to zinc contamination and β-D-glucosidase was the least sensitive, and therefore urease and phosphomonoesterase activities would be better bioindicators of soil contamination with zinc than β-D-glucosidase activity.

At the lowest dose of addition, Zn had little effect on the enzyme activities considered, but the highest doses caused a serious decrease in all the enzymatic activities.

The greatest amounts of DTPA-extractable zinc were observed in the soils with the lowest organic matter content and cation exchange capacity.

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References

Aoyama M, Itaya S (1995) Effects of copper on the metabolism of ^{14}C-labeled glucose in soil in relation to amendment with organic materials. Soil Sci Plant Nutr 41:245–252

Babich H, Stotzky G (1985) Heavy metal toxicity to microbe-mediated ecologic processes: A review and potential application to regulatory policies. Environ Res 36:111–137

Belyaeva ON, Haynes RJ, Birukova OA (2005) Barley yield and soil microbial and enzyme activities as affected by contamination of two soils with lead, zinc or copper. Biol Fertil Soils 41:85–94

Dick RP (1997) Soil enzyme activities as integrative indicators. In: Pankhurst CE, Doube BM, Gupta VVSR (eds) Biological indicators of soil health. CAB International, Wallingford, UK, pp 121–156

Eivazi F, Tabatabai MA (1990) Factors affecting glucosidase and galactosidase activities in soils. Soil Biol Biochem 22:891–897

FAO (1966) Guía para la Descripción de Perfiles de Suelo. FAO, Rome

Giller KE, Witter E, McGrath P (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol Biochem 30:1389–1414

Gillman GP, Bruce RC, Davey BG, Kimble JM, Searle PL, Skjemstad JO (1983) A comparison of methods used for determination of cation exchange capacity. Commun Soil Sci Plant Anal 14:1005–1014

Gil-Sotres F, Trasar-Cepeda C, Leirós MC, Seoane S (2005) Different approaches to evaluating soil quality using biochemical properties. Soil Biol Biochem 37:877–887

Guitián F, Carballas T (1976) Técnicas de Análisis de Suelos. Pico Sacro Editorial, Santiago de Compostela, Spain

Hemida SK, Omar SA, Abdel-Mallek AY (1997) Microbial populations and enzyme activity in soil treated with heavy metals. Water Air Soil Pollut 95:13–22

Juma NG, Tabatabai MA (1977) Effects of trace elements on phosphatase activity in soils. Soil Sci Soc Am J 41:343–346

Lindsay WL, Norvell WA (1978) Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Sci Soc Am J 42:421–428

McKeague JA, Day JH (1966) Dithionite and oxalate extractable Fe and Al as an aid in differentiating various classes of soils. Can J Soil Sci 46:13–22

Moreno JL, Hernández T, Pérez A, García C (2002) Toxicity of cadmium to soil microbial activity: effect of sewage slude addition to soil on the ecological dose. Appl Soil Ecol 21:149–158

Percival HJ, Speir TW, Parshotam A (1999) Soil solution chemistry of contrasting soils amended with heavy metals. Aust J Soil Res 37:993–1004

Smolders E, Buekers J, Oliver I, McLaurin J (2004) Soil properties affecting toxicity of zinc to soil microbial properties in laboratory-spiked and field-contaminated soils. Environ Toxicol Chem 23:2633–2640

Speir TW, Kettles HA, Percival HJ, Parshotam A (1999) Is soil acidification the cause of biochemical responses when soils are amended with heavy metal salts? Soil Biol Biochem 31:1953–1961

Speir TW, van Schaik AP, Hunter LC, Ryburn JL, Percival HJ (2007) Attempts to derive EC_{50} values for heavy metals from land-applied Cu-, Ni-, and Zn-spiked sewage sludge. Soil Biol Biochem 39:539–549

Stuczynski TI, McCarty GW, Siebielec G (2003) Response of soil microbiological activities to cadmium, lead, and zinc salt amendments. J Environmental Quality 32:1346–1355

Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2000) Biochemical properties of acid soils under climax vegetation (Atlantic oakwood) in an area of the European temperature humid zone (Galicia, N.W. Spain): Specific parameters. Soil Biol Biochem 32:747–755

Van Beelen P, Wouterse M, Posthuma L, Rutgers M (2004) Location-specific ecotoxicological risk assessment of metal-polluted soils. Environ Toxicol Chem 23:2769–2779

Welp G, Brümmer W (1997) Toxicity of increased amounts of chemicals and the dose-response curves for heterogeneous microbial populations in soil. Ecotoxicol Environ Saf 37:37–44

Welp G, Brümmer W (1999) Effects of organic pollutants on soil microbial activity: the influence of sorption, solubility, and speciation. Ecotoxicol Environ Saf 43:83–90

Evaluation of the Biological Activity in a Gypsiferous Soil Co-Amended with Residues

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Abstract The use of different types of residues for soil amendment is encouraged by environmental policies to improve waste management. These materials supply organic matter and nutrients to the soil and contribute the establishment of vegetal cover, which plays an important role in preventing erosion processes. The aim of this study was to evaluate the rehabilitation process of a degraded soil in a long-term field experiment located in Aranjuez (Madrid) and to assess the influence of the process on the biological activity of the soil. The soil was amended with recycled de-inking paper sludge (P) and was co-amended with either sewage sludge composted with pruning wastes (CP) or thermally dried sewage sludge (TD) in experimental plots (30 × 10 m). The vegetal cover was introduced by sowing a mixture of herbaceous species (grasses and legumes). Three years after the application, different effects on soil properties were observed. The recycled de-inking paper sludges (P) enhanced physical soil properties and increased organic matter. When they were combined with sewage sludge (composted or thermally dried), the treatments resulted in increased nutrient content in the soil and an important development of the vegetal cover when compared to the control soil. In general, the organic waste induced changes in soil enzyme activities (β-glucosidase, phosphatase alkaline, and urease) depending on the treatment applied. A decay in bacterial biodiversity as assessed through the PCR-DGEE profile was observed in the amended soil relative to the control soil.

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1 Introduction

Anthropic activities and climatic conditions in Mediterranean countries generate environmental impacts that create degraded sites, including uncovered soils. These sites must be rehabilitated to minimise erosion processes related to the loss of organic matter and nutrient soil content (Larney et al. 2000). A decrease in organic matter content affects the soil quality (Jones et al. 2004) and the decline of the vegetal cover. Therefore, the first step in restoring these degraded ecosystems is to improve the soil characteristics to facilitate the colonisation of native vegetation. Additionally, waste management policies encourage the use of different types of residues as soil amendments (Fytili and Zabaniotou 2008) to improve soil physicochemical properties and accelerate the vegetal cover development, which plays an important role in preventing erosion processes in semiarid ecosystems environments (Albadalejo et al. 1994; Alcañiz et al. 1999; Cuevas et al. 2000; García et al. 2000; Cabezas et al. 2004; Sastre-Conde et al. 2007). De-inking paper sludge have high organic matter content and improve the physical properties of soil (Chantigny et al. 1999), thereby restoring the soil biological activities (Chantigny et al. 2000; Fierro et al. 2000). Sewage sludge represents an important type of organic waste applied to agricultural soils. For the last 20 years, sewage sludge treatment has been studied as a method for increasing the biological activity of soil (Sastre et al. 1996; Sastre-Conde et al. 2003a; Sastre-Conde et al. 2007).

The composition and stabilisation of the exogenous organic matter applied to the soil significantly affects soil rehabilitation processes. The incorporation of organic wastes into the soil and the degradation of the soil organic matter by soil microorganisms are mediated by soil enzymes. Therefore, previous studies examined the importance of substrate utilisation for the maintenance of soil resilience and stability (Griffiths et al. 2004). Detailed studies of the soil enzymes involved in the soil degradation processes have been central to understanding the decomposition of the organic matter (Leinweber et al. 2008).

Biological and physicochemical parameters have been evaluated in soil restoration processes since the early 1990s (Nannipieri et al. 1990; Benthan et al. 1992; Pascual et al. 1999; Bonmatí et al. 2000; Ros et al. 2003; Bastida et al. 2008). In a greenhouse experiment studying the rehabilitation of soil with a combination of two practices (soil amended with sewage sludge and recovery with herbaceous plants), Sastre-Conde et al. (2007) found a close relationship between the effects of the rehabilitation processes on soil enzyme activities (alkaline phosphatase and β-glucosidase) and the mobilisation of nutrients through the plant. They suggested that soil enzyme activities were useful as indicators of the rehabilitation processes, particularly for processes that are responsible for the first vegetal cover (herbaceous plants). Nonetheless, the behaviour of plants, the types of shrubs that respond to the soil organic matter application and the effect of the application on the biological activity of soil are still unknown.

The aim of this study was to evaluate a long-term soil rehabilitation process with organic waste incorporation (recycled de-inking paper sludge, composted

Table 1 Soil properties

pH	E.C. dS m^{-1}	$CaCO_3$ %	$CaSO_4$	N	O.M.	P	Ca	Mg	Na	K
						mg kg^{-1}				
7.8	1.6	37	24.6	0.1	2.06	25	12970	630	15	211

sewage sludge and thermally dried sewage sludge). The selected soil was characterized by a high salt content and low stability of organic matter, making it particularly sensitive to erosion processes. The effects of the rehabilitation were examined by studying the biological activities of the soil as possible indicators of global changes in the soil-plant ecosystem. Therefore, the enzymatic activity of soil microorganisms and the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiles of 16S rRNA gene fragments of soil bacteria were evaluated during the rehabilitation process.

2 Materials and Methods

2.1 Soil

Experimental plots were located in a marginal area in Central Spain (Aranjuez). The soil in the study area was characterised as Typic Calcixerept (Soil Survey Staff 1998). Table 1 lists the chemical properties of the A horizon (0–25 cm). This type of soil is characterized by a high content of sulphates and very labile organic matter, which does not contribute to the formation of stable aggregates in soil. The assay was conducted in a model of experimental plots (30 × 10 m) in randomised block of three replicates, including the control treatment.

2.2 Organic Waste Characteristics

As organic amendments a residue from the industry recycled paper (P) was used both, alone or mixed with either compost of sewage sludge and pruning wastes (CP) or thermically dried sludge (TD). The chemical characteristics and heavy metal contents of the organic wastes are shown in Table 2. The doses of the different residues were established based on Spanish legislation (R.D. 1310/1990) (Table 3). Waste application was carried out in autumn, before seeding. Soil samples were taken 6 months, 1, 2 and 3 years after organic amendment. The study shows the results after three years in relation to the objective of a long term application.

Table 2 Organic wastes characteristics

Organic waste	Chemical characteristics						Exchangeable cation (mg kg^{-1})				Heavy metals (mg kg^{-1})					
	pH	EC dS m^{-1}	OM %	C/N	N %	P mg kg^{1}	Ca	Mg	Na	K	Ni	Pb	Cd	Cu	Cr	Zn
P	7.5	0.9	38.6	79.86	0.28	50	4218	203	576	39	13	74	0.3	145	9	68
CP	7.5	2.99	22.5	8.16	1.6	288	4705	883	352	2034	33	78	0.4	170	248	1602
TD	6.7	4.64	25.9	3.32	4.52	400	2770	970	552	974	73	219	1.9	385	280	1118

Table 3 Treatments used in the field experiment

Treatments	RDPS[1]	SSCP[2]	TDSS[3]
	(t ha^{-1} dry matter)		
C	0	0	0
P	100	0	0
P + CP	100	18.78	0
P + TD	100	0	18.78

[1] Recycled de-inking paper sludge
[2] Sewage sludge composted with pruning wastes
[3] Thermal dried sewage sludge

2.3 Plant Biomass

A mixture of grasses and legumes (100 and 200 kg of seeds ha^{-1}) were grown on soils three months after the amendment application. These herbaceous seeds were previously described in detail by Cabezas et al. (2004).

2.4 Analysis of Physicochemical Soil Characteristics

Soil properties were measured according to MAPA (1994). Electric conductivity (EC) and pH were measured at a 1:2.5 soil-to-water ratio. Organic matter and total nitrogen content were determined using Walkley–Black (1934) and Kjeldahl methods, respectively. Carbonate levels in the soil were measured using the Bernard Calcimeter method and sulphate content was determined in aqueous extract by gravimetry. Available nutrients were extracted with 0.1 N NH_4Ac at pH 7 and assessed using atomic absorption spectrometry (Varian, AA 240 FS). Water-holding capacity was measured using Richard Plates at 0.33 and 15 atm (MAPA 1994). Plant biomass was calculated using four representative subplots of 1 m^2 in each treatment.

2.5 Heavy Metal Analysis

After acid digestion of soil samples with HCl–HNO_3 using a microwave reaction system (Multiwave 3000, Anton Paar GmbH, Graz, Austria), total concentrations of Cd, Cr, Pb and Ni were analysed using inductively coupled plasma-atomic emission spectrometry (Varian Liberty AX, Victoria, Australia), and Zn and Cu concentrations were measured by atomic absorption spectrometry (Varian AA 240 FS).

2.6 Soil Enzyme Activities

The urease activity in the soil was analysed by a method proposed by Sastre-Conde and Lobo (2003b). Urea was used as a substrate and selective ammonium electrodes were used to determine activity levels. The glucosidase and alkaline

phosphatase activities were analysed according to García et al. (2003), and *p*-nitrophenil-*β*-D-glucopyranoside and *p*-nitrophenyl-phosphate were used as substrates. The *p*-nitrophenol content was determined by V-UV spectrophotometry at 400 nm.

2.7 Soil Respiration

Soil respiration was calculated by the amount of CO_2 released by glucose-induced respiration, which was assessed with a BACTRAC unit based on the impedance method (Walker et al. 2005). Previously, soil samples (sieved through 2-mm mesh and then dried) were incubated for 48 h.

2.8 DNA Extraction

Two plots per treatment were randomly selected for analysis of DNA from bulk soil samples. Briefly, 4 g of composite soil samples from each plot was re-suspended in 9 mL of phosphate buffer (5 mM) and treated in a Restcher MM200 blender (Restcher Gmb & Co, Haan, Germany) for 1 min at high speed. The supernatant was collected after centrifugation at low speed (2 min, 500 g). This step was repeated twice. Then, 50 μL of the combined supernatant was used for DNA extraction with a commercial kit (Mo Bio PowerSoil, Carlsbad, California) according to the manufacturer's instructions.

2.9 PCR-DGGE of Soil Microbial Community

The 16S rRNA gene of the bacterial community was amplified using GC-clamped 968f and 1401r primers (Heuer and Smalla 1997). PCR amplifications were performed in a reaction volume of 50 μL that contained the target DNA, 5 μL 10 × PCR buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.28 μg μL^{-1} bovine albumin serum, 20 pmol of each primer and 2.5 U of DNA polymerase (Biotools B&M Labs. Madrid, Spain). Touch-down PCR was performed following Rosado et al. (1996). After denaturing for 5 min at 94°C, the annealing temperature was initially set to 67°C and was decreased by 1°C every second cycle until it reached 57°C. Then, 10 additional cycles were run at 57°C. After each cycle, annealing was performed for 1 min. The separation of strands was carried out at 94°C for 1 min, and the extension was carried out at 72°C for 2 min.

The amplification of products of the expected size (approximately 450 bp) was verified by electrophoresis in a 1.5% w/v agarose gel. Next, DGGE of the amplified 16S rRNA gene sequence was performed with 6% acrylamide gels

containing a 40–60% gradient of the denaturants (100% defined as urea 7 M, 40% formamide). The complete PCR products were concentrated to a 15 μL final volume using a vacuum concentrator centrifuge (miVac DNAQuatro Concentrator, GeneVac LTD, Ipswich, UK) and loaded on gels, which were run for 18 h in an Ingeny device (Ingeny International, Goes, The Netherlands). Gels were stained for 45 min with ethidium bromide. The number of gel/lines replicas was three. The banding patterns were analysed using Alpha EaseFC software (Alpha Innotech, San Leandro, CA). The Shannon Index ($H = -\sum(n_i/N)\ \ln(n_i/N)$, where n_i is the peak height of the band i, i is the number of each band in the DGGE gel profile and N is the sum of peak heights of all bands in the DGGE gel profile) was calculated to estimate bacterial diversity based on the intensity and number of bands from PCR-DGGE profiles.

2.10 Statistical Analyses

ANOVA was performed on the biological variables of the soil, soil enzyme activities (β-glucosidase, alkaline phosphatase and urease), soil respiration and bacterial composition of the treated soil (P, P + CP and P + TD) in comparison to the control soil. The discrimination of similar groups was carried out using Duncan's analysis of variance of significant groups at 95% confidence levels. Biological parameters, vegetation analysis and water-holding capacity were analysed by a Pearson correlation analysis. All statistical analyses were performed using the statistical software SPSS (Version 13 for Windows).

3 Results and Discussion

3.1 Soil Properties

Soil chemical characteristics did not significantly differ among treatments, including unamended soil, three years after organic amendment (Table 4), although there was a slight increase in the percentage of organic matter and nutrients (especially potassium). However, the available phosphorous was significantly different for different treatments (statistical significance at $P < 0.05$). Thus, the amounts of available phosphorus in the control soil and the soil with recycled de-inking paper sludge were similar, but the soils that were co-amended with CP and TD showed the highest values.

The lack of significant changes in soil nutrients three years after the application of organic waste could be due to the two-phase degradation of organic matter incorporated into the soil, but also to the low sewage sludge doses applied in the coamended treatments. The initial phase of fresh organic matter decomposition in

Table 4 Soil chemical characteristics three years after organic amendments application to soil

	pH	EC dS m^{-1}	CO_3^- %	OM %	N %	P mg kg^{-1}	K mg kg^{-1}
C	7.61 ± 0.21^{a}	1.60 ± 0.29^{a}	23.31 ± 6.10^{a}	2.08 ± 0.73^{a}	0.18 ± 0.03^{a}	11.11 ± 5.69^{a}	172.17 ± 30.57^{a}
P	7.71 ± 0.27^{a}	1.68 ± 0.23^{a}	26.10 ± 5.17^{a}	3.01 ± 0.75^{a}	0.18 ± 0.03^{a}	11.78 ± 4.38^{ab}	182.00 ± 38.41^{a}
P + CP	7.68 ± 0.05^{a}	1.69 ± 0.07^{a}	24.99 ± 8.23^{a}	2.92 ± 0.45^{a}	0.16 ± 0.02^{a}	16.22 ± 4.94^{bc}	167.67 ± 64.90^{a}
P + TD	7.78 ± 0.15^{a}	1.65 ± 0.18^{a}	28.29 ± 4.79^{a}	3.26 ± 0.30^{a}	0.19 ± 0.01^{a}	17.89 ± 3.69^{c}	225.50 ± 26.27^{a}

Means followed by the same lower case letter are not significantly different at $p = 0.05$ LSD level

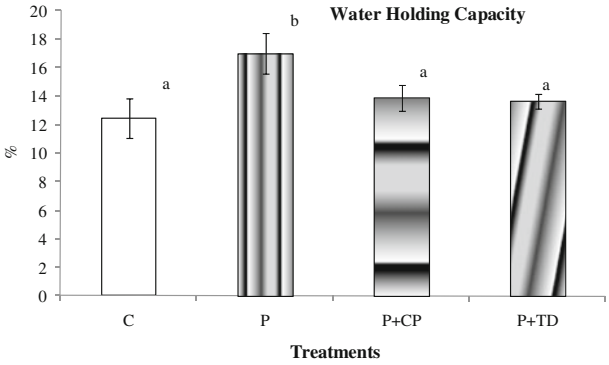

Fig. 1 Soil water holding capacity (WHC) three years after organic amendments application to soil

soil is rapid and mostly determined by free cellulose, which disappears after a short time. The second and slower degradation occurs when the cellulose is encrusted with lignin and lignocelluloses (Melillo et al. 1989). In this experiment and after a short time of amendment application, a significant increase in nutrient content was observed in the coamended soils according to the increase in the plant biomass (Cabezas et al. 2007). Then, the amendment application provokes an improvement in soil physical properties that leads to a best habitat to microorganism activity and consequently to the vegetal production. Similar results have been reported in long term field experiments using organic residues (Bastida 2008). Fierro et al. (2000) revealed a discrepancy in decomposition based on the two-part decay model, that could be responsible for the recycled paper residue decomposition in the experimental conditions. Chantigny et al. (1999, 2000) found that 40% of the added paper de-inking sludge remained in the soil in the form of acid-resistant carbon two years after the application. These authors suggested that when the application rates of de-inking paper sludge were high, soil conditions showed a nutrient-limiting state for the microbial activity responsible for carbon decomposition. Therefore, in this study, the incorporation of a large amount of nutrients in the co-amended soils did not seem to have an effect on the long-term soil chemical properties due to the incorporation of organic wastes. However, the soil that was co-amended seemed to experience faster organic matter decomposition which could be lead by soil microorganisms implied in its metabolism.

Other authors have corroborated the importance of soil properties and climatic conditions (Valauwe et al. 1997), as well the availability of nitrogen and phosphorus from endogenous or exogenous origins (Cheshire and Chapman 1996).

Three years after the incorporation of soil amendments, the water-holding capacity was significantly higher in the soil treated with the recycled de-inking paper sludge compared to the co-amended soil and control soil (Fig. 1). Chantigny et al. (1999) found similar results and reported that paper de-inking sludge markedly improved macroaggregate stability.

3.2 Soil Enzymes

The behaviour of the soil enzymes under different soil treatments varied according to the enzyme analysed. In general, the soils that were co-amended with P + CP had the lowest levels of enzyme activity (urease, β-glucosidase and alkaline phosphatase). These values were not significantly different from enzyme activities at the control soil (C) (Fig. 2). Urease activity increased significantly in soils that were co-amended with P + TD in comparison to the other treatments and the control soil (Fig. 2). Similar results were found for alkaline phosphatase, but there were no significant differences between the P + TD soil and the P soil. The highest β-glucosidase values were found in the soil amended with recycled de-inking paper sludge (P). The strong responses of the alkaline phosphatase and urease activities in the soil co-amended with P + TD could be due to the addition of the organic waste (TD) which had the highest nutrient content (N and P).

The enhancement in the response of the β-glucosidase in the soils with paper residues agreed with the improvement in the physical properties of the soil in these plots, as there was also an increase in the water-holding capacity, which should stimulate the soil biological activity through the microbial activity. Previous studies have reported a strong relationship between β-glucosidase and the soil bacteria population (Sastre-Conde et al. 2003a). Soil treated with recycled de-inking paper sludge showed the highest values of soil respiration (Fig. 3). However, this parameter was not significantly different in both co-amended soils (P + CP and P + TD) when compared to the P soil and the control soil (C).

3.3 Bacterial Communities in the Soil

The profiles of the bands in DGGE were related to the diversity of the bacterial communities in the soil. This process allowed for the comparison of the composition of the bacterial communities after the application of the different organic amendments (Fig. 4). Shannon Index values indicated a slight decrease in the bacterial diversity in the amended soil (Table 5). It appeared that each treatment introduced changes in the relative abundance of different bacterial strains. Several bands corresponding to original populations were not detected, whereas after treatment other strains appeared (arrows in Fig. 4). It is likely that the amendments provided new microorganisms to the soil or sources of organic matter that led to the rise of specific bacterial strains involved in the nutrient and biogeochemical cycles.

Kuan et al. (2006) studied the effect on the diversity of the bacterial communities in soils under different treatments, distinguishing significant difference in the bacterial communities under treated soils in comparison to untreated, but not between treatments. These results are in agreement with those obtained in this study.

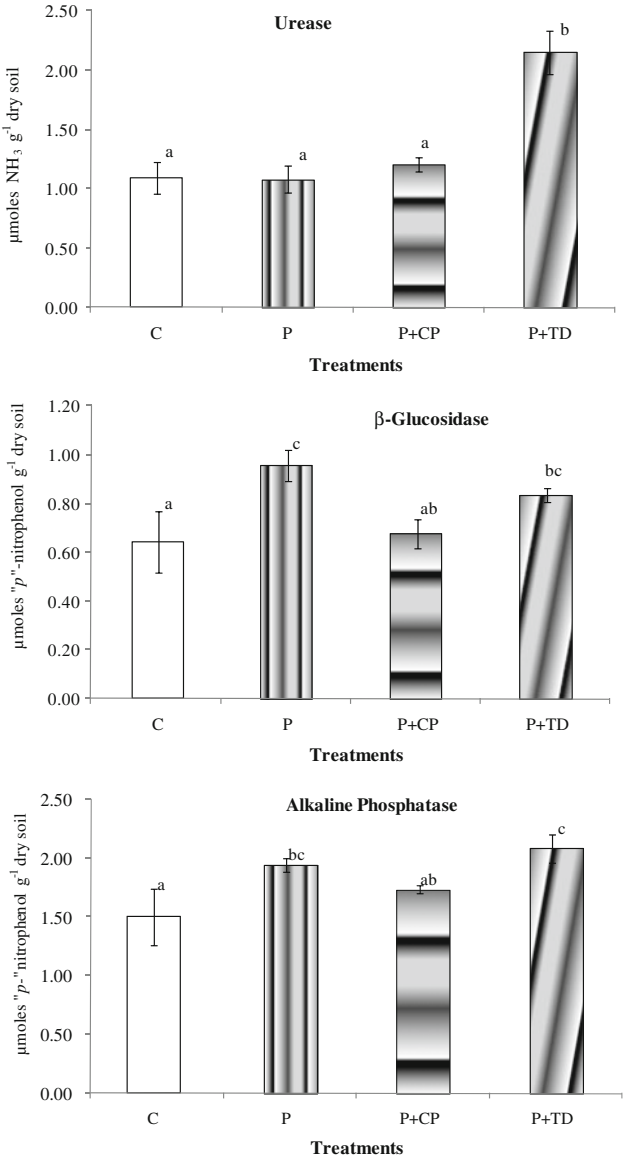

Fig. 2 Soil enzyme activity three years after organic amendments application to soil

3.4 Plant Biomass

The co-amended soils (P + CP, P + TD) showed the highest plant biomass (Fig. 5). Although there were no significant changes in the soil nutrients three years after organic waste application, the increase in the vegetal biomass could

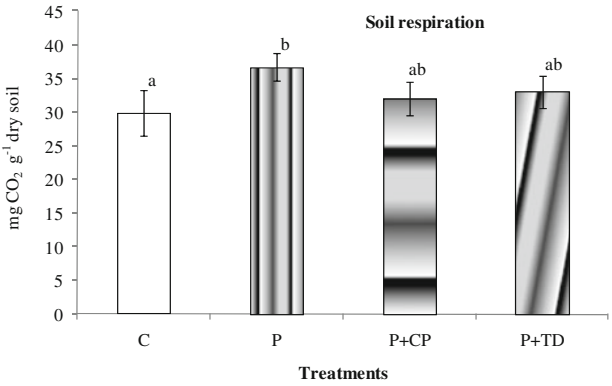

Fig. 3 Soil respiration three years after organic amendments application to soil

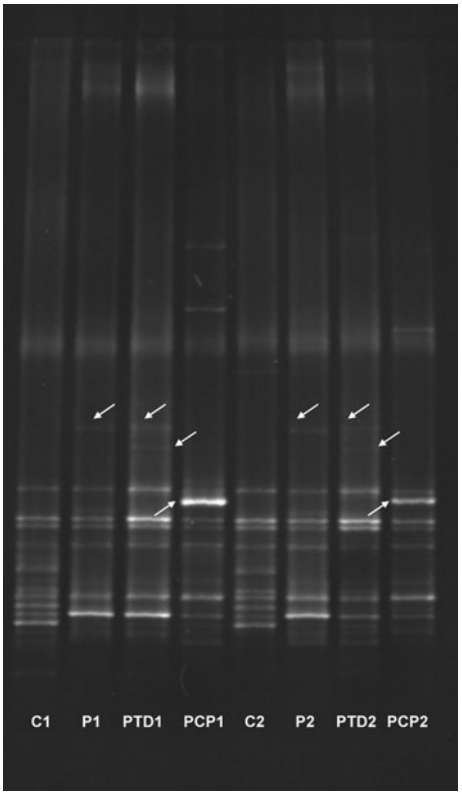

Fig. 4 DGGE profiles of bacterial community from soil amended with organic residues. None amendment (*C1, C2*), recycled de-inking paper sludge (*P*), recycled de-inking paper sludge + thermal dried sewage sludge (*PTD1* and *PTD2*), recycled de-inking paper sludge + sewage sludge composted with prunning wastes (*PCP1* and *PCP2*)

indicate a mobilisation of nutrients from the amended soil to plants. It appears that there was a significant effect on the soil nutrients in the co-amended soils from both organic wastes (CP and TD). Furthermore, the vegetal biomass depends on the nutrients supplied by the organic waste from sewage sludge (CP and TD).

Table 5 Values of Shannon diversity index calculated from PCR to DGGE data of bacterial community from soil amended with organic residues

Treatments	Shanon Index (H)
C	2.28 ± 0.08^{a}
P	1.85 ± 0.06^{b}
P + CP	1.96 ± 0.17^{b}
P + TD	1.99 ± 0.05^{b}

Means followed by the same lower case letter are not significantly different at $p < 0.05$ LSD level

Fierro et al. (2000) concluded that the nitrogen and phosphorus mineralising from decomposing sludge could regulate primary productivity in the early phases of revegetation of abandoned sands. In this study, higher concentrations of both nitrogen and phosphorus were observed in the soil that was amended with thermal dried sewage sludge (TD) (Table 4). Similarly, new bacterial strains appeared in the DGGE profiles (Fig. 4) that were not detected in the other treatments. Furthermore, the P + TD soil had higher biodiversity than the other treated soil (P and P + CP). It is likely that the highest soil biodiversity could be a direct or indirect effect of the highest percentage of vegetal cover and that high nutrient mobilisation from the organic matter incorporated in the soil is promoted by the micro- and macro-biota in the soil and the relationships with the plants' life cycles.

We observed a strong relationship between the changes promoted by the long term rehabilitation processes in the soil, enzyme activities (β-glucosidase and alkaline phosphatase) and the soil respiration in the correlation analysis between the soil parameters. (Table 6).

The most significant effect of treatment P (recycled de-inking paper sludge) is the improvement of water holding capacity, directly related to soil respiration and activity β-glucosidase. This is due to the importance of water in the soil to microbial activity.

Co-amendment treatments (P + CP and P + ST) provide available nutrients that improve soil fertility and thus increased plant biomass. In this regard, the combination of two different residues could allow a good strategy for soil rehabilitation in semi-arid conditions where water is a limiting factor.

4 Conclusions

After a three year rehabilitation process, the chemical characteristics of the soil only showed a slight improvement. Nevertheless, the application of recycled de-inking paper sludge had a positive effect on the water-holding capacity. Likewise, the application of this residue significantly affected specific biological parameters (β-glucosidase activity and soil respiration). An increase of nutrients in the soil that was co-amended with organic wastes (P + CP and P + TD) also increased biomass production. The soil co-amended with TD (P + TD) showed the most dramatic positive effect of all soil treatments studied, especially in terms

Table 6 Correlation analyses ($^{*}p < 0.05$, $^{**}p < 0.01$)

	Phosphatase	β-glucosidase	Urease	Soil respiration	Plant biomass
Phosphatase	1.00	0.726**	n.s	0.618**	n.s
β-glucosidase	0.726**	1.00	n.s	0.912**	n.s
Urease	n.s	n.s	1.00	n.s	n.s
Soil respiration	0.618**	0.912**	n.s	1.00	n.s
WHC	n.s	0.871**	n.s	0.914**	n.s
Plant biomass	n.s	n.s	n.s	n.s	1.00
pH	n.s	n.s	0.590**	n.s	n.s
EC	n.s	n.s	n.s	n.s	n.s
CO_3	0.687**	0.653**	n.s	0.636**	n.s
N	0.693**	n.s	n.s	n.s	n.s
C	0.626**	n.s	n.s	n.s	n.s
P	0.596**	n.s	n.s	n.s	0.651**
K	0.605**	n.s	n.s	n.s	n.s

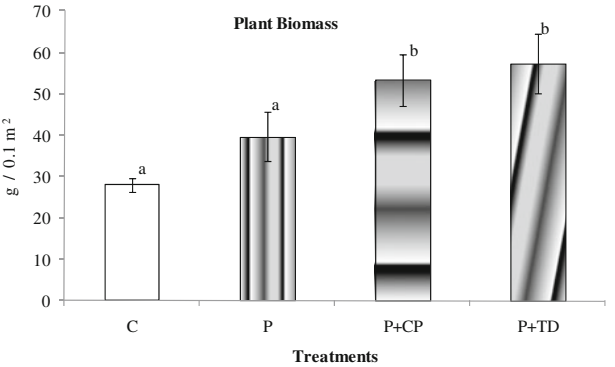

Fig. 5 Plant biomass in soil three years after residues application. Different letters are significative different to a $P < 0.05$

of soil urease and phosphatase activities. Changes in the PCR-DGGE profiles of the bacterial communities were found in response to the organic amendments applied to the soil. From the results of this study, we conclude that the application of the mixture of residues used in this experiment could constitute an adequate rehabilitation strategy by taking advantage of their different behaviours in the soil in comparison with the de-inking sludge treatment.

References

Albadalejo J, Stocking M, Diaz E, Castillo V (1994) Land rehabilitation by urban refuse amendments in a semi-arid environment: effect on soil chemical properties. Soil Technol 7:249–260

Alcañiz JM, Ortiz O, Sort X (1999) Suitability of some Mediterranean soils for recycling organic wastes in ecological restoration. In: Bech J (ed) 6th International meeting on soils with Mediterranean type of climate, Barcelona, Spain, pp 811–812

Bastida F (2008) Procesos de degradación y recuperación de suelos en zonas semiáridas. Establecimiento de un índice de degradación biológica de suelos. Tesis Doctoral, Universidad de Cartagena, Murcia, Spain, p 222

Bastida F, Kandeler E, Moreno JL, Ros M, García C, Hernández T (2008) Application of fresh and composted organic wastes modifies structure, size and activity of soil microbial community under semiarid climate. Appl Soil Ecol 40:318–329

Benthan H, Harris JA, Birch P, Short KC (1992) Habitat classification and soil restoration assessment using analysis of soil microbiological and physiochemical characteristics. J Appl Ecol 29:711–718

Bonmatí M, Jiménez P, Alvarez H, Calero E, Julià M, Morillo M, Nuñez E (2000) Evolución de actividades enzimáticas en el proceso restaurador de dos suelos procedentes de la explotación de canteras calcáreas en Cataluña utilizando altas dosis de lodos de depuradora. In: Garcia C, Hernandez MT (eds) Investigación y perspectivas de la enzimología de suelos en España. CSIC, Murcia, Spain, pp 209–293

Cabezas JG, Alonso J, Lobo MC (2004) Influence of organic amendments application in the establishment of herbaceous cover in degraded soil. In: Faz A, Ortiz R, García G (Eds) 4th International conference on land degradation, Cartagena, Murcia, Spain, p 379

Cabezas JG, Alonso J, Vicente MA, Valdazo J, Lobo MC (2007) Aplicación combinada de distintos tipos de residuos en un ensayo de campo de recuperación de suelos gypsiferos. In: Rodríguez A, Arbelo CD (Eds) Proceedings of III simposio sobre control de la degradación de suelos la desertificación, Fuerteventura, Spain, pp 379–380

Chantigny MH, Angers DA, Beauchamp CJ (1999) Aggregation and organic matter decomposition in soils amended with de-inking paper sludge. Soil Sci Soc Am J 63:1214–1221

Chantigny MH, Angers DA, Beauchamp CJ (2000) Active car-bon pool sand enzyme activities in soils amended with de-inking paper sludge. Can J Soil Sci 80:99–105

Cheshire MV, Chapman SJ (1996) Influence of the N and P status of plant material and of added N and P on the mineralization of ^{14}C-labelled ryegrass in soil. Biol Fertil Soils 21:166–170

Cuevas G, Blázquez R, Martinez F, Walter I (2000) Composted MSW effects on soil properties and native vegetation in a degraded semiarid shrubland. Compost Sci Utilization 8:303–309

Fierro A, Angers DA, Beauchamp CJ (2000) Decomposition of paper de-inking sludge in a sandpit minesoil during revegetation. Soil Biol Biochem 32:143–150

Fytili D, Zabaniotou A (2008) Utilization of sewage sludge in EU application of old and new methods-a review. Renew Sustainable Energy Rev 12:116–140

García S, Cuevas G, Martínez FE, Walter I (2000) Efecto sobre la cubierta vegetal de la aplicación de residuos orgánicos urbanos en un suelo degradado de la comunidad de Madrid. Ecología 14:79–87

García C, Gil F, Hernández T, Trasar C (2003) Técnicas de análisis de parámetros bioquímicos en suelos: medidas de actividades enzimáticas y biomasa microbiana. Editorial Mundi Prensa, Madrid, Spain

Griffiths BS, Kuan HL, Ritz K, Glover LA, JcCaig AE, Fenwick C (2004) The relationship between microbial community structure and functional stability tested experimentally in an upland pasture soil. Microbiol Ecol 47:104–113

Heuer H, Smalla K (1997) Aplication of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In: Van Elsas JD, Trevors JT, Wellington EMH (eds) Modern soil microbiology. Dekker, New York, pp 353–373

Jones RAJ, Yli-Halla M, Demetriades A, Leifeld J, Robert M (2004) Status and distribution of organic matter in Europe. In: Van-Camp L, Bujarrabal B, Gentil A-R, Jones RJA, Montanarella L, Olazabal C, Selvaradjou S-K (Eds) Reports of the technical working groups established under the thematic strategy for soil protection, Vol III. EUR 21329 EN/3. Office

for Official Publication of the European Communities, Luxembourg. http://ec.europ.eu/environment/soil/pdf/vol3.pdf
Kuan HL, Fenwick C, Glover LA, Griffiths BS, Ritz K (2006) Functional resilience of microbial communities from perturbed upland grassland soils to further persistent or transient stresses. Soil Biol Biochem 38:2300–2306
Larney FJ, Janzen HH, Olson BM, Lindwall CW (2000) Soil quality and productivity responses to simulated erosion and restorative amendments. Can J Soil Sci 80:515–522
Leinweber P, Jand G, Baum C, Eckhardt K-U, Kandeler E (2008) Stability and composition of soil organic matter respiration and soil enzyme activities. Soil Biol Biochem 40:1496–1505
MAPA (1994) Métodos oficiales de análisis (Oficial Methods of Analyses), vol III. Ministerio de Agricultura Pesca y Alimentación, Spain, pp 205–285
Melillo JM, Aber JD, Linkins AE, Ricca A, Fry B, Nadelhoffer KJ (1989) Carbon and nitrogen dynamics along the decay continuum: plant litter to soil organic matter. Plant Soil 115:189–198
Nannipieri P, Greco S, Ceccanti B (1990) Ecological significance of the biological activity of soil. In: Bollag J-M, Stotzky G (eds) Soil Biochem, vol 6. Marcel Dekker Inc, New York, pp 293–355
Pascual JA, García C, Hernández MT (1999) Lasting microbiological and biochemical effects on the addition of municipal solid waste to an arid soil. Biol Fertil Soils 30:1–6
Real Decreto (1990) R.D. 1310/1990 de 29 de octubre, por el que se regula la utilización de los lodos de depuración en el sector agrario, Spain. BOE n° 262, pp 32339–323340
Ros M, Hernández MT, García C (2003) Soil microbial activity after restoration of a semiarid soil by organic amendments. Soil Biol Biochem 35:463–469
Rosado AS, Seldin L, Wolters AC, van Elsas JD (1996) Quantitative 16S rDNA-targeted polymerase chain reaction and oligonucleotide hybridization for the detection of *Paenibacillus azotofixans* in soil and the wheat rhizosphere. FEMS Microbiol Ecol 19:153–164
Sastre I, Vicente MA, Lobo MC (1996) Influence of the application of sewage sludge on soil microbial activity. Bioresour Technol 57:19–23
Sastre-Conde I, Lobo MC (2003b) Determinación actividad ureasa en suelos. In: García C, Gil-Sotres F, Hernández MT, Trasar-Cepeda C (eds) Técnicas de análisis de parámetros bioquímicos de suelos. (Techniques of soil biochemical parameters analyze). Editorial Mundi Prensa, Madrid, pp 123–148
Sastre-Conde I, Vicente MA, Lobo MC (2003a) Contamination and environmental impact on soil biological activity. In: Lobo MC, Ibáñez JJ (eds) Preserving soil quality and soil biodiversity. The role of surrogate indicators. IMIA, Madrid, pp 99–117 CSIC
Sastre-Conde I, Cabezas JG, Guerrero AM, Vicente MA, Lobo MC (2007) Evaluation of the soil biological activity in a remediation soil assay using organic amendments and vegetal cover. Sci Total Environ 378:205–208
Soil Survey Staff (1998) Keys of soil taxonomy. US Department of Agriculture and Natural Research Conservation Services, USA
Valauwe B, Sanginga N, Merckx R (1997) Decomposition of four Leucaena and Senna pruning in alley cropping systems under sub-humid tropical conditions: the process and its modifiers. Soil Biol Biochem 29:131–137
Walker K, Ripandelli N, Flint S (2005) Rapid enumeration of *Bifidobacterium lactis* in milk powders using impedance. Int Dairy J 15:183–188
Walkley A, Black IA (1934) An examination of Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Sci 37:29–37

Evaluating the Restoration of Degraded Agricultural Soils Under Organic Fertilization

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Abstract Soil degradation is currently threatening the soil's capacity for crop production in many agricultural areas. In consequence, it is necessary to develop more sustainable agricultural practices that, among other aspects, preserve and improve soil quality. In this context, the objective of the current work was to evaluate the restoration of the quality for agriculture of a degraded soil under different managements. To this aim, a 4 year field study was carried out to compare the impact of two fertilization systems [organic (O) with cow slurry *vs*. inorganic (I) with NPK] on both soil health and corn yield under no-tillage. To assess soil health, a variety of soil chemical parameters and enzyme activities were measured. The application of fresh cow slurry mitigated soil acidity and enhanced organic matter content, leading to a higher activity of the soil microbial communities, in global terms. Most interestingly, all measured soil health parameters, together with corn yield values were condensed into a new agricultural Soil Quality Index (A-SQI). Prior to the treatments, organic and inorganic field plots showed A-SQI values of 13.8 and 13.4%, respectively (a contiguous native meadow was used as reference soil, i.e. values found in this reference soil were set to 100%). After 4 years, values of A-SQI were 67.6% for organic plots and 44.4% for inorganic plots. This study highlights the need to consider both soil health and crop yield when evaluating the long-term sustainability of agricultural managements. We conclude that the here proposed A-SQI index might be of great value for agroecosystem managers.

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1 Introduction

Agriculture not only feeds us but also may have a positive effect on environment, as it maximizes waste recycling, CO_2 fixation and O_2 production, among others. However, conventional intensive agriculture is considered an activity that causes air and water pollution, soil degradation, loss of biodiversity and, in general terms, an important environmental damage (Doran and Parkin 1994). Indeed, in approximately the last 50 years, the area of available agricultural land *per capita* has decreased by 50%, mostly due to population growth and degradation of agricultural soils (ISRIC 2004).

In this context, it is imperative to move towards an environmentally-friendly agriculture through, for instance, the incorporation of agricultural practices which reconcile crop yield with protection or restoration of soil health. In this respect, the application of livestock slurry as fertilizer helps to reuse a waste and increase soil organic matter (OM) content, having a beneficial effect on soil structure, water holding capacity, and nutrient availability of degraded agricultural soils (Li and Zhang 2007).

On the other hand, it is also necessary to develop techniques to monitorize soil restoration processes under different managements. In this regard, soil microbial communities provide us information relevant to the soil functioning, as they decompose soil OM, stabilize soil structure and, through its essential role in the cycling of elements, release nutrients for crop growth (Porazinska et al. 2003). Soil microbial properties are also becoming increasingly used as soil health indicators, due to their sensitivity to disturbances and capacity to integrate many environmental factors (Rodríguez-Loinaz et al. 2008). In particular, soil enzyme activities are good indicators of the functional status or condition of the soil ecosystem (Naseby and Lynch 2002) and can be condensed (together with other soil indicators) in soil quality indexes (Bloem et al. 2006).

The main objective of the current work was to study the effects of continued (4 year) organic fertilization (*vs.* mineral fertilization) in a degraded agricultural soil, in terms of both soil health and crop production. To this aim, we measured several chemical parameters and five enzyme activities, as well as corn yield. Finally, in an attempt to overcome the limitations of the Soil Quality Index (Bloem et al. 2006), which penalizes any type of variation from reference soil, we propose a new Agricultural Soil Quality Index (A-SQI) that incorporates all the measured parameters for evaluating the suitability of any practice in agricultural soils.

2 Materials and Methods

2.1 Location and Treatments

The study was carried out in Derio, Basque Country (Northern Spain) under a temperate and humid climate, with an annual mean temperature of 13.5°C and

a mean precipitation of 1,200 mm. The experimental field was established in a silty clay loam Epidystric Cambisol (FAO) that had been used as a forest nursery for more than 10 years. During this period, soil was subjected to intensive tillage to control weeds, and no fertilizers were applied. After this, the soil was intensively tilled during two more years in order to eliminate the remaining roots. Finally, just before the beginning of the study (spring 2005) the soil was almost devoid of plant cover, with 17.2 g organic matter kg^{-1} dry matter (DM) soil, moderately acid (pH = 5.1 in 1:2.5 water) and flooded due to its lack of structure and drainage capacity.

In this situation, a 4 year field assay was established to compare the impact of two different systems of fertilization [organic (O) *vs*. inorganic (I)] in forage corn crop. Each year, fertilization treatments consisted of 140 kg N ha^{-1}, 90 kg P ha^{-1} and 190 kg K ha^{-1} for corn and 150 kg N ha^{-1}, 60 kg P ha^{-1}, and 130 kg K ha^{-1} for Italian ryegrass, used as winter crop. Mineral fertilizers were applied in granular form as NH_4NO_3, P_2O_5, and KCl, respectively. Regarding organic plots, approximately 60,000 L ha^{-1} fresh cow slurry ($\sim$10% DM) were added for each crop to reach the N doses indicated above (slurry amounts fluctuated according to N concentration). Remainder P and K were added as mineral fertilizers. Both mineral fertilizers and cow slurry were applied manually to the soil surface a week before the direct sowing (no-tillage), which was carried out with a Semeato machine. Sowing density of corn was 100,000 plants ha^{-1}, with a row spacing of 70 cm.

A randomized complete block design with six replicates per treatment was established with each experimental plot measuring 5 × 3.5 m. An absolute control (reference point), consisting of a contiguous undisturbed native meadow, was also studied.

2.2 Sampling, Storage and Analysis

Before treatment application, a soil sampling was made to know the starting soil conditions and verify the homogeneity among experimental plots. After 4 years, soil was sampled again, during the maximum growth phase of corn, to assess the impact of treatments. In each sampling, fifteen soil samples (core diameter = 3 cm) were randomly collected from each plot to a depth of 0–25 cm and mixed together to give a composite sample. For chemical analysis, soils were air-dried at 30°C for 48 h, sieved to < 2 mm, and stored at room temperature. For analysis of enzyme activities, apart from those samples for dehydrogenase activity, soils were air-dried at 30°C for 48 h, sieved to < 2 mm and stored at 4°C until laboratory analysis. For dehydrogenase activity, soils were sieved to < 2 mm in fresh (field moisture) and then stored at 4°C.

Dehydrogenase enzyme activity (EC 1.1) was determined according to Taylor et al. (2002). A moist sample corresponding to 1 g DW soil was mixed with 0.4 mL of buffer (100 mM THAM, pH 7) and 0.4 mL of substrate [iodonitrotetrazolium chloride (0.5% w/v)]. The mixture was incubated at 25°C for 3 h and the reaction stopped with 8 mL of methanol. After centrifugation (3,500 g, 3 min), the

absorbance value of the samples was read at 490 nm. β-glucosidase (EC 3.2.1.21), arylsulphatase (EC 3.1.6.1) and acid phosphatase (EC 3.1.3.2) were determined according to Dick et al. (1996) and Taylor et al. (2002), as described in Epelde et al. (2008). Urease (EC 3.5.1.5) activity was determined according to Kandeler and Gerber (1988), as described in Rodríguez-Loinaz et al. (2008). Regarding chemical analyses, soil pH, organic matter (OM) content and cation exchange capacity (CEC) were determined following standard methods (MAPA 1994).

Finally, corn yield was estimated by cutting the two central rows at the end of corn growing season, at the fourth year. Harvested samples were oven-dried at 70°C for 72 h to calculate yield as tonnes DM ha^{-1}.

2.3 Data Analysis

Differences among treatments were analyzed by one-way ANOVA and Fisher's PLSD-test was used to establish the significance of the differences among means. Pearson's correlations were calculated between soil chemical parameters and enzyme activities. Microsoft Stat View Software (SAS Institute) was used for this analyses.

Sun-ray plots were used to provide a visual illustration of the overall impact of the treatments. From these sun-ray plots, the soil quality index (SQI) described by Bloem et al. (2006) was calculated using the average factorial deviation from the reference or "ideal" value (Ten Brink et al. 1991):

$$SQI = 10^{\log m - \frac{\sum_{i=1}^{n} |\log m - \log n_i|}{n}}$$

where m is the reference for soil quality (mean value of samples collected from Control "non disturbed" contiguous native meadow, set to 100%) and n are the measured values as percentages of the reference.

Here, we propose the A-SQI which takes into account the effects on both (i) soil health, estimated as described in Bloem et al. (2006) and (ii) soil productivity, estimated by the magnitude of the increase/decrease of a productivity parameter, as compared to the value for this parameter shown by another "reference soil for productivity" or "desired productivity":

$$SQI = 10^{\log m - \frac{\sum_{i=1}^{n} |\log m - \log n_i|}{n} + \log(P - P_m)}$$

where m and n are as described above for SQI, P_m is the "reference for productivity" and P are the measured values of a productivity parameter as percentages of this "reference for productivity". In this study, we used the mean value of corn yield of the most productive treatment in our experimental field as the "reference for productivity", which has been set to 100%.

3 Results and Discussion

3.1 Effects on Soil Properties and Corn Yield

Table 1 shows the effects of fertilization treatments on soil biological (*i.e.*, enzyme activities) and chemical properties, as well as on corn yield. As we expected, prior to the treatments there was no difference between organic and inorganic plots, confirming the homogeneity of the experimental field in origin. Most interestingly, at this point the values of the enzyme activities in the field plots were significantly lower in comparison with the ones of the undisturbed meadow (Control). This was probably due to the aggressive management of the experimental field before the beginning of the study (see Materials and Methods). Intensive tillage, apart from directly harming soil microorganisms (especially fungal hyphae), contributed to organic matter loss and soil acidification under humid climate (Table 1). It is well known that these factors affect the activity of those enzymes that catalyze the hydrolysis of organic compounds in soil (Dick et al. 1988).

Indeed, β-glucosidase (that hydrolyzes carbohydrates by splitting off the terminal β-D-glucose), acid phosphatase (which release PO_4^{-3} from organic P) and arylsulfatase were positively correlated with OM content (Table 2). Arylsulfatase was also positively correlated with soil pH, as well as urease (which uses urea as substrate for releasing CO_2 and NH_3) and dehydrogenase. So, it is not surprising that 4 years later, the increase of soil OM and pH (and CEC) due to no-tillage and slurry applications led to an enhancement of all the enzyme activities measured (Table 1). However, Christie and Beattie previously reported a decrease in soil pH as a result of long-term slurry amendments (1989). These apparently contradictory results are probably because our soils are more acidic, as soil acidification is mainly due to the oxidation of ammonium from slurry, and this process (nitrification) is slower in acidic soils. Besides, the pHs of the different slurries applied through these 4 year were next to 8.

In order to provide a visual illustration of the soil quality restoration as result of the treatments, a sun-ray plot is presented in Fig. 1. We observe that no-tillage cultivation for 4 years, both under organic and inorganic fertilization, led to a considerable enhancement of soil health indicators (we can not compare the productivity as we do not have the yield value at origin). Regarding cultivated plots, those subjected to organic fertilization showed higher values of all the parameters measured (except for CEC), so it might indicate an overall improvement of soil quality. In agreement with this, SQI and the here proposed A-SQI established significant differences between organic and inorganic treatments.

However, the differences between treatments according to A-SQI were higher than for SQI, as A-SQI takes into account the productivity (yield) as well as soil health indicators. In this respect, we consider that the capacity of soil to produce good yields is not only a soil health indicator *per se*, but also a key attribute of agricultural soils that is worth considering as importantly as the rest of soil

Table 1 Effect of treatments on biological parameters (enzyme activities and crop yield), soil chemical parameters (pH, OM and CEC) and soil quality indexes

	DH	Aryl-S	Acid-P	β-Glu	U	pH	OM	CEC	YIELD	SQI	A-SQI
Origin											
O	1.70^{A}	47.4^{A}	248.5^{A}	58.9^{A}	9.0^{A}	5.5^{A}	21.0^{A}	10.1^{A}	–	28.2^{A}	28.2^{A}
I	1.92^{A}	49.0^{A}	267.0^{A}	60.2^{A}	8.1^{A}	5.3^{A}	19.2^{A}	9.4^{A}	–	27.3^{A}	27.3^{A}
CTRL	12.9^{B}	402.1^{B}	951.2^{B}	464.4^{B}	19.7^{B}	6.0^{B}	30.1^{B}	12.0^{B}	–	–	–
After 4 years											
O	1.4^{A}	148.4^{B}	264.0^{B}	56.9^{B}	23.3^{B}	5.2^{B}	27.3^{B}	11.0^{A}	4.61^{B}	72.5^{B}	74.0^{B}
I	0.58^{A}	68.7^{A}	219.9^{A}	39.1^{A}	14.2^{A}	4.7^{A}	23.2^{A}	11.1^{A}	2.42^{A}	61.6^{A}	34.1^{A}
CTRL	0.59^{A}	353.6^{C}	295.6^{B}	49.5^{B}	13.4^{A}	5.5^{B}	29.9^{C}	11.7^{A}	–	–	–

Different letters in each column indicate significant differences between treatments ($P < 0.05$) within each sampling. Mean values ± SE ($n = 6$)

O organic fertilization; *I* inorganic fertilization; *CTRL* control undisturbed meadow (reference)

DH dehydrogenase (in mg INTF kg^{-1} h^{-1}); *Aryl-S* arylsulfatase, *Acid-P* acid phosphatase, *β-Glu* β-glucosidase (in mg *p*-nitrophenol kg^{-1} h^{-1}); *U* urease (in mg NH_4^+ -N kg^{-1} h^{-1})

OM (in g kg^{-1}); CEC (cmol(+) kg^{-1}); YIELD (t DM ha^{-1}); SQI (%); A-SQI (%)

Table 2 Pearson's correlations between soil chemical parameters (pH, OM and CEC) and soil biological parameters (enzyme activities)

Enzyme activities	pH	OM	CEC
Dehydrogenase	0.705^{**}	0.343	0.265
Arylsulphatase	0.651^{*}	0.767^{**}	−0.227
Acid phosphatase	0.284	0.543^{*}	−0.225
β-Glucosidase	0.443	0.769^{**}	−0.118
Urease	0.627^{*}	0.237	0.613

Asterisks represent significance level: * $P = 0.05$; ** $P = 0.01$

parameters together, within the framework of agroecosystems global sustainability. Besides, in previous studies (Mijangos et al. 2006, 2008) we have found that soil health and crop yield are not necessarily correlated, as crop yield is an integrative variable affected by many physical, chemical and biological factors.

On the other hand, corn yield, urease activity and, above all, dehydrogenase activity, were the most sensitive parameters to discriminate managements in this study. Dehydrogenase activity represents in true a multienzyme process that only occurs in viable microbial cells at each moment, so it was expected a marked response of this parameter. This sensitivity and rapid response has made dehydrogenase the most widely studied enzyme activity in soils (Burns and Dick 2002).

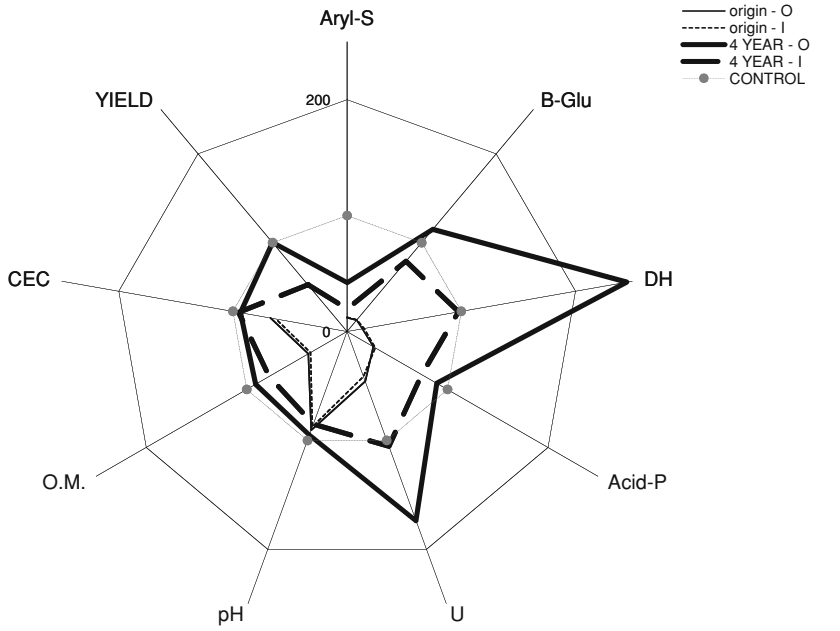

Fig. 1 Sun-ray plot of all measured variables for overall evaluation of soil quality. Mean values of soil parameters from the undisturbed native meadow and mean value of corn yield from the inorganic cultivated plots were used as references of soil health and soil productivity, respectively (set to 100%). O: organic fertilization; I: inorganic fertilization

4 Conclusions

In our study, 4 year cultivation by no-tillage mitigated soil acidity and enhanced organic matter content and cation exchange capacity of a degraded agricultural soil. These changes led to a higher activity of the soil microbial communities and a higher productivity, especially when combined with organic fertilization (cow slurry). All this parameters were integrated by the here proposed A-SQI, which indicated a significant restoration of soil quality. Regarding the studied soil properties, the enzyme activities urease and, above all, dehydrogenase, appeared to be the most sensitive parameters, showing their potential as indicators of changes induced by soil management.

References

Bloem J, Schouten AJ, Sørensen SJ, Rutgers M, van der Werf A, Breure AM (2006) Monitoring and evaluating soil quality. In: Bloem J, Hopkins DW, Benedetti A (eds) Microbiological Methods for Assessing Soil Quality. CAB International, Wallingford, UK, pp 23–49

Burns RG, Dick RP (2002) Enzymes in the environment, activity, ecology and applications. Marcel Dekker, New York, p 614

Christie P, Beattie JAM (1989) Grassland soil microbial biomass and accumulation of potentially toxic metals from long-term slurry applications. J Appl Ecol 26:597–612

Dick RP, Rasmussen PE, Kerle EA (1988) Influence of long-term residue management on soil enzyme activity in relation to soil chemical properties of a wheat-fallow system. Biol fertil soils 6:159–164

Dick RP, Breakwell DP, Turco RF (1996) Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In: Doran JW, Jones AJ (eds) Methods for assessing soil quality. Soil Science Society of America, Madison WI, pp 247–272

Doran JW, Parkin TB (1994) Defining and assessing soil quality. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (eds) Defining Soil Quality for a Sustainable Environment. Soil Science Society of America, Special Publication 35, Madison WI, pp 3–21

Epelde L, Becerril JM, Hernández-Allica J, Barrutia O, Garbisu C (2008) Functional diversity as indicator of the recovery of soil health derived from *Thlaspi caerulescens* growth and metal phytoextraction. Appl Soil Ecol 39:299–310

ISRIC (International Soil Reference Information Centre), 2004. Available from World Wide Web: http://lime.isric.nl/index.cfm?contentid=158

Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72

Li JT, Zhang B (2007) Paddy soil stability and mechanical properties as affected by long-term application of chemical fertilizer and animal manure in subtropical China. Pedosphere 17:568–579

MAPA (1994) Métodos Oficiales de Análisis, Plantas, productos orgánicos fertilizantes, suelos, aguas, productos fitosanitarios, fertilizantes inorgánicos, Vol. III. Publicaciones del Ministerio de Agricultura Pesca y Alimentación, Madrid, Spain, pp 205–324

Mijangos I, Pérez R, Albizu I, Garbisu C (2006) Effects of fertilization and tillage on soil biological parameters. Enzyme Microb Technol 40:100–106

Mijangos I, Albizu I, Epelde L, Pinto M, del Hierro O, Arriaga H, Garbisu C (2008). Effects of direct seeding and cow slurry application on corn production in fine-textured soils. In: Proceedings of the 22nd General Meeting of the European Grassland Federation, Uppsala, Sweden, pp. 613–615

Naseby DC, Lynch JM (2002) Enzymes and microorganisms in the rhizosphere. In: Burns RG, Dick RP (eds) Enzymes in the environment: Activity ecology and applications. Marcel Dekker, New York, pp 109–123

Porazinska DL, Bardgett RD, Blaauw MB, Hunt HW, Parsons AN, Seastedt TR, Wall DH (2003) Relationships at the aboveground–belowground interface: plants, soil biota, and soil processes. Ecol Monogr 73:377–395

Rodríguez-Loinaz G, Onaindia M, Amezaga I, Mijangos I, Garbisu C (2008) Relationship between vegetation diversity and soil functional diversity in native mixed-oak forests. Soil Biol Biochem 40:49–60

Taylor JP, Wilson B, Mills MS (2002) Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. Soil Biol Biochem 34:387–401

Ten Brink BJE, Hosper SH, Colijn F (1991) A quantitative method for description and assessment of ecosystems: the AMOEBA-aproach. Mar Pollut Bull 23:265–270

Laboratory Contamination with 2,4,5-Trichlorophenol: Effects on Some Enzymatic Activities in Two Forest and Two Agricultural Soils of Contrasting pH

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Abstract Previous studies have shown that different soils deteriorate to different degrees in response to the same amount of 2,4,5-trichlorophenol (2,4,5-TCP) and that enzymatic activities respond in different ways to contamination by this substance. Moreover, the results of these studies suggest that the different behaviour depends on the organic matter content and/or the pH of the soil. The present study was therefore carried out in order to provide further information about the response of soils of contrasting pH to contamination with 2,4,5-trichlorophenol. Two soils developed on limestone with a pH close to the pK of 2,4,5-TCP and two soils developed on acid materials were selected for the study. Soil samples were artificially contaminated with doses of 100 and 500 mg kg^{-1} of 2,4,5 trichlorophenol and after 72 h were analysed for some oxidoreductase and hydrolase enzyme activities. In general, the enzyme activities appeared to respond in different ways to contamination by trichlorophenol, being the hydrolytic enzymes, mainly invertase, those least affected by the presence of this contaminant. The effect of 2,4,5-TCP was higher in the soils developed on limestone than in the acid soils, thus supporting the idea that pH is one of the key factors that determine the effect of this contaminant on soils.

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1 Introduction

Contamination of soils and the consequent loss of quality is a serious environmental problem. Soils receive large amounts of xenobiotic substances, mostly as a result of human activities and these substances modify soil functions, sometimes irreversibly. The organic pesticides used in agriculture and silviculture are particularly important examples of xenobiotic substances because of their widespread use. With the aim of reducing the risk of soil contamination, the Spanish Government recently (BOE 18 Enero de 2005) published a Decree regarding contaminated soils (Real Decreto 9/2005), in which the concept of Generic Reference Levels (GRL) was defined (as the concentration of a substance in soil required for it to be considered as contaminated), GRL were established for different organic compounds in soils, and methods of quantifying the GRL were suggested for those substances not considered.

One group of substances considered in the Royal Decree is the chlorophenols, which include 2,4 dichlorophenol (2,4-DCP) and 2,4,5 trichlorophenol (2,4,5-TCP). These compounds are widely used in silviculture as antifungal agents, as they slow down the rotting of timber, and in agriculture as they are precursors of various herbicides. Previous studies involving application of these compounds to Galician soils have shown that the GRL established in the legislation are not appropriate for these soils, which can receive large amounts of the compounds without suffering any type of deterioration (Moscoso et al. 2007; Bello et al. 2007, 2008). These studies have also shown that the response of these soils to the presence of the compounds is difficult to predict as, on one hand, all biochemical indicators do not react in the same way, and, on the other, no edaphic property is clearly related to the potential for the soil to suffer deterioration due to the presence of the compounds. However, studies carried out so far have demonstrated that forest soils are much more resistant to degradation than agricultural soils, and that soil deterioration is correlated with soil pH (greater deterioration the higher the soil pH) and with the organic matter content (less deterioration the higher the organic matter content) (Moscoso et al. 2007; Bello et al. 2008). These results have led to the suggestion that the mechanism of action of these compounds on the soil requires ionization of the compounds and a low input of ionic forms to the interior of microorganisms, with the mobile ionized forms therefore regulated by both the soil pH and the organic matter content (Bello et al. 2008).

The aim of the present study was to test the accuracy of the previously mentioned ideas, by studying how one of these compounds, (2,4,5-TCP), affects two soils of very different pH. If the compound acts differently in relation to soil pH, further study of the role of pH in promoting the toxic effect of these compounds would be required with a larger number of soil samples.

2 Materials and Methods

Two study areas with different parent rock were selected to ensure that the soils would be of different pH. One area (M soils) was close to Mondoñedo (Lugo), where limestone parent material predominates (Vegadeo limestone); the other area

Table 1 Physiographic characteristics of the soils used in the study

	MF soil	MC soil	SF soil	SC soil
Location	Mondoñedo	Mondoñedo	Sobrado	Sobrado
Altitude (m.s.l.s.)	120	120	530	540
Longitude W	7° 19′ 30′	7° 19′ 25′	8° 03′ 20′	8° 03′ 15′
Latitude N	43° 28′ 05′	43° 28′ 10′	43° 01′ 23′	43° 01′ 10′
Parent material	Limestone	Limestone	Paragneiss	Paragneiss
Vegetation	Oak	Mixed grassland	Oak	Maize
Soil type[1]	Luvisol	Luvisol	Umbrisol	Regosol

[1] ISSS Working Group (1998)

was close to Sobrado dos Monxes, A Coruña (S soils), where the soils have developed over acid material (paragneiss). Two soils were sampled in each of the zones, one under forest vegetation of Atlantic oak *Quercus robur* L (soils MF and SF) and the other under agricultural management (soils MC and SC). In the Mondoñedo zone, the agricultural soil was under grassland (MC) and in the Sobrado zone, the soil was cropped with maize (SC). The physiographic characteristics of the 4 different types of soils used in the study are shown in Table 1.

In all cases the upper 0–10 cm of soil were sampled, after separating the fresh and partially decomposed plant remains layers (in the forest soils) and the layer containing the remains of organic fertilizers (in the cropped soils). At each sampling point 15–20 samples were collected, with a soil auger, and the samples were combined in the field. The composite samples were transported to the laboratory in insulated bags. In the laboratory the samples were sieved (<4 mm) and homogenized. An aliquot of the sample was air-dried for analysis of general properties. The rest of the material was stored at 4°C until the experiment was undertaken (always within 48 h of collecting the samples).

Aliquots of the fresh soils were artificially contaminated with two different doses of 2,4,5-trichlorophenol: 0 (control), 10 and 50 times the GRL established by the Spanish legislation (Real Decreto 9/2005), i.e. with amounts equivalent to 0, 100 and 500 mg of 2,4,5-TCP per kg of soil. As 2,4,5-trichlorophenol is sparingly soluble in water (Czaplicka 2004), it was first mixed with quartz sand (in the proportions required to generate the doses indicated below) before being added to the soil, and this mixture was then shaken for 48 h in a rotary shaker to achieve homogeneity (Moscoso et al. 2007). The soils were contaminated by addition of the sand/contaminant mixture to the moist soil (10 g of the mixture was added to the amount of moist soil equivalent to 100 g of dried soil, on an oven-dried soil basis, 105°C) to obtain the above indicated concentrations of contaminant, and enough water was added to maintain the system at optimal moisture content (water at field capacity); the mixture was then homogenized carefully. The soil moisture content was determined by oven-drying the soil at 105 °C for 24 h, to estimate the quantity of moist soil necessary to produce 100 g of dry soil. The mixtures were maintained at 20 °C for 72 h. This contact time was selected on the basis of the results of prior experiments that indicated that the major modifications in soil

properties are produced 72 h after contamination (Bello et al. 2008). Sand only (the same amount as added to the contaminated soils) was added to the control soils. After 72 h of incubation, the soils were analyzed to determine the activities of different oxidoreductase (catalase and dehydrogenase) and hydrolase (invertase and urease) enzymes.

The methods described by Guitián-Ojea and Carballas (1976) were used to determine the following soil properties: pH in water (1:2.5, soil:water ratio), pH in 1 M KCl (1:2.5, soil:solution ratio), total carbon content (by potassium dichromate oxidation), total nitrogen content (by Kjeldahl digestion), water retained at −33 kP pressure (field capacity) and particle size distribution (with a Robinson pipette and Calgon as dispersant); the texture of soils was determined following FAO (1966). Amorphous Al and Fe were extracted with 0.2 M ammonium oxalate/oxalic acid buffer of pH 3.0 (McKeague and Day 1966) and were determined by atomic absorption spectrometry (Varian SpectrAA 220 FS spectrometer, UK).

Dehydrogenase activity was determined using a modification of the method of von Mersi and Schinner (1991) described by Camiña et al. (1998), by incubating the samples with 98.87 mM iodonitrotetrazolium violet (INT) as substrate, for 1 h in 1 M TRIS–HCl buffer (pH 7.5). Catalase activity was determined according to the method of Trasar-Cepeda et al. (1999). Invertase activity was determined by incubating the samples with 35.06 mM saccharose in 2 M acetate buffer (pH 5.5) for 3 h, and measuring the released reducing sugars, following the method of Schinner and von Mersi (1990). Urease activity was determined by the method described in Nannipieri et al. (1980), by incubating the samples with 1065.6 mM urea as substrate, for 1.5 h in 0.2 M phosphate buffer (pH 8.0), and measuring the NH_4^+ released with an ammonia electrode. All determinations were performed in triplicate and for each soil sample the mean values of the three determinations (expressed on an oven-dried soil basis, 105°C), were calculated. Statistical analyses (means, deviations and significance tests) were performed with Statistica 6.0 (StatSoft®) for Windows (StatSoft Inc., 2001).

3 Results

3.1 General Soil Properties

The general properties of the 4 soils used in the experiment are shown in Table 2. The pH in H_2O of both of the Mondoñedo soils was slightly higher than 7, whereas the Sobrado soils were classified as strongly acidic, according to the categories described by Guitián-Ojea and Carballas (1976).

In both cases the forest soils appear to be better buffered that the cropped soils, possibly as a result of the higher organic matter content in the soils under climax vegetation (Trasar-Cepeda et al. 2008). The decrease in the amount of organic matter as a result of use was very important in the case of the Sobrado soils (10% of C for the SF soil and less than half for the SC soil) and of less quantitative

Table 2 General properties of the soils under study

	MF soil	MC soil	SF soil	SC soil
pH in H_2O	7.34 ± 0.01	7.70 ± 0.02	4.82 ± 0.02	4.84 ± 0.01
pH in KCl	6.50 ± 0.01	6.04 ± 0.02	4.22 ± 0.02	3.65 ± 0.01
Total carbon (%)	3.46 ± 0.17	2.54 ± 0.10	10.13 ± 0.19	4.67 ± 0.15
Total nitrogen (%)	0.34 ± 0.01	0.25 ± 0.01	0.88 ± 0.03	0.52 ± 0.01
C/N	10 ± 1	10 ± 0	12 ± 0	9 ± 0
Water field capacity (%)[1]	37 ± 1	39 ± 2	63 ± 3	39 ± 2
Sand (%)	34 ± 4	42 ± 3	50 ± 2	58 ± 4
Clay (%)	29 ± 4	14 ± 2	16 ± 4	15 ± 4
Textural class	Clay loam	Loam	Loam	Sandy loam
Fe_2O_3 (%)	0.63 ± 0.02	0.35 ± 0.02	1.19 ± 0.05	0.70 ± 0.01
Al_2O_3 (%)	1.12 ± 0.01	0.22 ± 0.02	2.83 ± 0.07	0.59 ± 0.02

[1] Water retained at −33 kPa pressure

importance, although also appreciable, in the Mondoñedo soils. There were also greater decreases in the total carbon than in total nitrogen, and therefore the C/N ratio was lower in the agricultural soils than in the forest soils. The decrease in the organic matter content as a result of agricultural use explains why the contents of extractable oxides were always lower in the agricultural soils than in the forest soils, as previously indicated (Trasar-Cepeda et al. 2008; Troitiño et al. 2008).

3.2 Activities of Oxidoreductase Enzymes

The highest value of catalase activity was observed in Mondoñedo forest (MF) soil and in both sites land use was found to generate a decrease in the value of this enzyme activity (Table 3).

As regards the effect of the contaminant on this activity, in both forest soils the presence of 2,4,5 trichlorophenol had very little effect on the value of the enzyme activity and the slight variations observed were inconsistent, so that they cannot be attributed to the presence of 2,4,5-TCP in soil. On the opposite, in both agricultural soils there was a clear decrease in the enzyme activity as the dose of trichlorophenol increased. This decrease was much more acute in MC than in SC soil, and only was significant in the former.

The MC and SF forest soils displayed the highest dehydrogenase activity (Table 3), which suggests the presence of more active microbial populations in these two soils than in SC and, especially, in MF soil. The trichlorophenol had an intense and significant effect on the activity of this enzyme in the Mondoñedo soils, in which the highest dose of contaminant (500 mg kg^{-1}) reduced the activity to less than 30% of that in the corresponding controls. In the Sobrado soils the highest dose of contaminant also reduced the level of this enzyme in both soils, although to a much lesser extent than in the Mondoñedo soils as the decrease only was significant for the highest dose. Interestingly, the dose of 100 mg kg^{-1} did not

Table 3 Values of oxidoreductase activities in soils affected by different doses of contaminant. For each soil and enzymatic activity, different letters indicate significant differences among the contaminant doses ($P \leq 0.05$)

Soil	Dose	Dehydrogenase[1]	Catalase[2]
MF soil	0	0.175 ± 0.000^{a}	3.415 ± 0.190^{a}
	100	0.143 ± 0.010^{b}	3.814 ± 0.180^{a}
	500	0.050 ± 0.000^{c}	3.565 ± 0.080^{a}
MC soil	0	0.450 ± 0.009^{a}	1.853 ± 0.154^{a}
	100	0.241 ± 0.001^{b}	1.633 ± 0.099^{a}
	500	0.130 ± 0.005^{c}	1.044 ± 0.117^{b}
SF soil	0	0.446 ± 0.004^{a}	1.700 ± 0.030^{a}
	100	0.447 ± 0.030^{a}	1.543 ± 0.110^{a}
	500	0.391 ± 0.010^{b}	1.818 ± 0.140^{a}
SC soil	0	0.325 ± 0.009^{a}	1.122 ± 0.132^{a}
	100	0.342 ± 0.018^{a}	1.096 ± 0.030^{a}
	500	0.218 ± 0.008^{b}	0.903 ± 0.064^{a}

[1] μmol INTF g^{-1} h^{-1}, [2] mmol H_2O_2 consumed g^{-1} h^{-1}

affect the dehydrogenase activity in the Sobrado soils, whereas it led to significant reductions in the Mondoñedo soils.

3.3 Hydrolase Enzyme Activities

The invertase activity differed in relation to land use in both sites. Thus, in the Sobrado soils the activity of this enzyme was higher in the SF soil than in the SC soil, whereas in the Mondoñedo soils the opposite was true, with an activity three times higher in the MC than in the MF soil. It was not possible to determine the invertase activity in MF soil contaminated with the 500 mg kg^{-1} dose. Independently of this, in all 4 soils, invertase was scarcely affected by the presence of the contaminant as the activity of this enzyme decreased only slightly in MC soil, whereas in the other three soils there was only little and not significant variation. The decreases in MC soil were significant and proportional to the dose of 2,4,5-TCP (Table 4).

Finally, the urease activity was much higher in both of the Sobrado soils than in the Mondoñedo soils. In the Mondoñedo soils, the urease activity was higher in the cropped than in the forest soil (almost three times higher), whereas in the Sobrado soils the values of this activity in the forest soil were much higher (more than 2 times) than in the cropped soil. The effect of the contaminant was also different in the two sites and for the two soil uses. In the SF soil, the contaminant caused a decrease in the activity of this enzyme that was in direct proportion to the dose of contaminant but only significant for the 500 mg kg^{-1} dose, whereas the activity varied at random in the SC soil, in which the lowest dose of the contaminant caused an increase in the activity of this enzyme and the highest dose resulted in a

Table 4 Values of hydrolase activities in soil affected by the different doses of contaminant. For each soil and enzymatic activity, different letters indicate significant differences among the contaminant doses ($P \leq 0.05$)

	Dose	Urease[1]	Invertase[2]
MF soil	0	0.93 ± 0.07^{a}	1.31 ± 0.00^{a}
	100	1.14 ± 0.07^{b}	1.27 ± 0.07^{a}
	500	1.05 ± 0.03^{ab}	n.d.
MC soil	0	3.04 ± 0.08^{a}	5.21 ± 0.10^{a}
	100	2.07 ± 0.11^{b}	4.29 ± 0.26^{b}
	500	1.32 ± 0.03^{c}	4.01 ± 0.27^{b}
SF soil	0	15.82 ± 1.11^{a}	5.67 ± 0.32^{a}
	100	15.14 ± 1.22^{a}	5.48 ± 0.37^{a}
	500	12.24 ± 0.36^{b}	5.44 ± 0.37^{a}
SC soil	0	6.56 ± 0.09^{a}	4.09 ± 0.05^{a}
	100	8.24 ± 0.70^{b}	4.18 ± 0.16^{a}
	500	5.73 ± 0.56^{a}	4.12 ± 0.17^{a}

[1] μmol NH_3 g^{-1} h^{-1}, [2] μmol glucose g^{-1} h^{-1}

decrease, with respect to the control values. In the Mondoñedo soils, the contaminant scarcely affected the enzyme in the MF soil, in which there was a slight although significant increase in activity, whereas in the MC soil there was a gradual and significant decrease in enzyme activity with increasing dose of the contaminant (Table 4).

4 Discussion

Given the different levels of enzyme activity in the soils, it is difficult to compare the results obtained by only considering the absolute values of activity. However, calculation of the values of the enzyme activities in contaminated soils as percentages of the corresponding activities in the uncontaminated or control soils, the patterns of responses become clearer (Figs. 1 and 2). Firstly, the enzyme activities appeared to respond in different ways to contamination by 2,4,5-trichlorophenol. Independently of the type of soil and management, the enzymes that were least affected by the presence of 2,4,5-TCP were the hydrolytic enzymes, mainly invertase, which was scarcely affected, even at the highest doses of contaminant (Fig. 2). This has been observed in a previous study (Bello et al. 2008) and clearly indicates that this enzyme activity cannot be considered as reliable in terms of assessing the degree of contamination of a soil, as it is not capable of reacting to the different doses of contaminant, one of the essential criteria for an edaphic property to be considered a good indicator of soil quality (Doran and Parkin 1994; Elliott 1997). In this case urease was also scarcely affected by the presence of trichlorophenol, although in previous studies this enzyme has been shown to be one of the best indicators of soil degradation by diverse organic compounds or

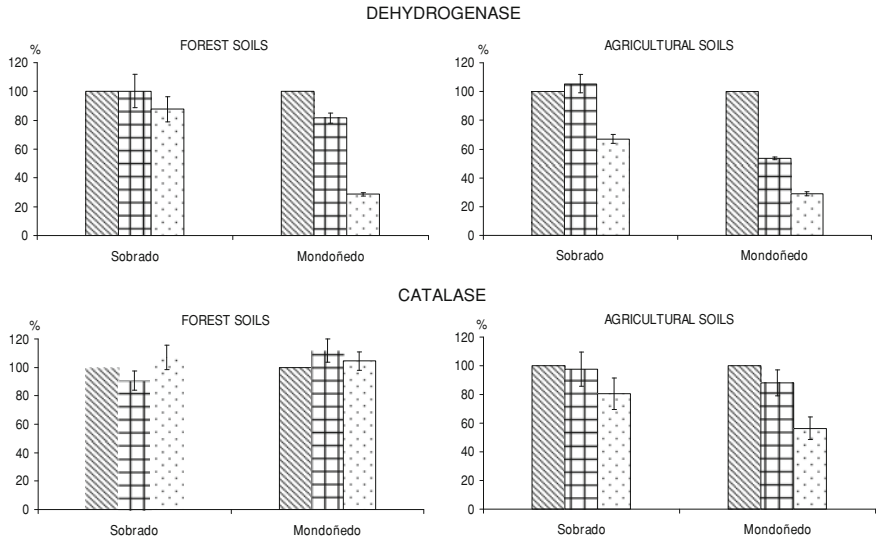

Fig. 1 Percentage activity of oxidoreductase enzymes in contaminated soil with respect to that in uncontaminated forest and agricultural soils, for alkaline (Mondoñedo) and acidic (Sobrado) soils (D0 D100 D500)

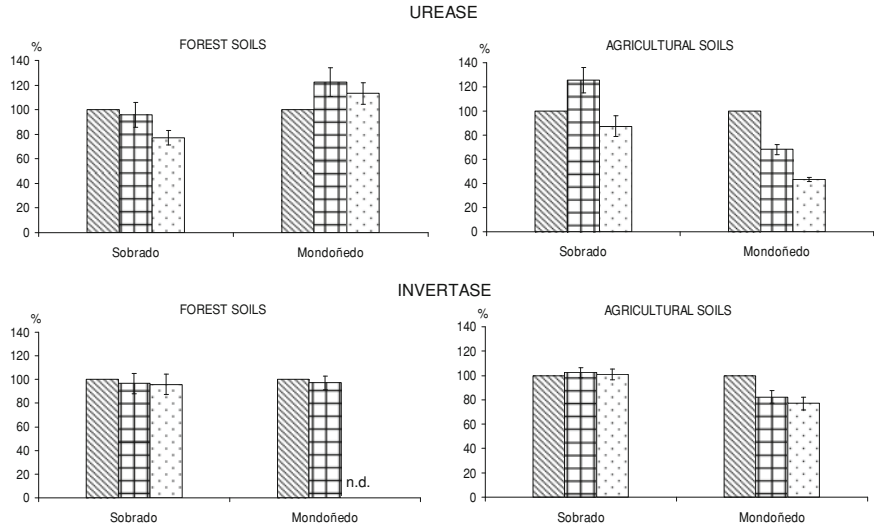

Fig. 2 Percentage activity of hydrolytic enzymes in contaminated soil with respect to that in uncontaminated forest and agricultural soils, for alkaline (Mondoñedo) and acidic (Sobrado) soils (n.d. = not determined) (D0 D100 D500)

heavy metals (Hinojosa et al. 2004; Bécaert et al. 2006; Bello et al. 2008). In the soils under study here, the urease activity scarcely varied in the forest soils, and a large decrease was only observed in soil MC, in which for the highest dose of contaminant, the urease activity was only 40% of that in the control soil. The behaviour of the oxidoreducatse enzymes was different. Although catalase activity was scarcely affected by the presence of the contaminant in the forest soils (Fig. 1), it was affected in the agricultural soils, particularly in soil MC, in which the highest dose of trichlorophenol resulted in a decrease in the activity to 60% of that initially present. Dehydrogenase was the most sensitive of all of the enzymes tested, as found in other studies (Tu 1992; Margesin et al. 2000; Trasar-Cepeda et al. 2000; Bello et al. 2008), although in both of the Sobrado soils, the dose of 100 mg kg^{-1} trichlorophenol had very little effect on dehydrogenase activity.

The fact that, in general, the soils under forest vegetation were less affected by 2,4,5-TCP than the agricultural soils suggests that the soil organic matter, which content is higher in forest than in agricultural soils (Table 1), is involved in the dynamics of the clorophenols in the soil. This influence is explained because both the non-dissociated and the dissociated forms of the 2,4,5-TCP would undergone some type of fixation process on the organic surfaces (Severtson and Banarjee 1996), and therefore limit the presence of this compound in the soil solution (Díez et al. 1999).

Nevertheless, which is important to note is that for all of the enzymes, and independently of the general effect that the soils may have suffered, the Mondoñedo soils, i.e. the least acidic soils, were most affected. In these soils, the percentage decrease in activity was greatest, and responses to the lowest concentrations were observed, unlike in the Sobrado soils, i.e. the most acidic soils. Independently of the fact that the other soil properties were different, these results appear to indicate that the dissociated forms of trichlorophenol cause the toxicity of the compound. The pK of trichlorophenol is 6.92 (Severtson and Banarjee 1996), and since a higher proportion of dissociated forms with respect to the non dissociated forms should occur the higher the pH of the soils (Shiu et al. 1994), it appears that the greater toxicity observed in the soils developed on limestone material is related to a greater dissociation of the trichlorophenol. It is possible that on acquiring the ionic form, the ionized compound can penetrate the interior of microbial cells thus causing the death of the organisms. This possible mechanism would also explain why an enzyme such as dehydrogenase, clearly related to the activity of active microorganisms (von Mersi and Schinner 1991; García and Hernández 1997), would be more affected than other enzymes by the presence of the contaminant.

In conclusion, the results suggest that an important part of the mechanism of action of trichlorophenol on the soil is due to dissociation of the compound and the appearance of anionic forms. This process is favoured by pH values that are close to the pK of dissociation of trichlorophenol, and therefore the effect of this compound on the soil will possibly be heightened in soils that have been treated with liming agents. Given that acidic Galician soils are often treated with this type of amendment, this practice will aggravate the risk of biological deterioration of soils and make them more vulnerable to the toxic effects of trichlorophenol.

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References

BOE, 18 Enero de (2005) Ministerio de la Presidencia, Real Decreto 9/2005, pp 1833–1843

Bécaert V, Samson R, Deschênes L (2006) Effect of 2,4-D contamination on soil functional stability evaluated using the relative soil stability index (RSI). Chemosphere 64:1713–1721

Bello D, Presas E, Leirós MC, Trasar-Cepeda C, Gil-Sotres F (2007) Soils contaminated with 2,4,5 TCP: ED_{50} values for biochemical properties and ecotoxicological tests. In: Sierra J, Martí E, Domene X (Eds) Proceedings international meeting soil and wetland ecotoxicology, Papers and abstracts, pp 1.2.4.-1–1.2.4.-7

Bello D, Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Evaluation of different tests for the diagnosis of soil contamination by 2,4,5-trichlorophenol (2,4,5-TCP). Environ Pollut 156:611–617

Camiña F, Trasar-Cepeda C, Gil-Sotres F, Leirós MC (1998) Measurement of dehydrogenase activity in acid soils rich in organic matter. Soil Biol Biochem 30:1005–1011

Czaplicka M (2004) Sources and transformations of chlorophenols in the natural environment. Sci Total Environ 322:21–39

Díez MC, Mora ML, Videla S (1999) Adsorption of phenolic compounds and color from bleached kraft mill effluent using allophonic compounds. Water Res 33:125–130

Doran JW, Parkin TB (1994) Defining and assessing soil quality. In: Doran JV, Coleman DC, Bezdicek DF, Stewart BA (eds) Defining soil quality for a sustainable environment. Soil Science Society of America, American Society of Agriculture, Madison, pp 3–21

Elliott ET (1997) Rationale for developing bioindicators of soil health. In: Pankhurst CE, Doube BM, Gupta VVSR (eds) Biological indicators of soil health. CAB International, Wellington, pp 49–78

FAO (1966) Guía para la Descripción de Perfiles de Suelo. FAO, Roma

García C, Hernández T (1997) Biological and biochemical indicators in derelict soils subject to erosion. Soil Biol Biochem 29:171–177

Guitián-Ojea F, Carballas T (1976) Técnicas de Análisis de Suelos. Pico Sacro Editorial, Santiago de Compostela, Spain

Hinojosa MB, García-Ruíz R, Viñegla B, Carreira JA (2004) Microbiological rates and enzyme activities as indicators of functionality in soils affected by the Aznalcóllar toxic spill. Soil Biol Biochem 36:1637–1644

ISSS Working Group, R.B., (1998). World reference base for soil resources: Introduction. International Society of Soil Science, International Soil Reference and Information Centre and Food and Agriculture Organization of the United Nations. Acco, Leuven, Belgium

Margesin R, Zimmerbauer A, Schinner F (2000) Monitoring of bioremediation by soil biological activities. Chemosphere 40:339–346

McKeague JA, Day JH (1966) Dithionite and oxalate extractable Fe and Al as an aid in differentiating various classes of soils. Can J Soil Sci 46:13–22

Moscoso F, Bouzas S, Gil-Sotres F, Leirós MC, Trasar-Cepeda C (2007) Suitability of the OECD tests to estimate contamination with 2,4 dichlorophenol of soils from Galicia (NW Spain). Sci Total Environ 378:58–62

Nannipieri P, Ceccanti B, Cervelli S, Matarese E (1980) Extraction of phosphatases, urease, proteases, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016

Schinner F, von Mersi W (1990) Xylanase-, CM-cellulase- and invertase activity in soil: an improved method. Soil Biol Biochem 22:511–515

Severtson SJ, Banarjee S (1996) Sorption of chlorophenols to wood pulp. Environ Sci Technol 30:1961–1969

Shiu W-Y, Ma K-Ch, Varhaníčová D, Mackay D (1994) Chlorophenols and alkylphenols: a rewiev and correlation of environmentally relevant properties and fate in an evaluative environmente. Chemosphere 29:1155–124

Trasar-Cepeda C, Camiña F, Leirós MC, Gil-Sotres F (1999) An improved method for measurement of catalase activity in soils. Soil Biol Biochem 31:483–485

Trasar-Cepeda C, Leirós MC, Seoane S, Gil-Sotres F (2000) Limitations of soil enzymes as indicators of soil pollution. Soil Biol Biochem 32:1867–1875

Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Hydrolytic enzyme activities in agricultural and forest soils. Some implications for their use as indicators of soil quality. Soil Biol Biochem 40:2146–2155

Troitiño F, Gil-Sotres F, Leirós MC, Trasar-Cepeda C (2008) Effect of land use on some soil properties in relation to the risk of soil P loss in Galicia (NW Spain). Land Degrad Dev 19:21–35

Tu CM (1992) Effect of three newer pesticides on microbial and enzymatic activities in soil. Bull Environ Contam Toxicol 49:120–128

von Mersi W, Schinner F (1991) An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol Fertil Soils 11:216–220

Temporal Changes in Some Enzymatic Activities in a Forest and an Agricultural Soils Artificially Contaminated with 2,4,5-Trichlorophenol

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Abstract The aim of the present study was to investigate the responses of two soils each contaminated with two different doses of 2,4,5-trichlorophenol (2,4,5-TCP), during a period of contact of 24 days, by determining any temporal changes in various enzyme activities. Two soils located close to each other but under different types of management (forest and agricultural) were selected for the study. Several subsamples of each of the soils were contaminated with doses of 2,4,5-TCP of 100 (D100) or 500 (D500) mg kg^{-1}. At 3, 10, 17 and 24 days (3, 10, 17 and 24 d) after addition of the contaminant, analyses were carried out to determine the activities of two hydrolytic (urease and invertase) and two oxidoreductase (catalase and dehydrogenase) enzymes in the contaminated and control soils. The results showed that although the response of the enzymes to the presence of 2,4,5-TCP was very variable, and in some cases almost negligible, the time of contact appeared to heighten the effect, particularly in the agricultural soil.

1 Introduction

Methodological-related problems in the diagnosis of soil degradation include determination of the time when analyses that indicate the existence of degradation should be carried out, and how to evaluate whether the degradation is transitory

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(i.e., caused by the immediate effect of the contaminant) or will persist in the long term. As regards the latter aspect, the persistence of the effect of the contaminant should be seen as the result of two processes: on one hand the capacity of the soil to recover, i.e., its resilience (Blum and Aguilar 1994) and on the other, the persistence of the contaminant in the soil, which will obviously depend on its physicochemical properties and the possibility that it will interact with the soil matrix. The latter aspect is essential when the contamination is generated by the application of highly volatile organic products.

The Royal Decree passed in Spain 2005 (Real Decreto 9/2005, BOE 18 January 2005) was important in terms of encouraging legislation aimed at the diagnosis of soil degradation and that ensures complete protection of soil as a resource (de Miguel Perales 2007). The decree defines Generic Reference Levels (GRL), provides a list of the values of these for a large group of substances and indicates the analytical procedures that should be used to diagnose soil quality and the protocol that should be followed when the contaminant is a compound not included in the list provided. Despite the great advance that these initiatives imply, because until now soil has remained unprotected by law, the present legislation has overlooked the time factor, even though many of the substances listed in the Annexes of the Decree are highly volatile organic substances, the persistence of which may be limited because of their high vapour pressure. The Decree makes no indication of when the analyses should be carried out or how often they should be repeated to test whether the initial degradation continues. This is important, as many of the substances considered act on edaphic microorganisms, basically causing biochemical and biological degradation of the soil, and therefore given the capacity of microbial populations to recover, it is possible that once the immediate effects of the contaminant have ceased, the affected properties will recover, thus modifying the initial diagnosis of the soil as contaminated, with the associated legal and economic effects.

Chlorophenols are one group of compounds included in the Decree; this group includes 2,4,5 trichlorophenol (2,4,5-TCP), a product that is widely used in silviculture and agriculture as a timber preservative and as a precursor in the synthesis of various herbicides. According to EPA documents (see, for example, http://msds.chem.ox.ac.uk/TR/2,4,5-trichlorophenol.html) this compound, dispensed as a white crystalline powder, has a fusion point of 68 °C and a vapour pressure of 1 mm Hg at 72 °C, which makes it particularly volatile. In fact, the EPA document indicates, amongst other properties, the irritant effect on humans of the compound due to its relatively high volatility. To date the effect of this compound on soil has been determined in our laboratories by considering a very short contact time (3 days) between soil and contaminant (Bello et al. 2007, 2008; Moscoso et al. 2007), but at present there is no information as regards the effects for longer exposure times. The aim of the present study was to obtain further information about the extent to which the time passed since addition of the contaminant to soil affects soil enzyme activities. For this, two soils representative of the region of Galicia were contaminated with two doses of 2,4,5-TCP and

periodically analysed for different enzymes amongst those usually used to diagnose soil contamination.

2 Materials and Methods

Samples were taken from the surface horizon (0–10 cm) of two soils at a site close to Santiago de Compostela (Cacheiras) under different types of use, one under forest vegetation of Atlantic oak *Quercus robur* L. (forest soil) and the other under agricultural management (agricultural soil); the soils are close to one another and both have developed over the same type of parent material.The physiographic characteristics of the soils are shown in Table 1. Composite samples were made by mixing (in the field) the various subsamples collected at different points in each plot, after removal of the förna and litter layer from the forest soil, and the layer containing remains of organic fertilizers and amendments from the agricultural soil. The soils were transported to the laboratory in insulated bags, sieved (<4 mm) and stored in the fridge at 4 °C until the beginning of the experiment. Portions of the samples were air-dried for analysis of general soil properties.

For the contamination experiment, aliquots of the soils were artificially contaminated with doses of 2,4,5-TCP equivalent to 100 (D100) and 500 mg kg^{-1} (D500) (i.e., doses that correspond to 10 and 50 times the value of the GRL considered in the legislation). Given the low solubility of the compound in water and to ensure good homogenization, the compound was added to the soil after mixing it with washed quartz sand (10 g of sand per 100 g of oven-dried soil). The amount of water required to maintain the mixture at optimal conditions of moisture (at water field capacity of the soil) was added. For each soil a similar mixture to the above was prepared, but with no contaminant, as a control. Once prepared, the control and contaminated samples were placed in appropriate polypropylene containers, sealed with Parafilm® to avoid moisture loss, and were maintained at 20 °C in controlled laboratory conditions. After 3, 10, 17 and 24 days the activities of two oxidoreductase enzymes (dehydrogenase and catalase) and two hydrolytic enzymes (urease and invertase) were determined.

The methods described by Guitián-Ojea and Carballas (1976) were used to determine the following soil properties: pH in water (1:2.5, soil:water ratio), pH in 1 M KCl (1:2.5, soil:solution ratio); total carbon content (by potassium dichromate oxidation); total nitrogen content (by Kjeldahl digestion); water retained at −33 kPa pressure (water field capacity) and particle size distribution (with a Robinson pipette and Calgon as dispersant); the textural class of soils was determined following FAO (1966). Amorphous Al and Fe were extracted with 0.2 M ammonium oxalate/oxalic acid buffer of pH 3.0 (McKeague and Day 1966) and were determined by atomic absorption spectrometry (Varian SpectrAA 220 FS spectrometer, UK).

Dehydrogenase activity was determined with 98.87 mM iodonitrotetrazolium violet (INT) as substrate, incubating with 1 M TRIS–HCl buffer (pH 7.5) for 1 h.

Table 1 Physiographic characteristics of the soils used in the study

Soil	Forest soil	Agricultrual soil
Location	Cacheiras	Cacheiras
Altitude	200 masl	180 masl
Longitude W	8° 33′ 20′	8° 33′ 30′
Latitude N	42° 49′ 22′	42° 49′ 00′
Parent material	Migmatitic granitoid	Migmatitic granitoid
Vegetation	Atlantic oakwood	Cultivated corn
Soil type[a]	Umbrisol	Regosol

[a] ISSS Working Group, 1998

The iodonitrotetrazolium formazan (INTF) produced was extracted with a mixture of ethanol and dimethylformamide, and measured at 490 nm (Camiña et al. 1998). Activity was quantified by reference to a calibration curve obtained using INTF standards incubated with soil under the same conditions as for the samples, and is expressed as μmol INTF g^{-1} h^{-1}. Catalase activity was determined by incubating the soil samples with 8.8 mM H_2O_2 for 10 min. The residual H_2O_2 was subjected to a reaction that yields a coloured product that absorbs at 505 nm and the activity, expressed as mmol H_2O_2 consumed g^{-1} h^{-1}, was calculated as the difference between the initial and the residual H_2O_2 (Trasar-Cepeda et al. 1999). Invertase activity was determined by incubating the samples with 35.06 mM saccharose in 2 M acetate buffer (pH 5.5) for 3 h, and measuring the released reducing sugars following the method of Schinner and von Mersi (1990). Urease activity was determined by the method described in Nannipieri et al. (1980), by incubating the samples with 1065.6 mM urea as substrate for 1.5 h in 0.2 M phosphate buffer (pH 8.0), and measuring the NH_4^+ released with an ammonia electrode. All determinations were performed in triplicate and for each soil sample the average values of the three determinations (expressed on an oven-dried soil basis, 105 °C), were calculated.

3 Results

3.1 Physical and Chemical Characteristics of the Soils

The values of the physical and chemical properties of both soils are shown in Table 2. Both are strongly acidic soils (particularly the forest soil) with quite different organic matter contents and sandy loam texture (the coarse sand fraction dominates in both case data not shown). As expected, the total carbon content was significantly lower in the agricultural than in the forest soil and the nitrogen content of the former was proportionally higher, so that the value of the C/N ratio in the agricultural soil was much lower than in the forest soil, developed under climax vegetation of Atlantic oak. The contents of Fe and Al amorphous oxides

Table 2 Physical and chemical properties of the soils under study

Parameters	Forest soil	Agricultural soil
pH in H_2O	4.23 ± 0.01	5 06 ± 0.03
pH in KCl	3.79 ± 0.03	4.25 ± 0.01
Total C (%)	4.60 ± 0.22	1.81 ± 0.11
Total N (%)	0.24 ± 0 00	0.13 ± 0.00
C/N	19 ± 1	14 ± 1
Water field capacity (%)	27 ± 2	21 ± 0
Sand (%)	80 ± 1	79 ± 4
Silt (%)	10 ± 2	9 ± 1
Clay (%)	10 ± 2	12 ± 4
Textural class	sandy loam	sandy loam
Fe_2O_3 (%)	0.47 ± 0.02	0.30 ± 0.02
Al_2O_3 (%)	0.55 ± 0.01	0.38 ± 0.01

extractable with pyrophosphate were slightly higher in the forest soil, as expected because of the higher organic matter content (Trasar-Cepeda et al. 2008; Troitiño et al. 2008).

3.2 Oxidoreductase Activities

The dehydrogenase activity in the uncontaminated forest soil was almost double that in the uncontaminated agricultural soil (Table 3), and in both the activity remained almost constant throughout the entire period of incubation. In the forest soil the contaminant only slightly affected the dehydrogenase activity at the highest dose, causing a slight gradual decrease in activity (Table 3). It was also observed that for the longest incubation time, the dose of 100 mg kg^{-1} appeared to cause a slight decrease in the activity of this enzyme. In the agricultural soil, both doses initially caused a large reduction in the activity, which was slightly greater at higher doses; the activity then did not change greatly throughout the rest of the incubation period maintaining the significant differences among both doses during the whole period, although there was a very slight trend of increasing values with the lower doses, and the opposite trend with the highest dose (Table 3).

As regards the catalase activity (Table 3), the value was again higher in the uncontaminated forest soil than in the uncontaminated agricultural soil and in both cases the value of the activity decreased clearly throughout the incubation period, mainly from 17 days onwards. The effect of the contaminant was similar in both soils, although was slightly more intense in the agricultural soil. The lowest dose (100 mg kg^{-1}) did not affect the catalase activity during the first days of the incubation, but from 10 days onwards there was a clear and significant effect. The highest dose (500 mg kg^{-1}) caused a significant decrease in the activity after 3 days in the agricultural and forest soil, and in both cases there was a large decrease in activity between 10 and 24 days of incubation.

Table 3 Values of the oxidoreductase activities of uncontaminated and contaminated soil samples throughout the experiment. Different capital letters indicate statistically significant differences among the contaminant doses for each soil and period of time ($P \leq 0.05$), while different lowercase letters indicate statistically significant differences throughout the incubation period for each contaminant dose ($P \leq 0.05$)

Days	Dehydrogenase activity[1]						Catalase activity[2]					
	Forest soil			Agricultural soil			Forest soil			Agricultural soil		
	D0	D100	D500	D0	D100	D500	D0	D100	D500	D0	D100	D500
3 d	$^{ab}0.29^{A}$	$^{ab}0.27^{A}$	$^{a}0.24^{B}$	$^{a}0.11^{A}$	$^{a}0.06^{B}$	$^{a}0.05^{C}$	$^{a}1.02^{A}$	$^{a}1.42^{B}$	$^{a}1.23^{AB}$	$^{a}0.69^{A}$	$^{a}0.65^{AB}$	$^{a}0.47^{B}$
10 d	$^{a}0.27^{A}$	$^{a}0.29^{A}$	$^{b}0.23^{B}$	$^{a}0.13^{A}$	$^{b}0.08^{B}$	$^{b}0.07^{C}$	$^{b}1.32^{A}$	$^{a}1.26^{A}$	$^{b}0.73^{B}$	$^{a}0.64^{A}$	$^{a}0.69^{A}$	$^{b}0.59^{B}$
17 d	$^{ab}0.29^{A}$	$^{ab}0.27^{A}$	$^{abc}0.22^{B}$	$^{a}0.12^{A}$	$^{b}0.08^{B}$	$^{a}0.05^{C}$	$^{a}0.97^{A}$	$^{b}0.37^{B}$	$^{c}0.32^{B}$	$^{a}0.52^{AB}$	$^{b}0.39^{A}$	n.d.
24 d	$^{b}0.30^{A}$	$^{b}0.25^{B}$	$^{c}0.21^{C}$	$^{a}0.11^{A}$	$^{b}0.07^{B}$	$^{a}0.04^{C}$	$^{c}0.59^{A}$	$^{c}0.73^{A}$	$^{d}0.38^{B}$	$^{b}0.30^{A}$	$^{c}0.12^{B}$	$^{c}0.10^{B}$

[1] μmol INTF g^{-1} h^{-1}, [2] mmol H_2O_2 consumed g^{-1} h^{-1}

Table 4 Values of the hydrolytic activities throughout the experiment. Different capital letters indicate statistically significant differences among the contaminant doses for each soil and period of time ($P \leq 0.05$), while different lower case letters indicate statistically significant differences throughout the incubation period for each contaminant dose ($P \leq 0.05$)

Days	Urease activity[1]						Invertase activity[2]					
	Forest soil			Agricultural soil			Forest soil			Agricultural soil		
	D0	D100	D500	D0	D100	D500	D0	D100	D500	D0	D100	D500
3 d	$^{a}8.25^{A}$	$^{a}8.67^{A}$	$^{a}6.43^{B}$	$^{a}1.83^{A}$	$^{a}2.00^{B}$	$^{a}1.28^{C}$	$^{a}1.27^{A}$	$^{a}1.40^{B}$	$^{ab}1.40^{B}$	$^{abc}0.93^{A}$	n.d	$^{a}0.51^{B}$
10 d	$^{b}5.94^{A}$	$^{b}5.95^{A}$	$^{b}5.06^{B}$	$^{b}1.53^{A}$	$^{bc}1.27$	$^{b}0.86^{B}$	$^{ac}1.34^{A}$	$^{b}1.02^{B}$	$^{a}1.29^{A}$	$^{ac}0.80^{A}$	$^{a}0.74^{A}$	$^{b}0.65^{B}$
17 d	$^{bc}5.41^{A}$	n.d	$^{b}4.90^{B}$	$^{a}1.89^{A}$	$^{b}1.36^{B}$	$^{b}0.82^{C}$	$^{bc}1.52^{AB}$	$^{c}1.62^{A}$	$^{ab}1.31^{B}$	$^{b}0.94^{A}$	$^{b}0.97^{A}$	$^{c}0.81^{B}$
24 d	$^{c}4.98^{A}$	$^{c}4.47^{A}$	$^{c}2.72^{B}$	$^{b}1.62^{A}$	$^{c}1.13^{B}$	$^{c}0.58^{C}$	$^{c}1.47^{A}$	$^{c}1.52^{A}$	$^{b}1.45^{A}$	$^{c}0.82^{A}$	$^{a}0.70^{A}$	$^{a}0.53^{B}$

[1] μmol NH_3 g^{-1} h^{-1}, [2] μmol glucose g^{-1} h^{-1}

3.3 Hydrolase Activities

The urease activity was much higher in the uncontaminated forest soil than in the uncontaminated agricultural soil, and also displayed a different pattern of activity throughout the period of incubation. The activity in the forest soil decreased gradually throughout the experiment, particularly between 3 and 10 days of incubation; in the agricultural soil the activity was almost constant throughout the period of incubation (Table 4). In the forest soil the lowest dose (100 mg kg^{-1}) did not affect significantly the value of this activity, which was rather similar to that in the control soil throughout the entire experiment, whereas the highest dose caused an initial slight but significant reduction in activity, which appeared to become significantly greater throughout the experiment. In the agricultural soil both doses caused a large decrease in activity, which was greater at higher doses, and this reduction continued throughout the incubation period, although it tended to stabilize with the lowest dose and the effect became more intense at the highest dose (Table 4).

The invertase activity (Table 4) was also higher in the uncontaminated forest soil than in the uncontaminated agricultural soil. In addition, in the forest soil the activity tended to increase with the incubation time, whereas in the agricultural soil it varied inconsistently throughout the period of incubation (Table 4).

In the forest soil none of the doses of contaminant appeared to have a clear effect on invertase activity, as the values were rather similar to the control values throughout the incubation period, and in any cases displayed random variations, possibly due to the heterogeneity of the sample. In the agricultural soil, the lower dose of 2,4,5-TCP did not appear to affect the activity. For the highest dose there was a significant initial large decrease in activity and the value obtained after 3 days of incubation tended to remain stable, although with the associated variations, possibly due, as indicated for the forest soil, to the heterogeneity of the sample.

4 Discussion

As indicated in other studies (Bello et al. 2007; Moscoso et al. 2007), the effect of 2,4,5-trichlorophenol on soil biochemical properties depends on both the enzyme considered and the characteristics of the soil. In this study, the soil that appeared most sensitive to the presence of the contaminant was the agricultural soil, and as indicated previously (Bello et al. 2008), it is possible that this sensitivity is linked to the decrease in the amount of organic matter that occurs in a soil as the result of continuous cultivation (Carter 1986; Janzen et al. 1992; Trasar-Cepeda et al. 2008). It should also be stressed that the most sensitive enzymes were the two oxidoreductase enzymes and urease, and the least sensitive invertase, as was also observed in previous studies (Bello et al. 2008). However, the effect that the time

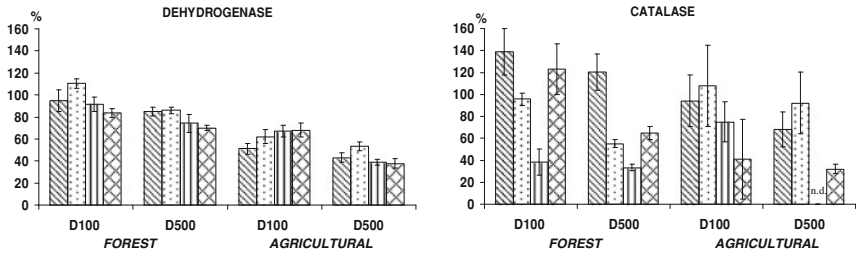

Fig. 1 Changes in the oxidoreductase activities in agricultural and forest soils contaminated with 2,4,5-TCP, expressed as a percentage with respect to the activity in the control soil at each measurement time during the incubation period (3 10 17 24 days)

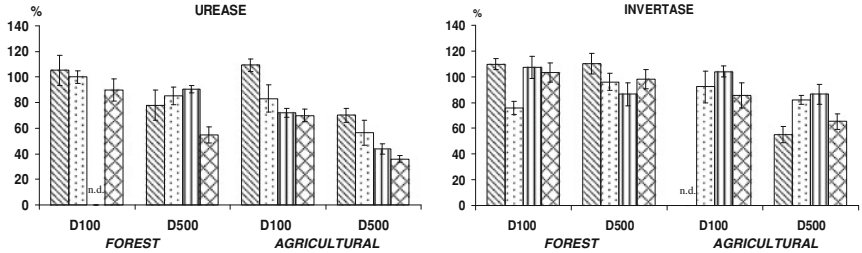

Fig. 2 Changes in the hydrolytic activities in the agricultural and forest soils contaminated with 2,4,5-TCP, expressed as a percentage with respect to the activity in the control soil at each measurement time during the incubation (3 10 17 24 days)

of contact between the contaminant and the soil has on the soil degradation does not appear too clear, as the biochemical properties in the control (uncontaminated) soils also vary throughout the incubation time, making it difficult to interpret the results, as it must be taken into account that the values obtained for the contaminated soils are due to both the action of the dose of contaminant added, and the variation that the soil will suffer as a result of the incubation. Despite these problems, when the values of the enzyme activities are expressed for each dose and time as a percentage of the values obtained for the control for the same time of incubation, clearer trends and differences were observed (Figs. 1 and 2). Thus, in general a great effect on the decrease in enzyme activity was usually produced in the first moments (first 3 days after addition of the contaminant) especially for the highest dose of 2,4,5-trichlorophenol and the agricultural soil, and the effect then remained practically the same and in many cases even increased, as a further decrease in the enzymatic activity occurred. This suggests that the compound has an initial action on the microorganisms and stabilized enzymes, and that there is then a residual effect that prevents, at least during the 24 days monitored, the recovery of the microbial populations.

The results indicate that the product remained in the soil matrix during the study period, and despite the relatively high volatility of the compound, certain soils

mechanisms appeared to favour the persistence of the product. It has been suggested that such mechanisms may include ionization of the compound and its persistence in the soil solution, or some adsorption process-possibly linked to the formation of homopolar bonds between the non dissociated molecule and the surfaces of the soil organic matter (Bollag et al. 1980). This pattern was clearly shown by dehydrogenase and urease activities, but the behaviour of catalase and invertase activities was clearly different. In general, catalase activity increased in the first moments in contaminated soils, but thereafter there was a continuous decline in the activity. This decrease was strongest in the forest soil than in the agricultural soil, although at the end of the incubation the activity strongly increased again in the former while still decreased in the agricultural soil. One possible explanation for this anomalous behaviour is that there may have been problems related to determination of catalase activity, as indicated in previous studies (Bello et al. 2008). Invertase activity was unaffected by the presence of 2,4,5-trichlorophenol during the whole incubation period. However, this behaviour was already expected, as in other studies invertase has also been shown to be relatively insensitive to the presence of the contaminant. This is possibly because it is usually a stabilized extracellular enzyme (Moorhead and Sinsabaugh 2000), and therefore the lack of inhibition may be associated with protection of the active centre, which is therefore not affected by the presence of the contaminant. The fact that this effect is less apparent in the agricultural soil than in the forest soil suggests that under normal conditions the active centre is provided some degree of protection by the soil organic matter.

5 Conclusions

The results of the study show that the biochemical degradation that soils undergo due to the presence of 2,4,5-trichlorophenol does not only occur at the moments immediately following the contamination, but that the effects are prolonged and increase over time, at least during a period of about one month. This persistence appears to be caused mainly by the incapability of the microbial populations to re-establish themselves in the soil, which suggests that the compound remains in the soil solution in forms that are toxic for microorganisms.

From the point of view of the diagnosis of contaminated soils, this suggests the need for periodic monitoring of soils contaminated by the product under consideration, and at the moment no one biochemical property can be recommended as a key parameter for use in the diagnosis of the recovery of contaminated soils.

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References

Bello D, Presas E, Leirós MC, Trasar-Cepeda C, Gil-Sotres F (2007). Soils contaminated with 2,4,5 TCP: ED_{50} values for biochemical properties and ecotoxicological tests. In: Sierra J, Martí E, Domene X (Eds.) Proceedings International Meeting Soil and Wetland Ecotoxicology, Papers and Abstracts, pp 1.2.4.-1-1.2.4.-7

Bello D, Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Evaluation of different tests for the diagnosis of soil contamination by 2,4,5-trichlorophenol (2,4,5-TCP). Environ Pollut 156:611–617

Blum WEH, Aguilar A (1994) A concept of sustainability and resilience based on soil functions. In: Greenland DJ, Szabolcs I (eds) Soil Resilience and Sustainable Land Use. CAB International, Wallingford, UK, pp 535–542

BOE, 18 Enero de (2005). Ministerio de la Presidencia, Real Decreto 9/2005, pp. 1833-1843

Bollag JM, Liu SY, Minard RD (1980) Cross-coupling of phenolic humus constituents and 2,4-dichlorophenol. Soil Sci Soc Am J 52:1366–1371

Camiña F, Trasar-Cepeda C, Gil-Sotres F, Leirós MC (1998) Measurement of dehydrogenase activity in acid soils rich in organic matter. Soil Biol Biochem 30:1005–1011

Carter MR (1986) Microbial biomass as an index for tillage-induced changes in soil microbial properties. Soil Till Res 7:29–40

de Miguel Perales C (2007) Régimen Jurídico Español de Suelos Contaminados. (Eds). Aranzadi SA, Zizur Menor, Navarra, Spain

FAO (1966) Guía para la Descripción de Perfiles de Suelo. FAO, Rome, Italy

Guitián-Ojea F, Carballas T (1976) Técnicas de Análisis de Suelos. Editorial Pico Sacro, Santiago de Compostela, Spain

ISSS Working Group, R.B., 1998. World Reference Base for Soil Resources: Introduction. International Society of Soil Science, International Soil Reference and Information Centre and Food and Agriculture Organization of the United Nations. Acco, Leuven, Belgium

Janzen HH, Campbell CA, Brandt SA, Lafond GP, Toronley-Smith L (1992) Light-fraction organic matter in soils from long–term crop rotations. Soil Sci Soc Am J 56:1799–1806

McKeague JA, Day JH (1966) Dithionite and oxalate extractable Fe and Al as an aid in differentiating various classes of soils. Can J Soil Sci 46:13–22

Moorhead DL, Sinsabaugh RL (2000) Simulated patterns of litter decay predict patterns of extracellular enzyme activities. Appl Soil Ecol 14:71–79

Moscoso F, Bouzas S, Gil-Sotres F, Leirós MC, Trasar-Cepeda C (2007) Suitability of the OECD tests to estimate contamination with 2,4 dichlorophenol of soils from Galicia (NW Spain). Sci Total Environ 378:58–62

Nannipieri P, Ceccanti B, Cervelli S, Matarese E (1980) Extraction of phosphatases, urease, proteases, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016

Schinner F, von Mersi W (1990) Xylanase-, CM-cellulase- and invertase activity in soil: an improved method. Soil Biol Biochem 22:511–515

Trasar-Cepeda C, Camiña F, Leirós MC, Gil-Sotres F (1999) An improved method for measurement of catalase activity in soils. Soil Biol Biochem 31:483–48

Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Hydrolytic enzyme activities in agricultural and forest soils, some implications for their use as indicators of soil quality. Soil Biol Biochem 40:2146–2155

Troitiño F, Gil-Sotres F, Leirós MC, Trasar-Cepeda C (2008) Effect of land use on some soil properties in relation to the risk of soil P loss in Galicia (NW Spain). Land Degrad Dev 19:21–35

Response of Biological Properties to the Application of Banvel® (2,4-D + MCPA + Dicamba) Herbicide in Soils Amended with Biostimulants

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Abstract In this paper the effect of Banvel® (2,4-D + MCPA + Dicamba) herbicide at a rate of 1.5 L ha^{-1} (manufactures rate recommended) in the biological properties of a Plagic Antrosol amended with four biostimulants (WCDS, wheat condensed distillers soluble; PA-HE, hydrolyzed poultry feathers; CGHE, carob germ enzymatic extract; and RB, rice bran extract) was studied. Seven hundred grams of soil were mixed with WCDS at a rate of 10%, CGHE at a rate of 4.7%, PA-HE at a rate of 4.3%, and RB at a rate of 4.4%, respectively, in order to apply the same amount of organic matter to the soil (16.38 g organic matter). An unamended polluted and amended non-polluted soil was used as control. For all treatments, the soil ergosterol, dehydrogenase, urease, and phosphatase activities were measured at two incubation times (1 and 60 days). The 16S rDNA-DGGE profiles in all treatments were determined at the beginning and end of the incubation period. The results indicated that at the end of the incubation period and compared with the control soil, the dehydrogenase, urease and phosphatase activities and ergosterol decreased 46.4, 17, 19.1 and 47.8%, respectively in the non-organically amended polluted soil. The application of organic matter to unpolluted soil increased phosphatase activity. However, this stimulation was higher in the soil amended with PA-HE, followed by RB, WCDS and CGHE. The application of herbicide to the amended soils decreased enzymatic activities and ergosterol content. However, these decreases were lower than those in the non-amended herbicide polluted soil. Possibly the low molecular weight proteins easily

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assimilated by soil microorganisms provided with the biostimulants are responsible for the lower inhibition of these enzyme activities and soil ergosterol in the amended soils. The 16S rDNA-DGGE profiles indicated that herbicide did not negatively affect soil bacterial biodiversity.

1 Introduction

Pesticides are important tools in agriculture that help to minimize economic losses caused by weeds, insects, and pathogens. Although their use has helped to increase crop yields and value, pesticides are recognized as a source of potential adverse environmental impacts and their persistence in surface and ground waters has grown considerably (Cox et al. 2001; Tejada 2009).

The actual agricultural activity requires the use of herbicides, and prevention of ground water pollution is much cheaper than restoring polluted aquifers. For this reason, it is of maximum interest the development of agricultural strategies directed to the decrease in pesticide movement (Cox et al. 1997).

Several bioremediation strategies have been proposed to reduce the presence of pesticides in soil from which they can reach groundwater, such as remediation by enhancing the microbial population able to degrade specifically the target compounds. This strategy has been approached by the addition of organic exogenous matter of different origin (Abdelhafid et al. 2000; Delgado-Moreno and Peña 2007, 2009). However, the influence of organic matter on soil properties and sorption process depends upon the type, amount and dominant components of the added organic materials (Tejada et al. 2007, 2008). This aspect is of great interest, since it supposes an important advance in the behaviour of the herbicides in the soil after the addition of different sources of organic matter.

New agricultural products are soil biostimulants (BS) which are organic products composed of peptides, amino acids, polysaccharides, humic acids and/or phytohormones, etc. (Parrado et al. 2008) which are also claimed to work through a series of widely varying mechanisms including activation of soil microbial activity and promotion or augmentation of the activities of critical soil enzymes, which would increase the microbial populations. Thus, these BS applied to soil could enhance herbicide degradation, being an experimental way for self-cleaning of polluted areas via the activities of indigenous degrading microorganisms.

Biological and biochemically mediated processes in soils are of paramount importance to ecosystem function. Many studies have shown that biological parameters have been used to assess soil quality and health as affected by agricultural practices (Zabaloy and Gómez 2008; Tejada 2009). In this respect, soil enzymes can be used as potential indicators of soil quality for sustainable management because they are sensitive to ecological stress and land management practices (Bandick and Dick 1999; Weaver et al. 2007).

The aim of this paper was to study the influence of different BS on a Banvel® (2,4-D + MCPA + Dicamba) herbicide polluted soil, by analyzing their effects

on soil enzymatic activities, ergosterol and soil bacterial community. Banvel® is a post-emergence herbicide for annual weed control broadleaves. This herbicide is widely used in crops such as wheat, barley, oats, and corn.

2 Materials and Methods

2.1 Soil and Biostimulants Characteristics

The soil used in this experiment is a Plagic Antrosol (FAO 1989). This soil had 529 ± 35 g kg^{-1} sand, 242 ± 19 g kg^{-1} silt and 229 ± 10 g kg^{-1} clay. Soil pH was 8.6 ± 0.2, 1.1 ± 0.3 g kg^{-1} organic matter and 0.4 ± 0.1 g kg^{-1} total N. Soil pH was determined in distilled water with a glass electrode (soil:H_2O ratio 1:2.5). Soil texture was determined by the Robinson's pipette method (SSEW 1982). Soil organic matter was determined by the method of Yeomans and Bremner (1988) and total N was determined by the Kjeldhal method (MAPA 1986).

The BS applied were: (1) wheat condensed distillers solubles (WCDS), a major by-product of ethanol fermentation provided by Abengoa-Bioenergy (Bioethanol Galicia**,** Teixero, Spain), (2) hydrolyzed poultry feathers (PA-HE), is an enzymatic water soluble extract from poultry feathers, (3) Carob germ enzymatic extract (CGHE), is an enzymatic water soluble extract from carob germ produced by enzymatic hydrolysis, (4) Rice bran extract (RB), obtained from rice bran flour by enzymatic hydrolysis. The enzymatic hydrolysis process for bioestimulants obtained is detailed in García-Martínez et al. (2010a, b).

The general properties of BS are shown in Table 1. Organic matter was determined by dry combustion. Total soluble carbohydrates were determined after extraction with a mixture of ethanol:water (2:3 v:v) for 2 h. After centrifugation at 4,000 *g*, the supernatant was filtered through no. 1 Whatman paper, and total soluble sugars were estimated colorimetrically by the phenol–sulfuric acid method, using a standard curve of glucose. The protein content was determined by multiplying the total nitrogen by a conversion factor of 6.25. Fats were determined gravimetrically after extraction with hexane for 12 h in a Soxhlet extractor. After nitric and perchloric acid digestion, P was determined by the Guitian and Carballas (1976) method, and K, Ca, Mg, Fe, Cu, Mn and Zn were measured by ICP-OES.

Molecular-mass distribution of protein (Table 2) in the samples was determined by size-exclusion chromatography using an ÄKTA-purifier (GE Healthcare), according to the procedure described by (Bautista et al. 1996), using a Superdex Peptide™ 10/300GL column (optimum separation range 0.1–7 kDa). Samples were centrifuged at 13,300 *g* for 15 min at 4°C to remove insoluble, and the supernatant was passed through a 0.2 μm filter and loaded into a 0.1 mL loop connected to an Äkta purifier system. The column was equilibrated, and eluted with 0.25 M Tris–HCl buffer (pH 7) in isocratic mode, at a flow-rate of

Table 1 Biostimulant chemical characteristics and standard error. Data are the means of four samples[1]

	WCDS	CGHE	PA-HE	RB
Organic matter (g kg^{-1})	234 ± 25^{a1}	492 ± 20^{b}	541 ± 43^{b}	530 ± 25^{b}
Total soluble carbohydrates (g kg^{-1})	49.8 ± 5.2^{c1}	38.0 ± 1.6^{c}	6.5 ± 1.9^{a}	19.0 ± 1.1^{b}
Proteins (g kg^{-1})	40.2 ± 3.8^{a1}	46.5 ± 1.2^{a}	83.0 ± 8.4^{b}	50.0 ± 2.3^{a}
Fats (g kg^{-1})	4.2 ± 0.4^{b1}	6.5 ± 0.2^{b}	2.0 ± 0.6^{a}	14.0 ± 0.9^{c}
N-Kjeldahl (g kg^{-1})	6.4 ± 1.8^{a1}	7.4 ± 1.2^{a}	13.3 ± 8.4^{b}	8.0 ± 2.3^{a}
P (g kg^{-1})	3.5 ± 0.2^{c1}	7.7 ± 0.6^{d}	0.50 ± 0.08^{a}	1.60 ± 0.24^{b}
K (g kg^{-1})	6.8 ± 0.6^{b1}	22.8 ± 1.2^{c}	0.86 ± 0.021^{a}	nd
Na (g kg^{-1})	0.82 ± 0.08^{a1}	0.9 ± 0.1^{a}	0.86 ± 0.24^{a}	nd
Ca (g kg^{-1})	≤0.5^{a1}	3.1 ± 0.2^{b}	0.78 ± 0.09^{a}	0.59 ± 0.10^{a}
Fe (g kg^{-1})	≤0.2^{b1}	≤0.1^{b}	≤0.05^{a}	≤0.02^{a}
Cu (g kg^{-1})	nd	≤0.05	nd	nd
Mg (g kg^{-1})	1.2 ± 0.21^{b1}	3.5 ± 0.2^{c}	≤0.32^{a}	0.79 ± 0.07^{b}
Mn (g kg^{-1})	≤0.05	nd	nd	nd
Zn (g kg^{-1})	≤0.05^{a1}	≤0.05^{a}	1.4 ± 0.14^{b}	nd

nd not determined; [1] Files (mean ± standard errors) followed by the same letter(s) are not significantly different ($P < 0.05$)

Table 2 Molecular weight (Daltons) distribution of biostimulants. Data are the means of four samples[1]

	WCDS	CGHE	PA-HE	RB
>10000	12.2 ± 1.3^{c1}	6.1 ± 1.7^{b}	2.8 ± 0.5^{a}	3.3 ± 0.9^{a}
10000–5000	5.7 ± 0.4^{b1}	4.8 ± 0.9^{b}	1.6 ± 0.3^{a}	2.8 ± 1.1^{a}
5000–1000	14.2 ± 2.6^{b1}	9.1 ± 1.1^{a}	8.3 ± 1.4^{a}	10.4 ± 1.9^{a}
1000–300	27.1 ± 3.1^{a1}	20.8 ± 2.3^{a}	20.4 ± 3.6^{a}	19.7 ± 2.6^{a}
<300	40.8 ± 6.9^{a1}	59.1 ± 7.6^{ab}	66.9 ± 8.8^{b}	63.8 ± 7.1^{b}

[1] Files (mean ± standard errors) followed by the same letter(s) are not significantly different ($P < 0.05$)

0.5 ml/min, and proteins/peptides were detected at 280 and 215 nm with a GE Healthcare UV900 module coupled to the column elution. A protein standard mixture (cytochrome C, 12,500 Da; aprotinin, 6,512 Da; vitamin B_{12}, 1,255 Da; Cytidine, 246 Da; glycine 75 Da) was used to cover the range of 100 Da–7,000 Da.

2.2 Incubation Procedure

Seven hundred grams of soil were pre-incubated at 25°C for 7 days at 30–40% of their water-holding capacity, according to Tejada (2009), prior to the treatments. After this pre-incubation period, soil samples were mixed with Banvel® at a rate of 1.5 L ha^{-1} (manufactures rate recommended) and treated with WCDS at a rate of 10%, CGHE at a rate of 4.7%, PA-HE at a rate of 4.3%, and RB at a rate of 4.4%, respectively, in order to applying the same amount of organic matter to the soil

(16.38 g organic matter). An unamended polluted soil and an amended non-polluted soil were used as controls.

The incubation treatments were detailed as follows:

(1) Control Soil, unamended and non herbicide polluted soil
(2) Soil + CGHE, non herbicide polluted soil amended with CGHE
(3) Soil + WCDS, non herbicide polluted soil amended with WCDS
(4) Soil + PA-HE, non herbicide polluted soil amended with PA-HE
(5) Soil + RB, non herbicide polluted soil amended with RB
(6) Soil + Banvel, non-amended soil polluted with herbicide
(7) Soil + CGHE + Banvel, herbicide polluted soil amended with CGHE
(8) Soil + WCDS + Banvel, herbicide polluted soil amended with WCDS
(9) Soil + PA-HE + Banvel, herbicide polluted soil amended with PA-HE
(10) Soil + RB + Banvel, herbicide polluted soil amended with RB

Triplicate treatments were incubated in darkness at 25°C in an incubation chamber for two different times (1 and 60 days). Soil sub-samples were stored in sealed polyethylene bags at 4°C for 15 days, until ergosterol and enzymatic activities analysis, whereas sub-samples for soil microbial community analysis were stored at −20°C.

3 Analytical Determinations

3.1 Soil Enzymatic Activities and Ergosterol

Soil dehydrogenase activity, determined by the reduction of *p*-iodonitrotetrazolium chloride (INT) to *p*-iodonitrotetrazolium formazan (INTF) was measured following the method reported by García et al. (1993). Soil urease activity was determined by the method of Kandeler and Gerber (1988), using urea as substrate. Phosphatase activity was measured using *p*-nitrophenylphosphate as substrate (Tabatabai and Bremner 1969). Ergosterol was extracted with ethanol for 30 min by oscillating shaking at 250 rev min^{-1} (Djajakirana et al. 1996). Ergosterol was determined by reversed-phase HPLC with 100% methanol as the mobile phase and detected at a wavelength of 282 nm. All biological parameters were measured in triplicate.

3.2 Extraction and Purification of DNA from Soil

A direct method was used to extract DNA from soil, using an UltraClean Soil DNA Kit (Mo Bio Laboratories, Solana Beach, Calif.). Samples of 1 g of soil were processed according to the manufacturer's instructions. Aliquots of DNA were analysed on 0.7% agarose gel containing 0.5 μg mL^{-1} of ethidium bromide and quantified spectrophotometrically.

3.3 Polymerase Chain Reaction

Soil DNA was amplified in a PCR Sprint thermocycler (Hybaid, Ashford, UK). Two sets of universal primers were used for bacteria rDNA. 968F-1401R eubacterial universal primers were used to amplify a 500 bp region of the 16S rDNA (Heuer and Smalla 1997). PCR amplicons were analysed by denaturing gradient gel electrophoresis (DGGE). Each PCR mixture contained 100 ng of DNA, 1 × reaction buffer implemented with 2.5 mM $MgCl_2$, 50 pmol of each primer, 0.2 mM of each dNTP, 3 Units *Taq*-polimerase (Euroclone) in a final volume of 50 μL. The PCR protocol for the 16S rDNA region targeting total bacteria consisted of 3 min at 95°C followed by 40 cycles each one consisting of a denaturing step (10 s at 95°C), primer annealing (20 s at 54°C) and an extension step (40 s at 72°C); a final extension step (10 min at 72°C) was finally carried out. Amplification products together with a Low Range ladder (1,000–80 bp, MBI Fermentas) were checked by electrophoresis on ethidium bromide stained 1.5% agarose gel run at 10 V cm^{-1} in 0.5 × TBE buffer.

3.4 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was carried out using the Bio-Rad Dcode system. PCR products (30 μL) were loaded into 6% polyacrylamide gel (acrylamide: bisacrylamide, 37:1) with a parallel gradient of 40% urea formamide on the top and 60% at the bottom of the gel (100% urea-formamide corresponded to 7 M urea and 40% v/v formamide) and run for 16 h at 5 V cm^{-1} at 60°C in 1 × TAE buffer. DNA band(s) were visualized by staining with SYBER green (Invitrogen) and photographed using a 1D Scientific Imaging System and a Kodak DC 290 Zoom Digital Camera (New Haven, Conn.).

The dominant bands of the obtained DGGE for each treatment were extracted from DGGE gels with sterile pipette tips. Later, these bands were used as template in a new amplification using the same primers. The resulting products of PCR were sequenced using the software ABI PRISM-377 DNA Sequencer (PerkinElmer). The sequences obtained were compared with 16S rRNA sequences available in the National Center for Biotechnology Information (NCBI) database, using the BLAST program (Basic Local Alignment Search Tool).

3.5 Statistical Analysis

Two-ways analysis of variance (ANOVA) was performed for all soil parameters, considering two variables involved (incubation time and treatments) using the Statgraphics Plus 2.1. The means were separated by the Tukey's test, considering a

significance level of $P < 0.05$ throughout the study. For the ANOVA, triplicate data were used for each treatment and every incubation day.

4 Results

4.1 Soil Enzymatic Activities and Ergosterol

Table 3 shows the evolution of soil enzymatic activities during the incubation period. The results indicated that at the end of the incubation period and compared with the control soil, the dehydrogenase activity significantly decreased (46.4%) in the non-amended polluted soil, reflecting the adverse effects of the herbicide on this intracellular activity. Also and compared with the control soil, in non-polluted soil the application of organic matter increased significantly the dehydrogenase activity. However, this increase depended on the kind of organic matter applied to the soil. In this respect and at the end of the incubation period, the dehydrogenase activity significantly increased 90.3, 88.8, 88.3 and 87% in soil amended with PA-HE, followed by RB, CGHE and WCDS, respectively. The application of herbicide to the amended soils decreased dehydrogenase activity. At the end of the incubation period, the dehydrogenase activity significantly decreased 39.4% for the Soil + RB + herbicide treatment (compared to the Soil + RB treatment), followed by 45% in the Soil + CGHE + herbicide treatment (compared to the Soil + CGHE treatment), 37.4% for the Soil + PA-HE + herbicide treatment (compared to the Soil + PA-HE treatment), and 47.7% for the Soil + WCDS + herbicide treatment (compared with the Soil + WCDS treatment).

Similarly to the dehydrogenase activity, urease activity decreased 17% in the non-amended polluted soil compared to the non-amended unpolluted soil (Table 3). However, this decrease was not statistically significant. Again, the application of organic matter to unpolluted soil significantly increased the urease activity. Also, the application of herbicide to the amended soils decreased urease activity. Compared to the Soil + organic matter treatments, this decrease was not statistically significant.

At the end of the experimental period and compared with the control soil, the phosphatase activity decreased 19.1% in the non-amended herbicide polluted soil (Table 3). Again, the application of organic matter to unpolluted soil increased the phosphatase activity. However, this stimulation was higher in the soil amended with PA-HE, followed by RB, CGHE and WCDS. The pollution of the organically-amended soils also produced an inhibition of this enzymatic activity. Also, it should be noted that the decrease of this activity with the addition of herbicide in the amended soils is similar to that of the unamended soil.

The ergosterol showed similar results to those obtained for soil enzyme activities (Table 3). At the end of the incubation period and compared to the control soil, ergosterol content significantly decreased by 47.8% in herbicide polluted soil.

Table 3 Evolution of enzymatic activities and ergosterol during the experimental for all experimental treatments

	Dehydrogenase activity (μg INTF g^{-1} h^{-1})		Urease activity (μmol NH_4^+ g^{-1} h^{-1})		Phosphatase activity (μmol PNP g^{-1} h^{-1})		Ergosterol (mAU mL^{-1})	
	Incubation days		Incubation days		Incubation days		Incubation days	
	1	60	1	60	1	60	1	60
Soil	3.0 ± 0.5^{b1}	2.8 ± 0.8^{b}	1.6 ± 0.3^{a1}	1.0 ± 0.2^{a}	12.0 ± 1.2^{a}	8.9 ± 1.5^{a}	2.3 ± 0.6^{b}	2.3 ± 0.4^{b}
Soil + CGHE	3.2 ± 0.4^{b}	24.0 ± 1.7^{d}	1.7 ± 0.3^{a}	4.6 ± 1.9^{b}	12.2 ± 1.0^{a}	27.8 ± 2.3^{bc}	2.4 ± 0.2^{b}	7.4 ± 1.4^{cd}
Soil + PA-HE	3.3 ± 0.4^{b}	28.9 ± 2.0^{e}	1.8 ± 0.4^{a}	5.3 ± 2.0^{b}	12.3 ± 1.1^{a}	31.9 ± 2.1^{c}	2.5 ± 0.3^{b}	9.4 ± 1.7^{d}
Soil + RB	3.3 ± 0.3^{b}	27.7 ± 1.8^{de}	1.8 ± 0.4^{a}	4.9 ± 1.5^{b}	12.3 ± 1.1^{a}	31.1 ± 2.8^{c}	2.5 ± 0.4^{b}	8.6 ± 1.5^{d}
Soil + WCDS	3.2 ± 0.4^{b}	21.6 ± 1.3^{d}	1.7 ± 0.5^{a}	4.1 ± 1.4^{b}	12.1 ± 1.2^{a}	26.9 ± 2.0^{b}	2.4 ± 0.6^{b}	6.7 ± 1.3^{c}
Soil + Herbicide	2.8 ± 0.3^{b}	1.5 ± 0.3^{a}	1.4 ± 0.2^{a}	0.83 ± 0.11^{a}	12.1 ± 1.5^{a}	7.2 ± 0.9^{a}	2.0 ± 0.5^{b}	1.2 ± 0.3^{a}
Soil + CGHE + Herbicide	3.0 ± 0.3^{b}	13.2 ± 1.1^{c}	1.6 ± 0.4^{a}	3.7 ± 1.0^{b}	11.9 ± 1.3^{a}	23.0 ± 1.5^{b}	2.4 ± 0.2^{b}	4.9 ± 1.2^{c}
Soil + PA-HE + Herbicide	3.1 ± 0.4^{b}	18.1 ± 1.6^{cd}	1.7 ± 0.4^{a}	4.2 ± 1.5^{b}	12.2 ± 1.2^{a}	25.1 ± 1.4^{b}	2.4 ± 0.2^{b}	6.4 ± 1.1^{c}
Soil + RB + Herbicide	3.1 ± 0.5^{b}	16.8 ± 1.3^{c}	1.7 ± 0.5^{a}	4.1 ± 1.7^{b}	12.2 ± 1.4^{a}	25.1 ± 2.1^{b}	2.3 ± 0.3^{b}	5.9 ± 1.4^{c}
Soil + WCDS + Herbicide	3.0 ± 0.5^{b}	11.3 ± 1.2^{c}	1.6 ± 0.3^{a}	3.4 ± 0.8^{b}	12.0 ± 1.1^{a}	21.4 ± 1.8^{b}	2.3 ± 0.4^{b}	4.0 ± 1.1^{c}

INTF 2-*p*-iodo-3-nitrophenyl formazan; *PNP* *p*-nitrophenol

[1] Column (mean ± standard errors) followed by the same letter(s) are not significantly different ($P < 0.05$)

Similar to the enzymatic activities, the application of organic matter to soil stimulated the soil ergosterol content. The application of herbicide in organic-amended soils decreased the ergosterol content. However, this decrease was lower than for the non-amended herbicide polluted soil. At the end of the incubation period, the ergosterol significantly decreased 31.4% for the Soil + RB + herbicide treatment (compared to the Soil + RB treatment), followed by 33.8% in the Soil + CGHE + herbicide treatment (compared to the Soil + CGHE treatment), 31.9% for the Soil + PA-HE + herbicide treatment (compared to the Soil + PA-HE treatment), and 40.3% for the Soil + WCDS + herbicide treatment (compared with the Soil + WCDS treatment).

4.2 Evolution of Soil Bacterial Community

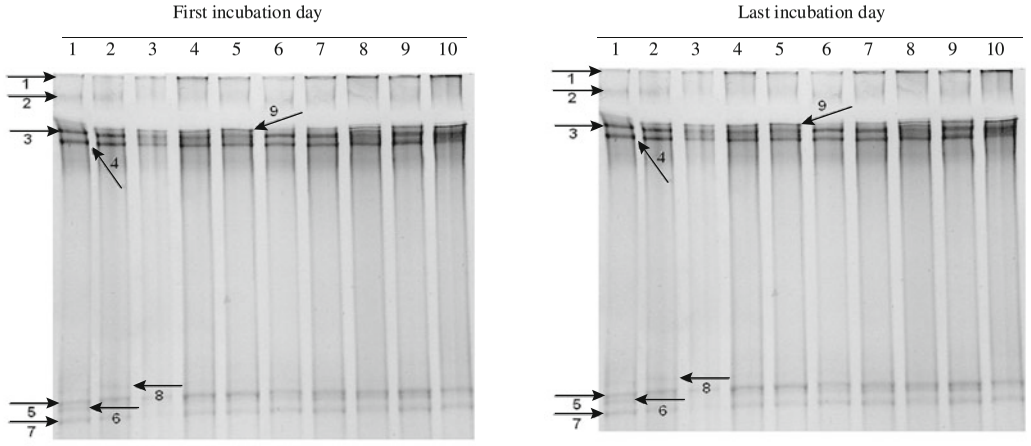
Figure 1 shows the PCR-DGGE fingerprinting and the sequenced bands and corresponding microorganisms for the treatments carried out and the beginning and end of the incubation period. The results show that matching bands were found in all treatments in both the final day as the initial phase. These results indicated that no changes occurred in the soil microbial community. DGGE analysis allowed the detection of 9-band (any band in some samples is not noticeable in this picture). Of these 9 bands were sequenced only 8, as it was impossible to determine the sequence of one of the bands cut. All of them showed a great similarity in sequence (89–98%) compared to the sequences available in databases.

5 Discussion

5.1 Soil Enzymatic Activities and Ergosterol

Our results indicated that Banvel® herbicide caused a toxic effect on some soil enzymatic activities and ergosterol. These results are in agreement with those obtained for other plaguicides such as benomyl, chlorothalonil, dinocap, chlorpyrifos, metsulfuron-methyl, 2,4-D, glyphosate, MCPA, diflufenican, etc. (Edwards 1989; Chen et al. 2001; Pandey and Singh 2004; Zabaloy and Gómez 2008; Černohlávková et al. 2009; Tejada 2009).

The application of organic matter to the unpolluted soil, increased the enzymatic activities and ergosterol. Soil microorganisms degrade organic matter through the production of diverse extracellular enzymes and for this reason after the application of BS to soil, the enzymatic activities and ergosterol increased. These results are in agreement with those of Arancon et al. (2006) and Tejada et al. (2007, 2008) who found an increase in enzymatic activities after the addition of different organic matter to soil. The incorporation of organic amendments to soil influences soil enzymatic activities and ergosterol because the added material may



DGGE Band	Access number	Similarity (%)	Database organism
B1	FJ006903.1	89	*Janthinobacterium sp.*
B2	-	-	Non determinated
B3	AB265150.1	98	*Sphingomonas sp*
B4	FJ392837.1	97	*Lysobacter sp EM 0470*
B5	AE005673.1	88	*Caulobacter crescentus CB15*
B6	DQ337559	98	*Pseudomonas sp BBCT*
B7	FJ373039.1	98	*Bacillus sp 57-3*
B8	AF368755.1	98	*Pseudomonas saccharaphila*
B9	AB264132.1	*95*	*Xantomonadaceae bacterium TDMA-47*

Fig. 1 16S rDNA-DGGE profiles and sequenced bands for all experimental treatments *Line 1*: Control soil; *Line 2*: Soil + CGHE; *Line 3*: Soil + PA-HE; *Line 4*: Soil + RB; *Line 5*: Soil + WCDS; *Line 6*: Soil + Herbicide; *Line 7*: Soil + CGHE + Herbicide; *Line 8*: Soil + PA-HE + Herbicide; *Line 9*: Soil + RB + Herbicide; *Line 10*: Soil + WCDS + Herbicide

contain intra- and extracellular enzymes and may also stimulate microbial activity in the soil (Pascual et al. 1998).

However, the increase in the soil enzymatic activities and ergosterol it is very different depending on the BS type applied to the soil. The protein molecular size distribution of the BS studied indicated that PA-HE had higher content of low molecular weight proteins. According to Parrado et al. (2008), the application to the soil of BS with high contents of low molecular weight proteins is of great agricultural interest, because these proteins can be directly assimilated by soil microorganisms, leading to higher stimulation of soil microbial community. Also, these authors suggested that the BS fat content also plays an essential role in nutrient and low molecular weight proteins absorption by soil microorganisms, indicating that a lower fat content favors nutrient and peptides sorption. Possibly for this reason soil enzyme activity and ergosterol content were higher in soil amended with PA-HE compared with other BS.

It is important to emphasize that after 1 incubation day, no significant effects were observed on the studied parameters in BS-amended soils. In this respect, García-Martinez et al. (2010a, b) found that BS induce a significant increase in soil enzyme activity from the fifth day of its application.

The application of organic matter to the herbicide polluted soil significantly decreased the inhibition of dehydrogenase activity and soil ergosterol content in soil. Our results suggest that the application of PA-HE + BS to the soil decreased to a higher extent this inhibition, compared with other BS applied to soil. The highest content of low molecular weight proteins in PA-HE easily assimilated by microorganisms may be responsible for this fact.

5.2 Evolution of Soil Bacterial Community

Our results suggest that Banvel® do not change the soil bacterial community. These results are in agreement with those obtained by Sigler and Turco (2002), who found that herbicides exert very little effect on soil bacterial communities, and in short-time are not toxic when were applied the correct rates. According to these authors, herbicides are not designed to inhibit soil microorganisms, but produce different effects on them. Also, these authors suggested that the inhibitory effect on soil bacterial community can occur when applications of herbicides were made continuously at the highest doses and not at the recommended doses. Therefore, the recommended dose of Banvel® herbicide may be toxic to soil microorganisms, decreasing their enzymatic activities. However, this dose is not toxic enough to kill bacteria and thus negatively affect the soil bacterial biodiversity.

However, some authors suggest that the impact of herbicides on soil microbial community can vary depending on the type of pesticide type, application rate, soil type, incubation conditions, etc. In this respect, Voets et al. (1974) show that the application of atrazine to soil significantly reduced the number of soil bacteria. However, Weaver et al. (2007) observed a slight increase in the soil bacteria

number after the application of 2,4-D, possibly due that this chemical element can be used as energy source by opportunistic bacteria.

6 Conclusions

It can be concluded that the Banvel® (2,4-D + MCPA + Dicamba) herbicide caused a negative effect on soil enzymatic activities and ergosterol content. However, this herbicide did not cause changes in soil bacterial community. The application of organic matter decreased the toxic action of herbicide on soil enzymatic activities (mainly in dehydrogenase activity) and ergosterol. However, this effect depended on the chemical composition of the organic matter. Comparing the biostimulants studied, PA-HE inhibited further the Banvel® toxic effect on enzyme activities and soil ergosterol, probably due to their higher content of lower molecular weight peptides, easily assimilated by soil microorganisms, and its lower fat content.

References

Abdelhafid R, Houot S, Barriuso E (2000) Dependence of atrazine degradation on C and N availability in adapted and non-adapted soils. Soil Biol Biochem 32:389–401

Arancon NQ, Edwars CA, Bierman CAP (2006) Influences of vermicomposts on field strawberries: Part 2 Effects on soil microbiological and chemical properties. Bioresour Technol 97:831–840

Bandick AK, Dick RP (1999) Field management effects on soil enzymes activities. Soil Biol Biochem 31:1471–1479

Bautista J, Hernández-Pinzón I, Alaiz M, Parrado J, Millán F (1996) Low molecular weight sunflower protein hydrolysate with low concentration in aromatic acids. J Agric Food Chem 44:967–971

Černohlávková J, Jarkovský J, Hofman J (2009) Effects of fungicides mancozeb and dinocap on carbon and nitrogen mineralization in soils. Ecotoxicol Environ Saf 72:80–85

Chen SK, Edwards CA, Subler S (2001) Effects of the fungicides benomyl, captan and chlorothalonil on soil microbial activity and nitrogen dynamics in laboratory conditions. Soil Biol Biochem 33:1971–1980

Cox L, Celis R, Hermosin MC, Becker A, Cornejo J (1997) Porosity and herbicide leaching in soils amended with olive-mill wastewater. Agric Ecosyst Environ 65:151–161

Cox L, Cecchi A, Celis R, Hermosín MC, Koskinen WC, Cornejo J (2001) Effect of exogenous carbon on movement of simazine and 2, 4-D in soils. Soil Sci Soc Am J 65:1688–1695

Delgado-Moreno L, Peña A (2007) Organic amendments from olive-mill wastes as a strategy to modify degradation of sulfonylurea herbicides in soil. J Agric Food Chem 55:6213–6218

Delgado-Moreno L, Peña A (2009) Compost and vermicompost of olive cake to bioremediate triazines-contaminated soil. Sci Total Environ 407:1489–1495

Djajakirana G, Loergensen RG, Meyer B (1996) Ergosterol and microbial biomass relationship in soil. Biol Fertil Soils 22:299–304

Edwards C (1989) Impact of herbicides on soil microcosms. Crit Rev Plant Sci 8:221–253

FAO (1989) Carte Mondiale des Sols Légende Révisée. FAO-UNESCO, Rome

García C, Hernández T, Costa F, Ceccanti B, Masciandaro G (1993) The dehydrogenase activity of soils and ecological marker in processes of perturbed system regeneration. In: XI International Symposium Environmental Biogeochemistry, Salamanca, Spain, pp. 89-100

García-Martínez AM, Tejada M, Díaz A, Bautista JD, Rodríguez B, Parrado J (2010a) Enzymatic production of an organic soil biostimulant from wheat-condensed distiller solubles: Effects on soil biochemistry and biodiversity. Process Biochem 45:1127–1133

García-Martínez AM, Tejada M, Díaz A, Rodríguez-Morgado B, Bautista JD, Parrado J (2010b) Enzymatic vegetable organic extracts as soil biochemical biostimulants and atrazine extenders. J Agric Food Chem 58:9697–9704

Guitián F, Carballas T (1976) Técnicas de Análisis de Suelos. Picro Sacro Editorial, Santiago de Compostela, Spain

Heuer H, Smalla K (1997) Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In: Van Elsas JD, Trevors JT, Wellington EMH (eds) Modern soil microbiology. Marcel Dekker, New York, pp 353–373

Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72

MAPA (1986) Métodos Oficiales de Análisis. Tomo I. Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Spain

Pandey S, Singh DK (2004) Total bacteria and fungal population after chlorpyrifos amd quinalphos treatments in groundnut (*Arachis hypogaea* L.) soil. Chemosphere 55:197–205

Parrado J, Bautista J, Romero EJ, García-Martínez AM, Friaza V, Tejada M (2008) Production of a carob enzymatic extract: potencial use as a biofertilizer. Bioresour Technol 99:2312–2318

Pascual JA, Hernández T, García C, Ayuso M (1998) Enzymatic activities in an arid soil amended with urban organic wastes: Laboratory experiment. Bioresour Technol 64:131–138

Sigler WV, Turco RF (2002) The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis. Appl Soil Ecol 21:107–118

Soil Survey of England and Wales (SSEW) (1982) Soil survey laboratory methods, Tech. Monogr. vol 6, Harpenden, UK

Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenol phosphate in assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Tejada M (2009) Evolution of soil biological properties after addition of glyphosate, diflufenican and glyphosate + diflufenican herbicides. Chemosphere 76:365–373

Tejada M, Hernández MT, García C (2007) Application of two organic wastes in a soil polluted by lead: Effects on the soil enzymatic activities. J Environ Qual 36:216–225

Tejada M, Moreno JL, Hernández MT, García C (2008) Soil amendments with organic wastes reduce the toxicity of nickel to soil enzyme activities. Eur J Soil Biol 44:129–140

Voets JP, Meershman P, Verstraete W (1974) Soil microbiological and biochemical effects of long-term atrazine applications. Soil Biol Biochem 6:149–152

Weaver MA, Krutz LJ, Zablotowicz RM, Reddy KN (2007) Effects of glyphosate on soil microbial communities and its mineralization in a Mississippi soil. Pest Manage Sci 63:388–393

Yeomans JC, Bremner JM (1988) A rapid and precise method for routine determination of organic carbon in soil. Commun Soil Sci Plant Anal 19:1467–1476

Zabaloy MC, Gómez MA (2008) Microbial respiration in soils of the Argentine Pampas after metsulfuron-methyl, 2, 4-D and glyphosate treatments. Commun Soil Sci Plant Anal 39:370–385

Effect of Natural Vegetation Strips and Herbicides on Enzyme Activities and Bacterial Diversity in Olive-Orchard Systems

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Abstract The use of vegetation strips in mountainous olive-orchards is of major importance in reducing soil erosion and minimizing the adverse effects of the herbicides transfer to surface waters. This study was carried out to evaluate the effects of natural vegetation strips and herbicides on soil enzyme activities and changes in bacterial community structures. Thus, four enzyme activities were determined in soil samples from different zones in an integrated olive-orchard system on slopes. Changes in microbial population were analysed by using PCR assays to target 16S rDNA genes and denaturing gradient gel electrophoresis. Soil samples were collected at a depth of 0–5 cm from the experimental plot upslope and downslope: (1) under the vegetation strips before and after application of glyphosate, (2) from bare soil between the strips and under the olive trees treated with oxyfluorfen and glyphosate, (3) native soil from a non-cultivated area. Enzyme activities and bacterial diversity were lower in soils from the olive-orchard system than in the native soil. In the olive-orchard system, the enzyme activities in soils from the strips were higher than in bare soils and soils under trees. In the soils under strips, the level of β-glucosidase and phosphatase activities increased after the application of glyphosate while dehydrogenase and urease activities decreased. The presence of natural vegetation in the strips as well as herbicide application modified the bacterial community structure and the biodiversity indices. Soil sampled in the downslope strip and after glyphosate addition showed the highest biodiversity values and dominance indices.

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1 Introduction

Given its economic, social and ecological importance, the cultivation of olive trees (*Olea europaea* L.) is a major agricultural activity in the Mediterranean basin. Spain, with 2,450,000 ha under cultivation, is the largest producer and exporter of olives and olive oil in the world. This crop is mainly located in the Andalusian region, where many orchards are found on hilly and mountainous land and poor soils. Soil erosion, which is one of the main problems with olive orchards in these areas, is aggravated by conventional tillage to reduce competition between trees and weeds for water uptake. In recent years, alternative and sustainable methods have been introduced to protect soil against erosion (Pastor et al. 1997). These methods include mechanical weed control, reduced tillage with or without weed control and the use of natural or sowed plant-cover strips, between rows of olive trees, introduced in the autumn and eliminated using herbicides in the spring.

The use of herbicides, which is essential for eliminating unwanted weeds and for a productive agriculture, has steadily increased. The intensive use of herbicides and the unintended consequences of their multiple applications has become an environmental concern due to the potential hazardous effects of these chemicals on the biological processes of soil and non-target organisms. Pre-and post-emergence herbicides, such as oxyfluorfen and glyphosate, are commonly used to control a wide range of weeds in extensive crop systems throughout the world, which include olive orchards under conventional, minimal and non-tillage management systems.

Oxyfluorfen and glyphosate are among the most common herbicides used by olive growers in Spain. Oxyfluorfen, a diphenyl ether herbicide (2-chloro-α,α,α,-trifluoro-*p*-tolyl-3-ethoxy-4-nitrophenyl ether) was introduced to control weeds in soybean, sugarcane, tea, cabbage, and garlic crops in Taiwan in 1979. The dissipation and mobility of oxyfluorfen have been subjected to very little study. Yen et al. (2003) have found that the half life of oxyfluorfen in soils ranges from 72 to 160 days and it is confined to the top layer of soil at a depth of a few centimetres. Oxyfluorfen could be converted into amino and acylamino derivatives through microbial activity (Chakraborty et al. 2002). Glyphosate is a non-selective systemic herbicide with a moderate persistence in soil and is mainly degraded by co-metabolic microbial processes (Strange-Hansen et al. 2004; Accinelli et al. 2005).

During herbicide application, much of these chemicals accumulate in the top-soil where most of the microbiological activities occur. Microorganisms degrade a variety of carbonaceous substances (including herbicides) to obtain energy and nutrients for their metabolisms. The microbial biomass content therefore increases, which has a positive effect on the transformation of plant nutrients in soils (Sandhu et al. 1990; Das et al. 2003). However, the adverse effect of herbicides on the growth and activity of beneficial microorganisms have also been reported (Selvamani and Sankaran 1993). These negative effects of herbicides on soil microorganisms and the associated transformation of plant nutrients also vary depending on the type of herbicide, microorganism and management practice.

Vegetation cover strips in the crop fields downslope can act as buffer zones that minimize the herbicide's adverse effects due to the multiplicity of physical, chemical and biological processes occurring in these areas. This could lead to an increase in microorganism activity and microbial biomass, as well as increased pesticide biodegradation (Benoit et al. 2000; Krutz et al. 2005).

It is well known that bacteria, fungi and their extracellular enzymes (Tabatabai 1994) are mainly responsible for biological changes that make nutrients available to plants and sustain soil functions. Soil microbial communities also play a critical role in soil recovery after degradation (Bending et al. 2000; Breure 2005). Thus, the measurement of the characteristics of the microbial community provides invaluable information on soil quality and sustainable agricultural management. One of the problems with using biological indicators is to determine which indicator responds to specific soil treatments and pollutants. To overcome this problem, the study of general biochemical properties such as microbial biomass carbon (Brookes 1985), eco-physiological quotients (Anderson and Domsch 1993) as well as specific hydrolytic soil enzymes relating to C, N and P cycles (Nannipieri et al. 1990) has been suggested. In this context, different molecular techniques have successfully been used to explore microbial diversity, identify microorganisms (Muyzer et al. 1993; Muyzer and Ramsing 1995; Borneman and Triplett 1997; Clement et al. 1998; Tiquia et al. 2002) and to investigate changes in soil microbial communities caused by organic and inorganic pollutants (Vivas et al. 2008).

The aim of this study is to evaluate the changes in enzyme activities and bacterial community structures in soils from olive-orchard systems on hill slopes, under integrated management with natural vegetation strips and treated with oxyfluorfen and glyphosate. To achieve this, dehydrogenase, β-glucosidase, alkaline phosphatase and urease enzyme activities were analyzed in soil located in different areas. The soil DNA from each soil sample was extracted, amplified with PCR technique targeting 16S rDNA genes, and its soil bacterial diversity analysed using denaturing gradient gel electrophoresis (DGGE).

2 Materials and Methods

2.1 Site Description and Soil Characteristics

The field experiment was conducted on an olive-orchard in the La Parra farm (Deifontes, Granada) in Southeast Spain (UTM coordinates X = 446,248.2; Y = 4,138,725.6) 835 m above sea level (ASL). Average annual precipitation is 460 mm, and over 85% of the total rainfall occurs between October and April. The experimental plot, with a Calcaric Cambisol (FAO 1989) located on a hillslope (5% gradient), covered an area of 338 m^2 (26 × 13 m) and contained

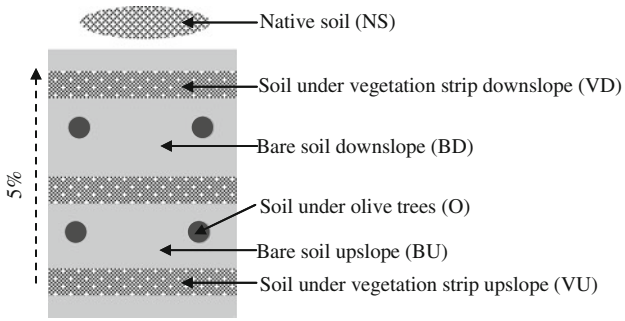

Fig. 1 Experimental plot showing the different soil samples

4 trees. This plot is part of an olive orchard containing 16-year-old olive trees (*Olea europaea* cv. Picual) at 9 × 9 m intervals. Oxyfluorfen and glyphosate were applied at 0.5 and 1 L ha^{-1} and 1.5 and 3 L ha^{-1}, respectively. The vegetation strips were treated with glyphosate at the end of spring, and a mixture of oxyfluorfen and glyphosate was applied to the bare soil and under the olive trees in winter.

2.2 Soil Sampling

Soil samples, in triplicate, were collected at a depth of 0–5 cm upslope and downslope in the experimental plot (Fig. 1): (1) under the natural vegetation strips upslope (VU) and downslope (VD) before the application of glyphosate, (2) under the natural vegetation strips 10 days after the application of glyphosate (g) (VUg and VDg), (3) from bare soil (B) between the vegetation strips treated with oxyfluorfen and glyphosate (og) upslope (BUog) and downslope (BDog) and iv) under the olive trees treated with oxyfluorfen and glyphosate (Oog). The native soil used for control purposes came from a non-cultivated area under oak trees near the experimental plot (NS). The three soil samples collected from each different zones were mixed in order to obtain a representative sample. Moist field sub-samples were kept at 4°C and processed within two months. Air-dried sub-samples were sieved (<2 mm) for chemical analysis.

2.3 Chemical Analysis

Particle-size distribution was determined by the pipette method (MAPA 1986). The pH and electrical conductivity was measured with a glass electrode using a 1:2.5 sample-water ratio. For the determination of $CaCO_3$, CO_2 released by addition of hydrochloric acid was measured by a calcimeter. The cation exchange

capacity (CEC) was determined by the ammonium acetate (pH 7) displacement method (MAPA 1986). The total organic carbon (OC) content was determined using the Walkley–Black wet dichromate oxidation method, and total N was determined by the macro-Kjeldahl digestion method (MAPA 1986). Available P and K were determined after extraction of soil with sodium bicarbonate and ammonium acetate, respectively (MAPA 1986). Water-soluble carbon (WSC) was determined in a 1:10 (w:v) water extract using potassium dichromate and sulphuric acid digestion (Sims and Haby 1971).

2.4 Enzyme Activities

Dehydrogenase activity (DH-ase) was determined by the reduction of 2-*p*-iodo-3-nitrophenyl 5-phenyl tetrazolium chloride to iodonitrophenyl formazan, which was measured in a spectrophotometer at 490 nm (García et al. 1997). *β*-Glucosidase (*β*-Glu) and phosphatase (Phos), using *p*-nitrophenyl-*β*-D-glucanopyranoside (PNG) and *p*-nitrophenyl phosphate (PNPP) as substrates respectively, were measured according to Nanniperi et al. (1982). The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm. Urease activity (Ure) was determined using urea as substrate and the product, NH_4^+, was measured using an ammonium-selective electrode (Nannipieri et al. 1982).

2.5 Soil-DNA Extraction and PCR-DGGE Analysis

The total DNA was extracted from soil sub-samples of 250 mg using the bead-beating method following the manufacturer's instructions for the MoBio Ultra-Clean Soil DNA Isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA). The DNA samples were checked for concentration and quality using the Nano-Drop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware; USA).

PCR-DGGE analysis was carried targeting 16S rDNA using the P1, P2 and P3 primers (TIB®MOLBIOL, Berlin, Germany) to amplify the V3 hypervariable region of 16S rDNA genes. The P3 primer contains the same sequence as P1 (Table 1) but with an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5′ end (Muyzer et al. 1993).

Two successive amplifications were carried out under the following conditions: a hot start of 5 min at 94°C; 19 cycles consisting of 94°C for 15 s, 65–55°C for 15 s, decreasing the temperature by 0.5°C each cycle (touchdown), and 72°C for 30 s; 14 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s; and a final 10 min stage at 72°C. The total reaction mixture for the first PCR consisted in the following ingredients: 1 μL volume (approx. 10 ng) of extracted DNA, 1 μM primer P1, 1 μM primer P2, 10 μL Eppendorf® Master Mix (2.5X) and sterile Milli-Q water to a final volume of 25 μL. The second amplification was

Table 1 Nucleotide sequences of the used primers

Primer	Nucleotide sequence
1	5′-CCTACGGGAGGCAGCAG-3
2	5′-ATTACCGCGGCTGCTGG-3′
3	5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′

performed by using 1 μl of the product of the first reaction as a template, where primers P2 and P3 were used under conditions similar to those described above. PCR products were analysed by electrophoresis in 2% agarose gels stained with ethidium bromide.

DGGE analyses were conducted using 20 μl of this latter PCR product loaded into a 40–65% urea-formamide-polyacrylamide gel. An INGENYphorU System (Ingeny International BV, The Netherlands) was run at 75 V for 17 h at 58°C to separate the fragments. Gels were stained with the Bio-Rad Silver Stain according to the standard DNA-staining protocol and photographed under UV light ($\lambda = 254$ nm) using an UVItec Gel Documentation system (UVItec Ltd, Cambridge, UK). The band patterns were compared in different lanes using UVImap Analysis software (UVItec Ltd, Cambridge, UK). The lanes were normalized in order to contain the same amount of total signal after background subtraction, and the gel images were straightened and aligned to produce a densitometric curve. Band positions were converted to Rf values of between 0 and 1, and profile similarity was calculated by determining the Nei and Li similarity coefficients (Nei and Li 1979) for the total number of lane patterns from the DGGE gel. The similarity coefficients thus calculated were then used to construct a dendrogram by applying the unweighted pair-group method with arithmetical averages (UPGMA).

The species richness on DGGE gels *(R)* was calculated as the mean number of bands presents (Yáñez-Ruiz et al. 2007). The Shannon (H') and Simpson (D) indices were used to evaluate the biodiversity and predominance of microbial groups in the soil samples (Magurran 1988). For these analyses, each band was presumed to represent the ability of that bacterial species to be amplified. The intensity of the bands was reflected as peak heights in the densitometric curve. The Shannon H' and Simpson D indexes were calculated from the following equations:

$$H' = -\sum(P_i \log P_i); \quad D = \sum P_i^2$$

where, $Pi = ni/N$; n_i = height of peak and N = sum of all peak heights in the curve.

2.6 Statistical Analysis

All data measured in triplicate was subjected to variance analysis, and Duncan's Multiple Range Test was used on a *post-hoc* basis to separate the means with an overall significance level of 0.05. A correlation matrix of the data was also

calculated for determining the relationship between chemical and biochemical parameters. The significant levels reported are based on Student's distribution. All statistical analyses were conducted using STATGRAPHICS Plus (Statistical Graphics Corp., Princenton, NJ).

3 Results

The clay, CEC, OC, available K and WSC contents in the soils under the vegetation strips collected downslope (VD and VDg) were significantly higher than those found under vegetation strips upslope (VU and VUg) (Table 2). After the application of glyphosate, the VUg and VDg soils showed significantly lower OC, N, and CEC values but higher EC than the corresponding soil samples not treated with ghyphosate (VU and VD). Furthermore, bare soil samples from upslope and downslope BUog and BDog soils had lower OC, N and EC values than those soils sampled from under the vegetal strips (V). These lower values could be mainly explained by the absence of vegetation on the bare soil. In the soil samples under the olive trees from the Oog soil, only WSC contents were higher than those for the V and B soil samples. Native soil samples (NS) recorded the lowest carbonate content but the highest clay, CEC, OC, N, available K and WSC contents in comparison with the other sampled soils.

Dehydrogenase activity (DH-ase) ranged from 0.03 to 0.2 μg INTF g^{-1} h^{-1} (Fig. 2). This enzyme activity was closely correlated ($P < 0.05$) with the OC, N and urease activity of the soils (Table 3). Native soil NS and soil under the vegetation strip not treated with glyphosate (VD and VU) showed the highest DH-ase values. The application of glyphosate to the natural vegetation strips, VUg and VDg, significantly reduced this enzyme activity. This reduction was comparatively more pronounced in the soil samples taken from upslope strips (60%). The bare soils, BU and BD, treated with oxyfluorfen and glyphosate showed the lowest DH-ase values.

Total β-glucosidase and phosphatase activities, which were correlated between themselves (Table 3), were lower in the soils without vegetal cover treated with oxyfluorfen and glyphosate (BUog, BDog and Oog) (Fig. 2). However, levels of these hydrolases were higher in soils under natural vegetal strips, especially after the application of glyphosate (VUg and VDg). These results suggest that the glyphosate herbicide influences these microbial activities although this was not the case for oxyfluorfen. Furthermore, the slope of the soil also had a noticeable impact on the phosphatase activities measured in vegetated strips, where VD and VDg showed higher levels of activity than VU and VUg.

In all the soil samples from the agricultural plot, urease activity was significantly lower than in the native soil (NS) (Fig. 2). After glyphosate application, this enzymatic activity was not significantly affected, unlike with the other hydrolytic enzymes. In bare soils treated with oxyfluorfen and glyphosate (BUog and BDog), hardly any of this enzyme activity was detected.

Table 2 Selected properties of the different soil from the experimental plot

	Sand (g kg^{-1})	Silt	Clay	pH	EC (µS cm^{-1})	$CaCO_3$ (mg kg^{-1})	CEC ($cmol_c$ kg^{-1})	OC (g kg^{-1})	N (g kg^{-1})	Available		WSC (mg kg^{-1})
										P (mg kg^{-1})	K (mg kg^{-1})	
VU	120^{cd}	590^{a}	290^{d}	8.4^{ab}	102^{bc}	550^{a}	19^{d}	7.6^{c}	1.1^{b}	12^{cd}	285^{e}	190^{e}
VD	140^{b}	490^{de}	370^{b}	8.5^{a}	101^{bc}	450^{b}	24^{b}	9.8^{b}	1.1^{b}	13^{c}	400^{c}	223^{d}
VUg	180^{a}	540^{bc}	280^{d}	8.5^{a}	136^{a}	590^{a}	17^{e}	6.5^{de}	0.9^{d}	9^{cd}	280^{e}	138^{f}
VDg	130^{bc}	560^{ab}	310^{c}	8.4^{ab}	144^{a}	480^{b}	22^{c}	7^{cd}	1^{c}	9^{cd}	330^{d}	400^{c}
BUog	110^{d}	540^{bc}	350^{b}	8.7^{a}	93^{cd}	560^{a}	19^{d}	4.6^{f}	0.7^{e}	14^{c}	265^{e}	200^{e}
BDog	130^{bc}	480^{e}	390^{b}	8.7^{a}	86^{d}	460^{b}	22^{c}	5.7^{e}	0.9^{d}	11^{cd}	285^{e}	118^{f}
Oog	120^{cd}	520^{cd}	360^{b}	8.5^{a}	111^{b}	470^{b}	22^{c}	6.2^{de}	0.9^{d}	34^{a}	464^{b}	594^{b}
NS	120^{cd}	400^{f}	480^{b}	8.2^{b}	142^{a}	280^{c}	31^{a}	22.7^{a}	2.5^{a}	23^{b}	1013^{a}	629^{a}

V soil under the vegetation strip, *B* bare soil, *O* soil under olive trees, *NS* native soil, *U* and *D* samples from upslope and downslope soil, respectively. Samples treated with glyphosate were marked g and those with oxyfluorfen were marked o. In each parameter, different letters indicate significant differences ($P < 0.05$)

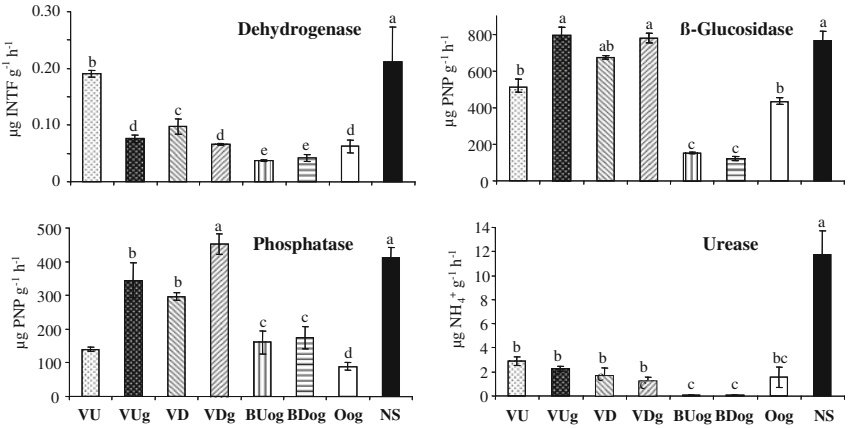

Fig. 2 Soil enzyme activities in samples from the experimental plot. The letters above the columns indicate the least significant differences ($P < 0.05$)

Table 3 Correlation coefficients between soil chemical properties and soil enzymes activities in samples from the experimental plot. The correlation values are significant at $P < 0.05$

	pH	CE	CEC	OC	N	P	K	WSC	DH-ase	*β*-Glu	Phos	Ure
pH	1.00											
CE	–	1.00										
CEC	–	–	1.00									
OC	–	–	0.89	1.00								
N	–	–	0.87	0.99	1.00							
P	–	–	–	–	–	1.00						
K	–	–	0.91	0.96	0.95	–	1.00					
WSC	–	–	–	–	–	0.71	0.78	1.00				
DH-ase	–0.93	–	–	0.77	0.79	–	–	–	1.00			
ß-Glu	–	0.86	–	–	–	–	–	–	–	1.00		
Phos	–	0.81	–	–	–	–	–	–	–	0.77	1.00	
Ure	–	–	–	0.97	0.98	–	0.94	–	0.82	–	–	1.00

The structure of the bacterial-community profiles was determined on the basis of amplified soil DNA from DGGE (Fig. 3a and b). Two main clusters were observed using UPGMA dendrogram analysis. The first cluster was found in the soil samples taken from under the vegetation strip (VU, VD, VUg, VDg) and under olive trees (O). Nevertheless, we found changes in the structure of the bacterial community (Fig. 3a) due to the application of glyphosate to the vegetal cover (VUg, VDg). The soil under the vegetation strip downslope before application of glyphosate (line VD) and the soil under the vegetation strip upslope after application of glyphosate (line VUg) were very similar, as shown by the homology dendogram (UPGMA). Although the soil under the olive trees was grouped in the same cluster, the profiling was quite different (line Oog) when compared with soil from vegetal strips. The second cluster included both bare soils treated with

Table 4 Species richness (R), Shannon (H') and Simpson (D) diversity indexes values from DGGE profiles of soil samples from the experimental plot

	VU	VUg	VD	VDg	BUog	BDog	Oog	NS
R	29	24	24	39	23	31	30	45
H'	3.260	3.070	3.076	4.960	2.987	3.055	3.510	4.440
D	0.040	0.048	0.049	0.083	0.052	0.041	0.091	0.050

oxyfluorfen and glyphosate(BUog and BDog) and the native soil (line NS) which showed slight homology.

The number of species can be estimated on the basis of the total number of bands found in the DGGE profile, as each band in the DGGE gel derives from a single phylogenetically different population. The richness index *(R)* was calculated for all the DGGE patterns (Table 4). Richness ranged from significantly higher values for the native soil, with an R value of 45, to low values for the bare soil upslope treated with oxyfluorfen and glyphosate (BUog) with an R value of 23.

The general biodiversity index (H′) and the concentration of dominance (D) were calculated according to the band numbers and the bands' relative intensities on a gel track (Table 4). The natural soil (NS) had the widest microbial diversity (4.440) associated with a low concentration of dominance (0.050). Nevertheless, the soil under the vegetation strip downslope after application of glyphosate (VDg) exhibited the highest diversity index (4.960) associated with a high dominance index (0.083) followed by the soil under olive trees. The bare soil samples collected upslope and treated with oxyfluorfen and glyphosate (BUog) showed the lowest biodiversity (2.987) and relative high dominance (0.052) indices. Both soil biodiversity and dominance concentration therefore varied significantly with glyphosate applications, the presence or absence of natural vegetation cover and the position of the soil sample on the experimental plot slope.

4 Discussion

Soil enzyme activities were influenced by the changes recorded in the properties of the soil samples collected from different locations in this integrated olive-orchard system. Despite the scarce correlation between enzyme activities and soil properties (Table 3), it was observed that the cultivation systems applied reduced the organic carbon content of the soil in the olive-orchard. This reduction reached the 77% in the bare soil and 58% under the vegetative strips in the native soil (Table 2). The relatively higher level of the soil OC content under vegetal cover could stimulate microbial activity, resulting in a higher level of dehydrogenase activity than in the bare soil. Nevertheless, the water-soluble carbon, which is closely related to microbial activity, showed an opposite trend related to the DH-ase activity in the soil under vegetation strips (Table 2 and Fig. 2). On the whole, DH-ase activity levels were very low in all cases, especially in the bare soil with the lowest OC, N, and WSC content.

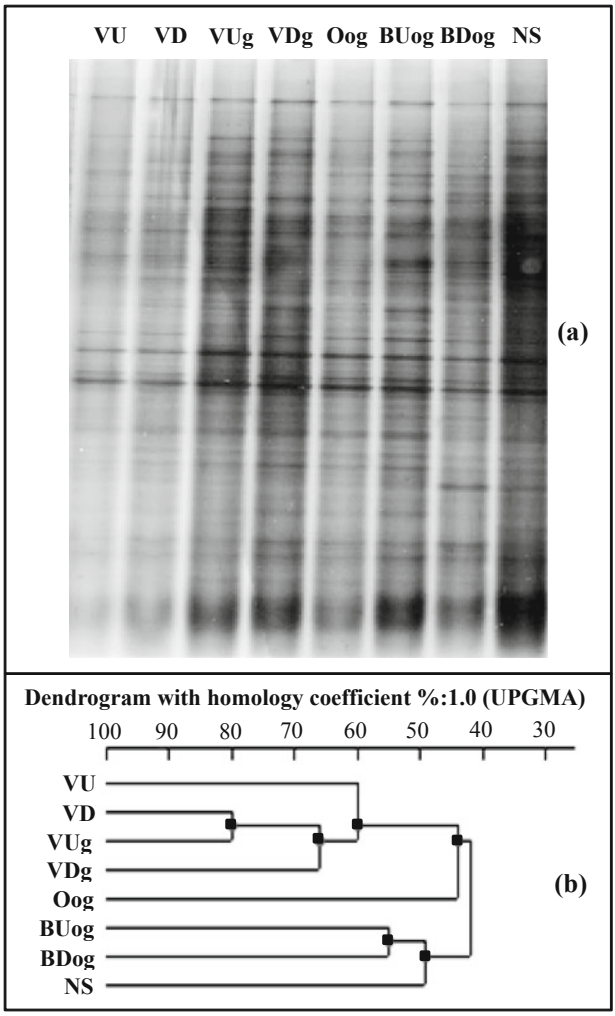

Fig. 3 DGGE profiles of bacterial communities (**a**), and cluster analysis (UPGMA, Nei and Li's similarity coefficients) (**b**), of molecular banding patterns generated by DGGE of soil samples from experimental plot

Dehydrogenase activity is often used as a measure of any disruption caused by pesticides, trace elements or management practices (Reddy and Faza 1989; Wilke 1991; Frank and Malkomes 1993) as well as a direct measure of microbial activity in the soil (Skujins 1978; Trevors 1984). Dehydrogenase which varied according to pesticide doses, decreased at high dosages (Baruah and Mishra 1986). Accinelli et al. (2002) found that both gluphosinate-ammonium and glyphosate at doses ranging from 20 to 200 mg kg^{-1} soil had a positive effect on microbial activity as measured by respiration and DH-ase. On the other hand, Lupwayi et al. (2007) have observed a

decrease in soil DH-ase after addition of glyphosate. In our study, the application of glyphosate to the natural vegetation inhibited dehydrogenase activity, which caused a reduction in biological activity in the soil under the vegetal strips.

As hydrolases are inducible enzymes, their activity is regulated by the presence of available substrates (Burns 1982). The increased activity of β-glucosidase and phosphatase in soils under vegetation strips could therefore correspond to a higher level of hydrolytic activity caused by nutrient availability (phosphates and carbon compounds) for microorganisms and plants (Mader et al. 2002). Also, the increase in both enzyme activities after the application of glyphosate indicates that this herbicide may stimulate population responsible for the production of these enzyme activities, as described by Sannino and Gianfreda (2001), Accinelli et al. (2002) and Araujo et al. (2003). Nevertheless, in the bare soil and under the olive trees, the application of oxyfluorfen and glyphosate seems to have an inhibitory effect on soil microbial activities. In mineral soils, Beste et al. (1983) reported that microbial degradation was not a major cause of oxyfluorfen's breakdown and half life was of 30–40 days. In organic soils, Frank et al. (1991) have shown that no oxyfluorfen residues were found in drainage water from the treated area.

The absence of urease activity in bare soil and its low level of activity in the other types of soil, with the exception of native soil, is not easily explained. It is commonly accepted that every soil has a stable level of urease activity that is determined by the ability of its constituents to provide protection to this enzyme (Zanthua and Bremner 1976). Other studies have suggested that a considerable amount of an enzyme's total activity (including urease) in soil can be ascribed to an enzymatic fraction located in cells or attached to or within cell debris (Nannipieri 1994; Sannino and Gianfreda 2001). Thus, the low soil OC concentration and low DH-ase could explain these very low urease activities especially in bare soils.

Glyphosate is an organophosphonate that can be used as a source of P, C or N by either gram-positive or gram-negative bacteria (van Eerd et al. 2003). Ratcliff et al. (2006) have reported a transient increase in fungal propagules and no effect on culturable bacteria after a 50 mg kg^{-1} addition of glyphosate, while a 100-fold higher dosage enriched culturable bacteria and the bacterial:fungal ratio. It is likely that this herbicide provides nutrients for heterotrophic bacteria growth. This phenomenon was even more powerful in soils with a history of glyphosate application (Zabaloy et al. 2008; Araujo et al. 2003). On the other hand, in soils with no previous applications, when treated with a low-dose of the herbicide the fungal counts were higher. Assuming a bacterial role in glyphosate dissipation, Gimsing et al. (2004) found a close correlation between glyphosate mineralization rates and *Pseudomonas spp*. counts for five different Danish soils. Two soils with high glyphosate mineralization rates also showed high CFU counts (Gimsing et al. 2004). Weaver et al. (2007) have observed a transient increase in the abundance of the 16:1ω7c fatty acid, a biomarker associated with gram-negative bacteria, in response to the in vitro addition of glyphosate (47 mg kg^{-1}). Using 16S rDNA-PCR coupled with DGGE, Accinelli et al. (2007) observed that a 1 mg kg^{-1} dose of glyphosate had no detectable effect on soil microbial community structure four weeks after treatment. In our study, the application of glyphosate to natural

vegetation increased the concentration of dominance (D) and the general biodiversity index (H'), which correlated with the increase in glucosidase and phosphatase. The effects of glyphosate on bacterial density are clearly dose-dependant and highly temporal and could be explained by a rapid enrichment of opportunistic copiotrophic bacteria that use the compound as a nutrient and/or C (Ratcliff et al. 2006). Further analyses are required to explain the divergence observed in the bacterial community structure among the different soil treatments and locations.

5 Conclusions

The information presented in this multidisciplinary study, which integrates physical, chemical, biochemical and microbiological analysis of soils, provides a sound basis for the evaluation of the incidence of natural vegetation strips and herbicides application under an olive-orchard management system on hillslopes.

Although the levels of soil enzymes activities were low, due to scarce soil organic content, they, in general, were enhanced in soils under the natural vegetation strips.

Glyphosate, with a chemical structure more labile than oxyflourfen, appears to be a C and P source for soil microorganisms, and, therefore, β-glucosidase and phosphatase activities increased after its application.

The presence of the natural vegetation strips, as well as the application of glyphosate or oxyfluorfen modified the soil bacterial community structure and the biodiversity and dominance indexes. These indexes were higher after elimination with glyphosate of the natural vegetation strips located at down slope.

The methodological approach was sufficiently sensitive to detect changes in biochemical parameters and in diverse bacterial microbial communities among soil samples subjected to different treatments, providing information on the extent and duration of herbicide impact and natural vegetation attenuation in a olive-orchard management system on hillslopes.

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References

Accinelli C, Screpanti C, Dinelli G, Vicari A (2002) Short-time effects of pure and formulated herbicides on soil microbial activity and biomass. Int J Environ Anal Chem 82:519–527

Accinelli C, Koskinen WC, Seebinger JD, Vicari A, Sadowsky MJ (2005) Effects of incorporated corn residues on glyphosate mineralization and sorption in soil. J Agric Food Chem 53:4110–4117

Accinelli C, Koskinen WC, Becker JM, Sadowsky MJ (2007) Environmental fate of two sulfonamide antimicrobial agents in soil. J Agric Food Chem 55:2677–2682

Anderson TH, Domsch KH (1993) The metabolic quotient CO_2 (qCO_2) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils. Soil Biol Biochem 25:393–395

Araujo ASF, Abarkeli RB, Monteiro RTR (2003) Effect of glyphosate an the microbial activity of two Brazilian soils. Chemosphere 52:799–804

Baruah M, Mishra RR (1986) Effect of herbicides butachlor, 2, 4-D and oxyfluorfen on enzyme activities and CO2 evolution in submerged paddy field soil. Plant Soil 96:287–291

Bending GD, Putland C, Rayns F (2000) Changes in microbial community metabolism and labile organic matter fractions as early indicators of the impact of management on soil biological quality. Biol Fertil Soils 31:78–84

Benoit P, Barriuso E, Vidon P, Réal B (2000) Isoproturon movement and dissipation in undisturbed soil cores from a grassed buffer strip. Agronomie 20:297–307

Beste CE, Humburg NE, Kempen HM, Miller GR, Radke RO, Riggleman JD, Stritzke JF (1983) Herbicide Handbook of the Weed Science Society of America, 5th edn, Weed Society of America. Champaign Ill, p 68130

Borneman J, Triplett WE (1997) Rapid and direct method for the extraction of RNA from soil. Soil Biol Biochem 29:1621–1624

Breure AM (2005) Ecological soil monitoring and quality assessment. In: Doelman P, Eijsackers HJP (eds) Vital soil: function value and properties. Elsevier Amsterdam, The Netherlands, pp 281–305

Brookes PC (1985) Microbial biomass and activity measurements in soil. J Sci Food Agric 36:269–271

Burns RG (1982) Enzyme activity in soil: location and possible role in microbial ecology. Soil Biol Biochem 14:423–427

Chakraborty SK, Bhattacharyya A, Chowdhury A (2002) Degradation of oxyfluorfen by *Azotobacter chroococcum* (Beijerink). Bull Environ Contam Toxicol 69:203–209

Clement BG, Kehl LE, DeBord KL, Kitts CL (1998) Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for comparison of complex bacterial communities. J Microbiol Meth 31:135–142

Das AC, Debnath A, Mukherjee D (2003) Effect of the herbicides oxadiazon and oxyfluorfen on phosphates solubilizing microorganisms and their persistence in rice fields. Chemosphere 53:217–221

FAO (1989) Mapa Mundial de Suelos Leyenda Revisada. FAO, Rome, Italy

Frank T, Malkomes HP (1993) Influence of temperature on microbial activities and their reaction to the herbicide Goltix in different soils under laboratory conditions. ZBL Mikrobiol 148:403–412

Frank R, Clegg S, Ritcey G (1991) Disappearance of oxyfluorfen (Goal) from onions and organic soils. Bull Environ Contam Toxicol 46:485–491

García C, Hernández T, Costa F (1997) Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun Soil Sci Plant Anal 28:123–134

Gimsing AL, Borggaard OK, Jacobsen OS, Aamand J, Sørensen J (2004) Chemical and microbiological soil characteristics controlling glyphosate mineralisation in Danish surface soils. Appl Soil Ecol 27:233–242

Krutz LJ, Senseman SA, Zablotowicz RM, Matocha MA (2005) Reducing herbicide runoff from agricultural fields with vegetative filter strips: a review. Weed Sci 53:353–367

Lupwayi NZ, Hanson KG, Harker KN, Clayton GW, Blackshaw RE, O'Donovan JT, Johnson EN, Gan Y, Irvine RB, Monreal MA (2007) Soil microbial biomass, functional diversity and enzyme activity in glyphosate-resistant wheat-canola rotations under low-disturbance direct seeding and conventional. Soil Biol Biochem 39:1418–1427

Mader P, Fliebach A, Dubois D, Gunst L, Fried P, Niggli U (2002) Soil fertility and biodiversity in organic farming. Science 296:1694–1697

Magurran AE (1988) Ecological diversity and its measurement. Princeton University Press, Princeton, NJ, USA

MAPA (1986) Métodos Oficiales de Análisis. Tomo III. Plantas, productos orgánicos fertilizantes, suelos, agua, productos fitosanitarios y fertilizantes inorgánicos. Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain

Muyzer G, Ramsing NB (1995) Molecular methods to study the organization of microbial communities. Water Sci Technol 32:1–9

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analyses of polymerase chain reaction-amplified genes for 16S rRNA. Appl Environ Microbiol 59:695–700

Nannipieri P (1994) The potential use of soil enzymes as indicators of productivity, sustainability and pollution. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Management in sustainable farming systems. CSIRO, East Melbourne, Australia, pp 238–244

Nannipieri P, Ceccanti B, Conti C, Bianchi D (1982) Hydrolases extracted from soil: their properties and activities. Soil Biol Biochem 14:257–263

Nannipieri P, Grego S, Ceccanti B (1990) Ecological significance of the biological activity in soil. In: Bollag J-M, Stotzky G (eds) Soil biochemistry, vol 6. Marcel Dekker, New York, pp 293–355

Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Pastor M, Castro J, Vega V, Humanes MD (1997) Sistemas de manejos de suelo. In: Barranco D, Fernández RE, Rallo L (eds) El Cultivo del Olivo. Editorial Mundi Prensa, Madrid, Spain, pp 190–228

Ratcliff AW, Busse MD, Shestak CJ (2006) Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. Appl Soil Ecol 34:114–124

Reddy GB, Faza A (1989) Dehydrogenase activity in sludge amended soil. Soil Biol Biochem 21:327–331

Sandhu KS, Josi AK, Chahal VPS, Singh HD (1990) Rhizosphere microflora as affected by different herbicidal treatments in carrot crops. J Res Uunjab Agric Univ 27:41–45

Sannino F, Gianfreda L (2001) Pesticides influence on soil enzymatic activities. Chemosphere 45:417–425

Selvamani S, Sankaran S (1993) Soil microbial population as affected by herbicides. Madras Agric J 80:397–399

Sims JR, Haby VA (1971) Simplified colorimetric determination of soil organic matter. Soil Sci 112:137–141

Skujins J (1978) Soil enzymology and fertility index-a fallacy? History of abiontic soil enzyme research. In: Burns RG (ed) Soil enzymes. Academic Press, London, UK, pp 1–49

Strange-Hansen R, Holm PE, Jacobsen OS, Jacobsen CS (2004) Sorption, mineralization and mobility of N-(phosphonomethyl) glycine (glyphosate) in five different types of gravel. Pest Manag Sci 60:570–578

Tabatabai MA (1994) Soil enzymes. In: Weaver RW, Angle S, Bottomley P (eds) Methods of soil analysis. Part 2 microbiological and biochemical methods. Soil Science Society of America, Madison, WI, pp 775–883

Tiquia SM, Lloyd J, Herms DA, Hoitink HAJ, Michael FC Jr (2002) Effects of mulching and fertilization on soil nutrients, microbial activity and rhizosphere bacterial community structure determined by analysis of TRFLPs of PCR-amplified 16S rRNA genes. Appl Soil Ecol 21:31–48

Trevors JT (1984) Dehydrogenase activity in soil: A comparison between the INT and TTC assay. Soil Biol Biochem 16:673–674

Van Eerd LL, Hoagland RE, Zablotowicz RM, Hall JC (2003) Pesticide metabolism in plants and microorganisms. Weed Sci 51:472–495

Vivas A, Moreno B, Coral del Val C, Macci C, Masciandaro G, Benítez E (2008) Metabolic and bacterial diversity in soils historically contaminated by heavy metals and hydrocarbons. J Environ Monitor 10:1287–1296

Weaver MA, Krutz LJ, Zablotowicz RM, Reddy KN (2007) Effects of glyphosate on soil microbial communities and its mineralization in a Mississippi soil. Pest Manag Sci 63:388–393

Wilke BM (1991) Effect of single and successive additions of cadmium, nickel and zinc on carbon dioxide evolution and dehydrogenase activity in a sandy Luvisol. Biol Fertil Soils 11:34–37

Yáñez-Ruiz DR, Williams S, Newbold CJ (2007) The effect of absence of protozoa on rumen biohydrogenation and the fatty acid composition of lamb muscle. Br J Nutr 97:938–948

Yen JH, Sheu WS, Wang YS (2003) Dissipation of the herbicide oxyfluorfen in subtropical soils and its potential to contaminate groundwater. Ecotoxicol Environ Saf 54:151–156

Zabaloy MC, Garland JL, Gomez MA (2008) An integrated approach to evaluate the impacts of the herbicides glyphosate, 2, 4-D and metsulfuron-methyl on soil microbial communities in the Pampas region, Argentina. Appl Soil Ecol 40:1–12

Zanthua MI, Bremner J (1976) Production and persistence of urease activity in soils. Soil Biol Biochem 8:369–374

Amendment of Soils with Composted Sewage Sludge. Long Term Effects on C and N Transformation

Jordi Sierra, Neus Roig, Esther Martí, Martí Nadal and Marta Schuhmacher

Abstract This work aims to evaluate the effects of the current common agricultural use of composted sewage sludge on 5 crop soils, focusing on some chemical, biochemical and ecotoxicological properties related to organic matter mineralization and microbial activity. Therefore, pH, EC_{25}, oxidizable and dissolved carbon, total Kjeldahl N, soluble phenolic compounds, specific UV-absorbance$_{254,}$ ammonium and nitrates, dehydrogenase and urease activity have been determined. Soil microbial activity has been assessed by respirometry (cumulative, basal and substrate induced respiration, respiratory activation quotient) and by potential nitrification, according to OECD guidelines. The analyses have been done after a minimum of 6 months since sludge amendment to avoid the initial enhancement due to recent organic matter supply.

The sludge compost enhances some soil properties, as soluble organic matter, carbon mineralization, with general increases in the microbial activity and the active biomass and decreases in the respiratory activation quotients in most soils.

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Nitrogen mineralization has been negatively affected in some cases; nitrification and urease activity have decreased in two soils, probably due to the repeated application of sludge, more than to the total amount used. Dehydrogenase activity results are variable. In the poorer native soils it has increased, negatively correlated with the estimated active biomass and positively correlated with the respiratory activation quotient. Insufficiently controlled and repeated compost amending could be the cause of a C and N cycles imbalance, producing a decrease of the C/N ratio that can involve risk for waters due to an excess of mineral nitrogen.

1 Introduction

The use of sewage sludge in agriculture is increasing. About 2.7 million tonnes of dry matter, representing about 38% of total sludge production in the EU, are used yearly in agriculture (E. C. 2001). Regulations made on this topic have been taking into account heavy metal concentrations for sewage sludge as well as for soil, and maximum annual heavy metals loads through the application of sewage sludge. However, in the established environmental policies other important issues have not been considered till now. For instance, persistent organic pollutants concentration and the global toxicity of the sludge due to the pollutants load. In fact, many groups of chemicals can interact and have a notable influence on soil functioning and biodiversity (Schowanek et al. 2004; Ros et al. 2006), not only the heavy metal pollution.

Sewage sludge is constituted mainly by organic matter, macro and micronutrients, micropollutants and micro and mesofauna. Sludge amendments improve soil properties such as organic matter, nutrient contents, soil porosity, bulk density, aggregate stability and water holding capacity (Singh and Agrawal 2008). Sludge materials, as organic amendments, cause initial enhancement in the soil microbial activity and biomass as well as soil biochemical activity, due to higher organic matter and nutrients availability (Marschner et al. 2003).

Among the main soil functions involving soil microorganisms, C and N cycles deserve special attention, as its performance contributes to a good ecological equilibrium (Ros et al. 2006). Also, plant growth is often limited by nutrients, especially N, and water supply. The purpose of this work is to characterize several soil properties (pH, EC_{25}, oxidizable and dissolved carbon, total Kjeldahl nitrogen, soluble phenolic compounds, ammonium and nitrate, specific UV-absorbance$_{254}$, dehydrogenase and urease activities) of 5 soils that have been amended with sewage sludge. Moreover, the effects of the soil sludge amendment on the carbon mineralization process assessed by respirometry (basal and substrate induced respiration), have been determined, as well as the effects of the soil sludge amendment on the nitrogen cycle by potential nitrification and urease activity.

Table 1 Soil amendment details

Soil sample	Sludge dose Mg ha^{-1} $year^{-1}$	No. amendments	Last amendment before sampling (years)	Soil tilling	Sludge homogeneity
A-AM	50	1	1.5	No	Irregular/non-incorporated
B-AM	80	2	1.5	Yes	Regular/well incorporated
C-AM	30	5	0.5	Yes	Irregular/ incorporated
D-AM	60	2	0.5	Yes	Regular/well incorporated
E-AM	40	6	0.5	No	Irregular/non-incorporated

2 Materials and Methods

Soil samples (A, B, C, D, E) have been taken from five plots of agricultural exploitations from Montroig del Camp, Tarragona, Spain. The studied soils correspond to olive and carob crops that had been receiving composted sludges as a current practice. Soils A, B, C and D have been developed on calcareous materials, Soil E is developed on granodiorite and alkaline granite materials. The sampling of the amended soils (indicated by AM in the sample references) has been done at least 6 months after the sludge application, during the summer dry period, except for two plots in which the amendment was done 18 months before. Control soil samples (indicated by CT in the samples reference) have also been taken, from nearby areas with the same soil without sludge application. In some plots the incorporation of sludge in the soil matrix or in surface was not homogeneous. This has been taken into account in the sampling protocol by taking regular grid subsamples from each plot and analyzing them separately. The details regarding plots amendment, annual amount of waste used, number of applications, time elapsed since the last application, and homogeneity is shown in Table 1. The control soil for samples A and B is the same one. Samples A-AM and B-AM correspond to the same original soil with different application and tillage operation types. The reference for this control soil is AB-CT. For the rest of controls the references are C-CT, D-CT and E-CT.

The residual material used to amend the soils was a composted sludge, coming from the wastewater treatment plant of Vila-seca (Tarragona). Its main properties are shown in Table 2. All heavy metal concentrations and organic pollutants quantified are well below the limit values established in the Spanish regulations and in the 3rd draft of the working document on sludge of the corresponding EC Directive. In soil E only the two last amendments were done with this material. The first four applications were done with non-composted sludge. Regarding ecotoxicological parameters, this compost can be classified as non-phytotoxic (Zucconi et al. 1981) and has low toxicity to bacteria.

Table 2 Main properties of the composted sludge used

Physico-chemical parameters	
Dry matter	80.3%
pH	7.19
Electrical conductivity (1:5)	11.61 dS m^{-1}
Chemical parameters	
Total-N	3.41% dm
NH_4^+-N	1.35% dm
Organic-N	2.06% dm
P (P_2O_5)	6.39% dm
K (K_2O)	0.62% dm
Organic matter characterization	
Organic matter	50.8% dm
Resistant organic matter	26.0% dm
Stability degree	51.2% dm
Non-hydrolysable N	1.67% dm
C/N ratio	7.2
Soluble phenol compounds	209 mg kg^{-1} dm
Potentially toxic elements	
Cr	82 mg kg^{-1} dm
Ni	32 mg kg^{-1} dm
Pb	89 mg kg^{-1} dm
Cu	438 mg kg^{-1} dm
Zn	1,068 mg kg^{-1} dm
Hg	2.0 mg kg^{-1} dm
Cd	1.4 mg kg^{-1} dm
Organic pollutants	
AOX	360 mg kg^{-1} dm
LAS	1,245 mg LSS kg^{-1} dm
Nonylphenols	42.92 mg kg^{-1} dm
PAHs	0.88 mg kg^{-1} dm
PCBs	0.12 mg kg^{-1} dm
Toxic equivalents dioxin-like PCBs	653 ng kg^{-1}dm
Ecotoxicological parameters	
Germination Index (1:5 aq. extract) *Allium cepa*	88.9%
Germination Index (1:5 aq. extract) *Loliun perenne*	81.1%
(Microtox®) EC_{50} (1:5 aq. extract) extract	270 mg sludge
(Microtox®) EC_{50} (acetone/hexane-DMSO extract)	16.1 mg sludge

The following parameters have been determined in the amended and control soil samples: electrical conductivity and pH in 1:2.5 water extracts, pH in 1:2.5 (w/v) 1 M KCl; oxidizable carbon, according to Walkley–Black method (Page et al. 1982); Dissolved Organic Carbon (TOC determination by AJ analyzer Multi N/C 3100 after inorganic carbon elimination), specific UV absorbance (254 nm), and phenolic compounds concentration Folin–Ciocalteau method (Box 1983) in 1:5 (w/v) 0.01 N $CaCl_2$ extracts; total Kjeldahl nitrogen; ammonium and nitrate in 1:10 1 M KCl; urease activity (Kandeler and Gerber 1988), dehydrogenase activity (García et al.

2003). Also, the soils basal respiration has been measured as follows: after a pre-incubation at the field soil humidity, samples were incubated in closed reactors with the water content at 60% of the soil water holding capacity, a temperature of 30°C, and darkness throughout the incubation.

The microbial activity was analyzed incubating soil samples (25 g) in manometric respirometers, which allow the determination of sample oxygen consumption (Oxitop®, WTW). The humidity was adjusted to the 60% of the respective soil water holding capacity. Samples were kept at 25°C for 21 days in darkness in a temperature controlled incubator. The cumulative oxygen consumption (Cumulative respiration, CR) was periodically registered throughout the incubation period. Once the incubation was completed, Substrate induced respiration (SIR) was determined, by the quantification of the average oxygen consumed shortly after a substrate addition, when the respiration is fairly constant (12 h) after substrate addition (aqueous solution equivalent to 4000 mg glucose per kg of soil) to the incubated samples (OECD 2000b; ISO 2002).

Basal Respiration rate (BR) was estimated as the average hourly respiration rate over the last 5 days of incubation when the respiration was stable (ISO 2002). The Respiratory activation quotient (Q_R) was calculated by dividing the BR rate by the SIR rate according to ISO 17155 (ISO 2002). All experiments were done in triplicate. Nitrogen transformation test (OECD 2000a) was performed according to OECD 216 guideline, keeping the same incubation conditions as in the respirometric experiment during 4 weeks.

To determine the statistical significance of the differences between amended soils and controls, an ANOVA followed by Duncan's *post-hoc* test ($P < 0.05$) was done with the results of the experiments. Pearson correlations between parameters have been done ($P < 0.01$; or $P < 0.05$) in order to see relationships between the different parameters measured.

3 Results and Discussion

The results of the soil parameters quantification are shown in Table 3. According to the parent materials, soil AB-CT, C-CT and D-CT have alkaline pH and fine texture. Soil E-CT has neutral-slightly acidic pH and its texture is coarser than in the other soils. All the control soils have low salinity and organic matter content, and suitable C/N ratios.

In the soils, the composted waste application produces a general effect of compensation of the pH values. The salinity increases with the application of the waste, but the increases never reach values that could be hazardous for the vegetal development or other soil functions as microbial activity and organic matter mineralization.

Though the sampling was done at least 6 months after the last composted sludge application most soils show an increase in the organic matter concentration, and especially in total N, and ammonium and nitrate, compared to the respective controls. There is a consequent decrease in the C/N ratios of the soils, reaching

Table 3 Mean values of pH, Electrical conductivity (EC), Oxidizable carbon (OC), Total Kjeldahl nitrogen (TKN), Ammonium, Dissolved organic carbon (DOC), Specific UV absorbance 254 nm (SUVA) and soluble phenolic compounds concentration in control and amended soils

	AB-CT	A-AM	B-AM	C-CT	C-AM	D-CT	D-AM	E-CT	E-AM
pH H_2O	7.80	7.73	7.68	7.87	7.65	8.00	7.78	6.94	7.25
pH KCl	7.45	7.49	7.36	7.58	7.35	7.35	7.66	6.37	6.65
EC_{25} (μS cm^{-1})	98.7	236.0*	190.0*	128.3	288.0*	217.0	272.0*	46.1	89.8
Carbonates[a]	++	++	++	++	++	+++	+++	–	–
OC (%)	1.59	1.96*	2.60*	1.89	2.58*	0.88	1.79*	0.61	1.38*
TKN (%)	0.159	0.204*	0.372*	0.19	0.389*	0.07	0.230*	0.057	0.158*
C/N	10.0	9.6	7.0*	9.9	6.6*	12.1	7.8*	10.6	8.8*
NH_4^+-N (mg kg^{-1})	2.7	11.0*	8.0*	8.3	9.6	3.2	9.5*	2.8	11.1*
NO_3^--N (mg kg^{-1})	7.3	16.5*	22.1*	9.7	39.5*	6.1	34.4*	8.7	21.7*
DOC (mg C kg^{-1})	92.8	87.0	186.7*	79.8	310.8*	31.6	160.6*	35.9	125.9*
SUVA (L mg^{-1} C m^{-1})	0.93	2.65*	4.19*	3.24	4.16*	0.86	3.86*	3.34	4.21*
Phenols (mg kg^{-1})	1.1	2.2	7.5*	3.6	11.8*	3.3	10.7*	1.8	7.2*

* Significant differences between amended and control samples ($P < 0.05$)

[a] Field semiquantitative determination of carbonates: ++: 10-20%; +++20–30% carbonates; –: below detection limit

quite low values. There is an increase in soil organic carbon, expectable after the addition of an organic material agreeing with other studies (Marschner et al. 2003).

The soluble organic matter notably increases in the soils that have been amended with the compost, in any dose and management type. It presents high degree of aromaticity, as it can be deduced from SUVA results. The aromaticity of the organic matter increases with its maturity degree (Stevenson 1982; Surampalli and Tyagi 2004). The SUVA values have been used to denote the quality of the dissolved organic matter (Jaffrain et al. 2007), so it seems that the contribution with the composted sludge may enhance the quality of this property. However, the differences between amended samples and controls depend on the original soil and type of management. The aromaticity of the organic matter improves the soil ability to adsorb organic pollutants (de Paolis and Kukkonen 1997), this is especially important in soils subjected to anthropic influences. Among the aromatic constituents, free phenolic compounds are of special interest. They undergo considerable increases in the amended soils compared to controls. Whereas aromaticity increases with the maturity of organic matter, free phenolic molecules allow some reactivity of this soil component. This is important for soil pollution compensation. However, these compounds may have a notable degree of toxicity, being antimicrobial molecules. In the compost sample the levels of nonylphenols and AOX are notable, though just below the regulation limits. It is important, so, to take into account not only inorganic elements as sludge born pollutants but also organic molecules, and the ability of the soils to compensate this type of pollution.

Total Nitrogen increases in all samples due to the application of composted sludge, parallel to the organic matter inputs. This effect can be seen even much time after the application. In fact, the purpose of adding such materials to soils is to

Table 4 Cumulative respiration (CR), Basal respiration (BR), Substrate induced respiration (SIR) and Respiratory activation quotient (Q_R) of control and amended soils and the percentage of variation related to control samples

Soil sample	CR (mg O_2 100 g^{-1}) 21 days	% to control	BR (mg O_2 kg^{-1} h^{-1})	% to control	SIR (mg O_2 kg^{-1} h^{-1})	% to control	Q_R	% to control
AB-CT	98.2	0	1.30	0	7.40	0	0.186	0
A-AM	107.0	+9.0	1.55*	+19.2	7.13	−3.8	0.217	+16.6
B-AM	149.0*	+51.7	2.51*	+93.0	29.04*	+392.4	0.086	−53.5
C-CT	191.8	0	2.30	0	26.83	0	0.086	0
C-AM	192.5	+0.3	2.64	+14.7	36.49*	+36.0	0.072	−16.3
D-CT	85.4	0	1.43	0	6.05	0	0.236	0
D-AM	193.7*	+126.8	2.27*	+58.8	10.85*	+79.3	0.209	−11.4
E-CT	39.9	0	0.34	0	18.83	0	0.018	0
E-AM	101.4*	+154.1	1.25*	+267.6	35.83*	+90.3	0.034	+88.9

* Significant differences between amended and control samples ($P < 0.05$)

enhance their fertility, regarding organic matter and nutrients. As a consequence of nitrogen inputs, the C/N ratios decrease comparing to control, reaching in some cases excessively low values, as in samples B-AM, C-AM and D-AM. Comparing to the sludge and original soils, the C/N ratios get even lower that what could be foreseen. Generally speaking, nitrogen concentrations are still higher in the samples in which the application of waste is more recent. The decreases in the C/N ratios are usually due to excessive doses or frequency of the amendments. The lowest suitable C/N ratio varies depending on the authors, but it is usually established about 8 or 9 (Gagnard et al. 1988; Saña et al. 1996). From an environmental point of view, the decrease of C/N ratios represents a strong hazard for the water environment, due to the diffuse pollution by mineral nitrogen. For example, the amended soils in this work may have an average excess of 4000 kg N ha^{-1} compared to the controls.

Ammonium and nitrate concentrations also tend to increase in the amended soils, due to the long term mineralization of the waste organic nitrogen. Also, negative correlations have been found between C/N ratios and soluble mineral nitrogen forms, with correlation coefficients of 0.448 and 0.509 ($P < 0.05$). The mobilization of mineral nitrogen after organic matter decomposition may enhance vegetal nutrition, but also negative consequences on water pollution due to mineral nitrogen leaching have to be taken into account. This should be especially important in the E soil, because of its *a priori* lower exchange capacity, and coarse texture, which makes it more susceptible to nitrogen losses.

Regarding the microbial activity (Table 4), the CR increases in all samples; this parameter shows the overall soil state (microbiota and nutrient availability). Organic matter addition often leads to a rapid increase of soil respiration, linked with the availability of organic matter (Marschner et al. 2003). In the case of the soils with the lowest organic matter concentration (D, E) the increases are above 100%. These two samples correspond to the soils with the highest initial C/N ratio.

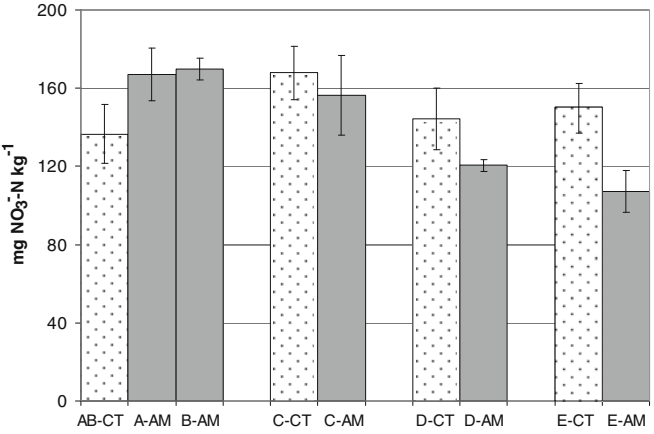

Fig. 1 Nitrogen transformation test. Total nitrates released during the incubation period

The BR increases in all the amended soils. It represents the real biological activity, considered when the labile carbon source is exhausted and the respiratory activity is stabilized. This increase in the activity is parallel to an increase in SIR values. This later parameter represents the active biomass (ISO 2002). In this work, CR and BR are positively correlated to DOC (0.578 and 0.566, $P < 0.01$) and oxidizable carbon (0.750 and 0.694 $P < 0.01$), which are brought with the sludge amendment. The microbial respiration activity is affected by several parameters, as water content, temperature, the quality of the soil organic matter, contaminants, i.e. (Eisentraeger et al. 2005). SIR values, which are sensitive indicators of toxicity in soils (Martí et al. 2007), are also higher in the amended samples, so no toxicity can be found in the amended soils, even considering the pollutants load and the time elapsed since the application, which might allow the exhibition of long term effects after initial enhancement due to the organic matter increase (Montserrat et al. 2006). Usually, in the presence of toxicants, the initial increases of CR are due to the consumption of labile organic matter from the dead organisms and/or the presence of organic matter in the waste, in this case, the toxicity may be revealed by a decrease in SIR values. The Q_R measures the relationship of the activity of microbiota and the number of active microorganisms, serving as an indicator of stress of soil microbiota, Q_R values above 0.3 may indicate soil microbiota stress in polluted soils (ISO 2002). However, increased Q_R may occur for two reasons. On one hand, they are an indicator of bioavailable carbon sources. On the other hand, increased Q_R may indicate the presence of contaminants that are not biodegradable, e.g., heavy metals (Eisentraeger et al. 2005). In this work the estimated values of Q_R are below this limit, the application of waste generally decreases the Q_R, and so the stress level, except for the acidic soil, which show significant increase of about a 90%, this is the less active native soil, has lower pH and coarse texture, and low exchange capacity. It has received high amounts of waste and not all the sludge received was previously stabilized by composting processes.

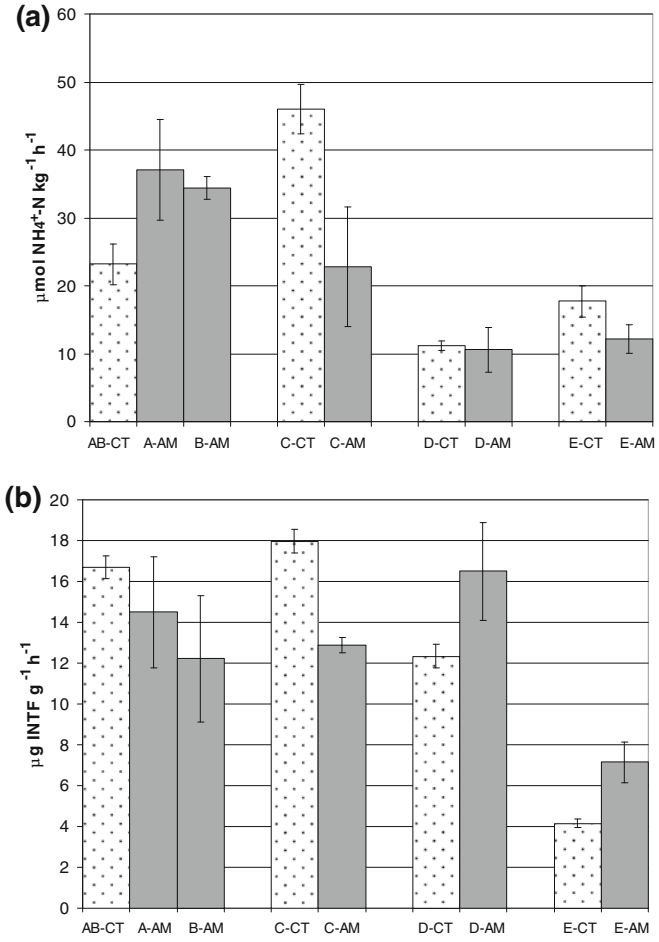

Fig. 2 Urease activity (UA) (**a**), and dehydrogenase activity (DHA) (**b**), of samples

Nitrogen transformation assay results are shown in Fig. 1. The amendment of AB-CT with composted sludge produces an increase in the potential nitrogen mineralization. In soils C, D and E nitrogen mineralization tends to decrease due to the compost application, being significant in the last two samples. The inhibitory effect is produced in the amended soils coming from native soils with poorest biological activity (D and E).

Urease (UA) and dehydrogenase activity (DHA) results are shown in Fig. 2. UA values are similar to those of N mineralization. So, it seems that this may be one of the limiting steps in the nitrogen mineralization process. The decrease in the UA compared to the control soils seems to be depending on the time elapsed since the last waste application and on the frequency of application more than on the dose applied. We have found negative correlations between the differences in

UA of amended and control samples and the frequency of applications (−0.545; $P < 0.01$). Some authors report that UA decrease in soils where repeated applications of ammonium are produced, related with the by-products of the microbial assimilation of inorganic forms of nitrogen (McCarty et al. 1992). Soils C and E (with significant inhibition in UA) have received 5 and 6 applications of compost, respectively. The effects on UA due to a recent use of wastes might be related to the inhibition caused by the release of pollutants during the sludge mineralization (Kizilkaya and Bayrakli 2005). UA has been reported as a sensitive parameter for metal pollution toxicity (García et al. 2003). However, some authors have reported increases in the UA related to the increase of toxic metals (Moreno et al. 1999). This enzymatic activity is not affected by microbial biomass (García et al. 2003), and this has also been observed in this work, as no significant correlations have been found between this parameter and those corresponding to the microbial activity or biomass.

Dehydrogenase is an intracellular enzyme involved in microbial O_2 metabolism. This activity depends on the metabolic state of the soil biota and may be a good indicator of soil microbial activity in semiarid areas (García et al. 1994). The amendment with composted sludge produces different effects (increase or inhibition) in DHA, depending on the soils tested. DHA has been most related to an enhancement in the microbial processes and biomass, and to increases in the organic matter concentration in soils (García et al. 2003). The effect of sludges on soil DHA is not always consistent among different studies. Metal polluted sludges may increase or decrease DHA. It is important to note that DHA activity is enhanced if the rate of sewage sludge addition to soil is limited, as high doses of amendment may reverse the DHA effect. However, the microbial activity is usually stimulated by sludge applications, even in the presence of metals over controlled concentrations (Obbard et al. 1994; Reddy and Faza 1989). The results in this work show some inhibitions in samples that have shown general increases in the microbial activity, and increases in other samples. No significant correlations have been found between DHA and most parameters, except for two of them, SIR and Q_R, with correlation coefficients of −0.398 and 0.408, respectively ($P < 0.05$). So, the DHA increases seem to be related, in this case, to "stress parameters," as SIR values decrease in the presence of toxicants, and Q_R increases in the same situation (ISO 2002).

4 Conclusions

The application of compost on soils, as it is known, enhances some soil properties. Increases of soluble organic matter concentrations, together with its aromaticity and phenolic compounds content, have been found.

Carbon mineralization seems to be enhanced due to the sludge compost application. This can be observed as general increases in the microbial activity and the active biomass and decreases in the respiratory activation quotients except for

one of the soil samples. This indicates little negative effects on the aerobic heterotrophic microbiota, even if long time has elapsed since the last sludge application and an eventual toxicity effect could be revealed due to the accumulation of pollutants.

On the other side nitrogen mineralization processes have been more negatively affected than carbon transformations. Nitrification and urease activity have decreased in two of the soils. This is probably due to the repeated application of sludge, more than to the total amount used. Dehydrogenase activity results are variable in the different soils assayed. In the poorest native soils this enzymatic activity has increased, negatively correlated with the estimated active biomass and positively correlated with the respiratory activation quotient. Such effects can be related to a certain degree of stress in the soils, so further research should be done on this issue.

The repeated and not sufficiently controlled (high doses also) compost amending could be the cause of the imbalance between C and N cycles producing a decrease of the C/N ratio that can involve significant risk of water contamination by excess of mineral nitrogen.

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References

Box JD (1983) Investigation of the folin-ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. Water Res 17:511–525

De Paolis F, Kukkonen J (1997) Binding of organic pollutants to humic and fulvic acids: influence of pH and the structure of humic material. Chemosphere 34:1693–1704

E. C. (European Commission) 2001 Disposal and recycling routes for sewage sludge. Scientific and technical sub-component report. DG Environment-B/2. Luxembourg. European Communities

Eisentraeger A, Hund-Rinke K, Roembke J (2005) Assessment of ecotoxicity of contaminated soil using bioassays. In: Margesin R, Schinner F (eds) Manual for soil analysis–monitoring and assessing soil bioremediation. Book series: soil biology, vol 5. Springer, Berlin Heidelberg, Germany

Gagnard J, Huguet C, Ryser JP (1988) L'Analyse du Sol et du Végétal dans la Conduite de la Fertilisation: le Contrôle de la Qualité des Fruits. Organisation Internationale de la Lutte Biologique et Integrée. Paris

García C, Gil F, Hernández MT, Trasar C (2003) Técnicas de Análisis de Parámetros Bioquímicos en Suelos: Medidas de Actividades Enzimáticas y Biomasa Microbiana. Editorial Mundi-Prensa, Madrid, Spain

García C, Hernández T, Costa F (1994) Microbial activity in soils under mediterranean environmental conditions. Soil Biol Biochem 26:457–466

ISO, 2002. Soil quality. Determination of abundance and activity of soil microflora using respiration curves. Reference number ISO 17155:2002 (E). Switzerland

Jaffrain J, Gérard F, Meyer M, Ranger J (2007) Assessing the quality of dissolved organic matter in forest soils using ultraviolet absorption spectrophotometry. Soil Sci Soc Am J 71:1851–1858

Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72

Kizilkaya R, Bayrakli B (2005) Effects of N-enriched sewage sludge on soil enzyme activities. Appl Soil Ecol 30:3–192

Marschner P, Kandeler E, Marschner B (2003) Structure and function of the soil microbial community in a long-term fertilizer experiment. Soil Biol Biochem 35:453–461

Martí E, Sánchez M, Sierra J, Cruañas R, Garau MA (2007) Ecotoxicological tests assessment of soils polluted by chromium (VI) and pentachlorophenol. Sci Total Environ 378:53–57

McCarty GW, Shogren DR, Bremmer JM (1992) Regulation of urease production in soil by microbial assimilation of nitrogen. Biol Fertil Soils 12:261–264

Montserrat G, Martí M, Sierra J, Garau MA, Cruañas R (2006) Discriminating inhibitory from enhancing effects in respirometry assays from metal polluted-sewage sludge amended soils. Appl Soil Ecol 34:52–61

Moreno JL, Hernandez MT, Garcia C (1999) Effects of a Cd-contaminated sewage sludge compost on dynamics of organic matter and microbial activity in an arid soil. Biol Fertil Soil 28:230–237

Obbard JP, Sauerbeck D, Jones KC (1994) Dehydrogenase activity of the microbial biomass in soils from a field experiment amended with metal contaminated sewage sludges. Sci Total Environ 142:157–162

OECD (2000a) OECD Guidelines for the testing of chemicals. 216. Soil microorganisms: nitrogen transformation test

OECD (2000b) OECD Guidelines for the testing of chemicals. 217. Soil microorganisms: Carbon transformation test

Page AL, Miller RH, Kenney DR 1982 Methods of soil analysis. Part 2. Chemical and microbiological properties. 2nd Edition, Agronomy series n. 9. American Society of Agronomy-Soil Science Society of America, Madison, WI, USA

Reddy GB, Faza A (1989) Dehydrogenase activity in sludge amended soil. Soil Biol Biochem 21:2–327

Ros M, Pascual JA, García C, Hernández MT, Insam H (2006) Hydrolase activities, microbial biomass and bacterial community in a soil after long-term amendment with different composts. Soil Biol Biochem 38:3443–3452

Saña J, Moré JC, Cohí A (1996) La Gestión de la Fertilidad de los Suelos. Publicaciones del Ministerio de Agricultura, Pesca y Alimentación. Madrid, Spain

Schowanek D, Carr R, David H, Douben P, Hall J, Kirchmann H, Patria L, Segui P, Smith S, Webb S (2004) A risk-based methodology for deriving quality standards for organic contaminants in sewage sludge for use in agriculture-conceptual framework. Regul Toxicol Pharm 40:227–251

Singh RP, Agrawal M (2008) Potential benefits and risks of land application of sewage sludge. Waste Manage 28:347–358

Stevenson FJ (1982) Humus chemistry. Genesis, composition, reactions. Wiley, NY, USA

Surampalli RK, Tyagi KD 2004 Advances in water and wastewater treatment. American society of civil engineering, Reston, VA, USA

Zucconi F, Pera A, Forte H, de Bertoli M (1981) Evaluating toxicity of immature compost. Biocycle 22:54–57

Part III
New Applications of Enzymes and Other Molecular Techniques

Fingerprinting the Microbial Communities in Organic Wastes Using Oligonucleotide Microarrays and Real-Time PCR

Marta Goberna, Maria Gadermaier, Michael A. Schoen, Daniel Sperl, Ingrid H. Franke-Whittle, Bernhard Wett and Heribert Insam

Abstract Unravelling the prokaryotic diversity in organic wastes is among the challenges for optimising biological waste treatment. For a faster analysis of the microbiota involved in the recycling of biodegradable wastes, we have recently developed two oligonucleotide microarrays targeting the 16S rRNA gene of prokaryotes involved both in aerobic (COMPOCHIP) and anaerobic (ANAEROCHIP) processes. The COMPOCHIP includes 369 probes for bacteria and has proven successful in discriminating the microbial communities in composts produced from different residues and at diverse maturation stages. The first version of the ANAEROCHIP includes 103 probes targeting most lineages of methanogenic archaea involved in anaerobic digestion under mesophilic and thermophilic conditions. The information generated by the microarrays can be used as a basis for conducting quantitative assays. We have designed primers and optimised SYBR Green I based assays for most genera of methanogens detectable with the ANAEROCHIP. Combining both techniques, we have investigated the methanogenic communities during the start-up of cattle manure fed digesters and during a co-digestion assay of manure and olive mill wastes. We suggest a new approach for fingerprinting the microbial communities in organic wastes: a fast screening of the prokaryotic diversity using custom-made oligonucleotide microarrays followed by the quantification of specific targets through real-time PCR.

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1 Introduction

Prokaryotes drive the decomposition of organic wastes in aerobic and anaerobic environments. Molecular tools developed during the last two decades have helped in unravelling the microbial communities involved in biological waste treatment and fingerprinting them is nowadays a routine practice in many laboratories worldwide (Insam et al. 2010). Most studies that attempt to screen the prokaryotic diversity target the 16S rRNA gene and involve as starting steps the direct DNA extraction from samples and PCR amplification with universal or group-specific primers. The PCR amplified genes can be separated using a wealth of techniques, the most common involving the construction of clone libraries or the electrophoretic separation based on sequence or restriction site differences (Talbot et al. 2008). These approaches include a final sequencing step if the organisms detected are to be phylogenetically identified. These strategies, though successful in analysing the community structure, are time-consuming and only allow a low sample throughput. Massive sequencing technologies solve such problems and are becoming more common as they get increasingly inexpensive, but require a deep data analysis which is not effective for every purpose (e.g. detection of well known organisms in a certain environment). Here, we suggest fingerprinting the microbial communities in organic wastes using newly devised oligonucleotide microarrays for the fast detection of selected taxa.

Microarrays consist of a solid matrix holding up to tens of thousands of DNA probes targeting complementary known sequences attached in a precise location, allowing the simultaneous hybridisation with fluorescently labelled DNA from complex samples (Schadt and Zhou 2006). Hence, a broad array of microbes inhabiting a particular environment can be detected in a single assay. Microarrays can be designed to target functional genes, such as those involved in C, N, S and P cycling, metal resistance and contaminant degradation (He et al. 2007). However, most of the recently developed microarrays are based on the rRNA genes or their flanking internal transcribed spacer (ITS) sequences. These phylogenetic arrays have been created targeting: 1) classes at diverse taxonomic levels, e.g. the class Alphaproteobacteria (Sanguin et al. 2006), the genus *Kitasatospora* (Günther et al. 2006); 2) functional groups, e.g. sulphate-reducing prokaryotes (Loy et al. 2002), methanotrophs (Stralis-Pavese et al. 2004); and 3) specific habitats, such as soils contaminated with hexachlorocyclohexane (Neufeld et al. 2006). Within the third category, our group has designed the COMPOCHIP, a microarray targeting the 16S rRNA gene of bacteria involved in composting, which can discriminate the microbial communities in composts produced from different residues and composts at diverse maturation stages (Franke-Whittle et al. 2009a). More recently, we have developed the ANAEROCHIP, which targets prokaryotes involved in the anaerobic digestion of organic wastes (Franke-Whittle et al. 2009b). This chapter summarises the design and application of this type of phylogenetic microarray, taking as a model our newest device.

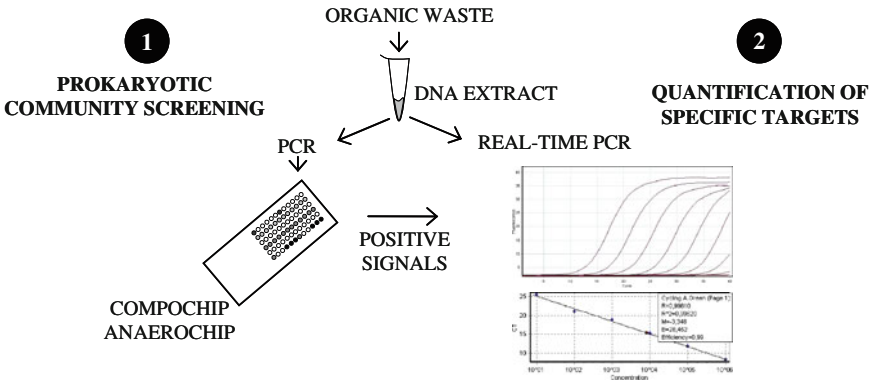

Fig. 1 How to fingerprint microbial communities in organic wastes

Phylogenetic microarrays permit a strikingly fast detection of the presence/absence of the targeted microbes. An estimate of their abundance is also possible from the fluorescence intensity. However, the quantitative ability of DNA microarrays is limited due to PCR bias towards the amplification of dominant DNA populations (Felske et al. 1998) and to differences in hybridisation efficiencies among oligonucleotide probes (Schadt and Zhou 2006; Wagner et al. 2007). Nonetheless, the information generated by a microarray can be used as a basis for conducting quantitative assays with other techniques (Fig. 1; Wagner et al. 2007). In order to measure the 16S rRNA gene copy numbers of the organisms detectable with the ANAEROCHIP, we designed genus-specific primers and optimised real-time quantitative PCR assays. Among the real-time PCR platforms available, we selected the use of SYBR Green I, a fluorophore that non-specifically binds all molecules of double stranded DNA, since it allows a relatively inexpensive and fast processing of the samples (Smith 2005). Combining our custom-made microarray for anaerobic environments and real-time PCR quantification of defined targets, we have thoroughly investigated the methane-producing communities during the start-up of cattle manure fed reactors and during a co-digestion assay of manure and olive mill waste.

2 Materials and Methods

2.1 Design and Application of the ANAEROCHIP

The design and application of the ANAEROCHIP was described by Franke-Whittle et al. (2009b) and is summarised in Fig. 2 (steps 1–5). Briefly, the target organisms to be included in the microarray were selected after 1) an intensive literature review, and 2) the construction of clone libraries after amplification of the 16S rRNA gene from environmental samples (Franke-Whittle et al. 2009b;

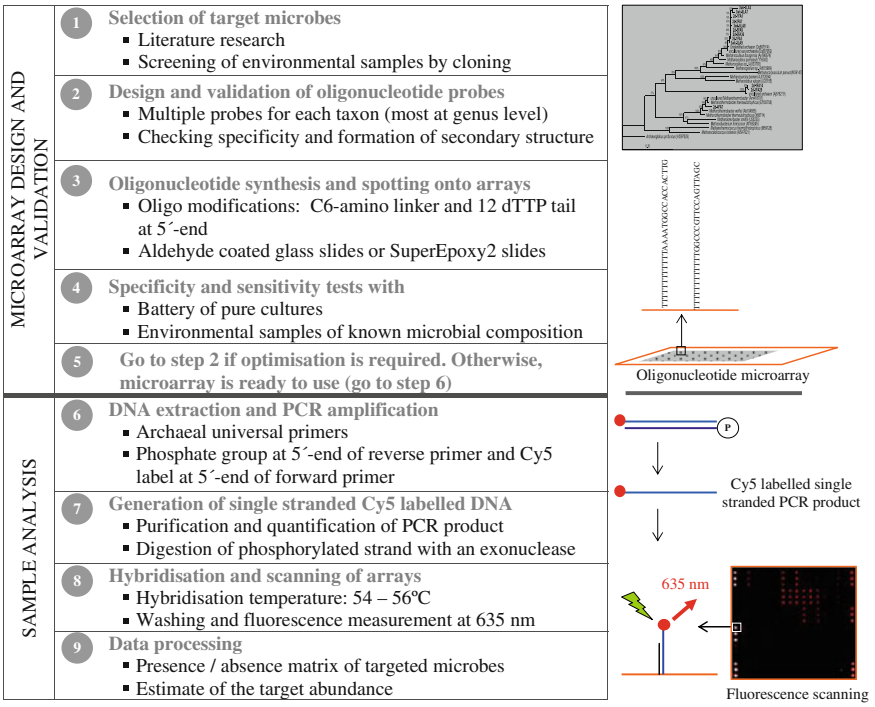

Fig. 2 Work-flow for the design and application of the ANAEROCHIP

Goberna et al. 2009). Probes specific for the 16S rRNA gene of each target organism were designed using the ARB software package (Ludwig et al. 2004), and their specificity and potential formation of secondary structures tested *in silico*. The oligonucleotides were synthesised with a terminal C6-aminolinker and 12 dTTP tail at the 5′-end before attachment to either aldehyde coated glass slides or SuperEpoxy2 slides. Finally, array specificity and sensitivity were tested using pure cultures and environmental samples of known microbial composition-e.g. from cloning-in order to select the final set of probes more efficient in the detection of the targets, and define the optimal hybridisation conditions.

Screening the prokaryotic diversity of anaerobic sludge using microarrays requires the generation of single-stranded fluorescently labelled PCR products (steps 6–9 in Fig. 2). Sludge DNA was submitted to PCR amplification of the 16S rRNA gene using universal archaeal primers (Table 1). The forward primers were synthesised with a Cy5 fluorophore attached to their 5′-end and the reverse primers were phosphorylated at their 5′-end. PCR amplification reactions and thermal cycling were performed as in Franke-Whittle et al. (2009b). PCR products were checked on 1% agarose gels, purified and quantified. The phosphorylated DNA strands of

Table 1 PCR primers targeting archaea and several methanogenic genera

Target	Primer	Sequence (5′–3′)	AT (°C)[1]	Ref. [2]
Archaea	109F	ACKGCTCAGTAACACGT		[1]
	934R	GTGCTCCCCCGCCAATTCCT		
Methanosarcina	240F	CCTATCAGGTAGTAGTGGGTGTAAT	64	[2]
	589R	CCCGGAGGACTGACCAAA		
Methanosaeta	MS1b	CCGGCCGGATAAGTCTCTTGA	60	[3]
	SAE835R	GACAACGGTCGCACCGTGGCC		
Methanoculleus	298F	GGAGCAAGAGCCCGGAGT	65	[2]
	586R	CCAAGAGACTTAACAACCCA		
Methanobrevibacter	210F	TTTCGCCTAAGGATGGGTCT	59	[4]
	367R	CGATTTCTCACATTGCGGAG		after [5]
Methanobacterium	fMbium	CGTTCGTAGCCGGCYTGA	58	[5]
	748R	TTCGTTACTCACCGTCAGGT		[4]
Methanosphaera	594F	TAAGTCTTTGGTGAAAGCTT	61	[4]
	747R	GTTACTCACCGTCAAGAT		
Methanocorpusculum	193F	TCCTCGAAAGATCCGTC	58	[4]
	488R	CTGCCCTTTCTTCACATA		

[1] Real-time PCR annealing temperature; [2] [1] Grosskopf et al. (1998); [2] Franke-Whittle et al. (2009c); [3] Shigematsu et al. (2003); [4] Goberna et al. (2010a); [5] Skillman et al. (2004)

1000 ng of purified PCR products were digested by incubation with lambda exonuclease. The Cy5-labelled single-stranded PCR products were hybridised with the microarray at 54–56°C for 4 h. Slides were then washed, air dried and scanned at 635 nm with a Scan Array G_x microarray scanner (Perkin Elmer, USA). The signal-to-noise ratio (SNR) was calculated for each probe using the formula:

$$\mathrm{SNR} = \frac{F635_P - (F635_{NB} - B635_{NB})}{B635_P}, \quad (1)$$

where $F635_P$ and $F635_{NB}$ denote the median fluorescent signals at 635 nm for each probe (P) and the non-binding control (*NB*), and $B635_P$ and $B635_{NB}$ the corresponding background signals. SNR $\geq$ 2 were considered positive signals (Loy et al. 2002).

2.2 Development and Application of Real-Time PCR Assays

Sludge DNA was subjected to real-time quantitative PCR amplification to quantify several groups of methanogens included on the ANAEROCHIP using a SYBR Green I commercial kit. Figure 3 covers the steps followed to develop real-time PCR assays. Primers were either obtained from the literature or designed using the

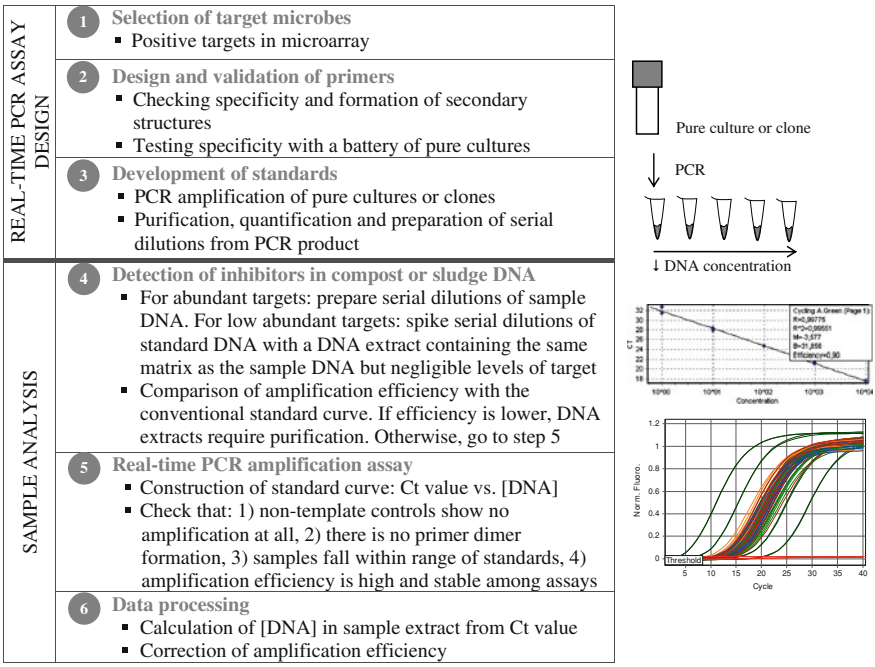

Fig. 3 Work-flow for the design and application of real-time PCR assays for quantifying microbes in organic wastes

ARB software package (Ludwig et al. 2004). Standard curves were constructed using 16S rRNA gene PCR amplified products from pure cultures or environmental clones using the genus-specific primers in Table 1. End-point PCR amplifications were performed as in Franke-Whittle et al. (2009c) and Goberna et al. (2010a). PCR products were purified and quantified in triplicate with PicoGreen® (Invitrogen, USA) using low autofluorescent microtiter plates and stored in low DNA binding tubes at −20°C. The total number of copies of the 16S rRNA gene in the stock DNA was calculated from the length of the amplified fragment and the DNA concentration, assuming an average weight of 650 Daltons for each single base pair. From this standard solution with a known number of gene copies, serial dilutions were prepared in DNAase free 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to cover a range of 10^9 to 1 gene copies per μL. These dilutions were freshly made for each assay.

Real-time PCR amplifications were conducted using the Quantimix Easy SYG Kit (Biotools, Spain) and performed in a Rotor-Gene™ 6000 (Corbett Life Sciences, Australia) in 20 μL volumes, with each standard reaction mix containing a final concentration of 1× Quantimix reaction premix, 100 nM each primer, 0.4 mg mL^{-1} BSA and distilled water. Two μL sludge DNA were applied directly to the PCR reaction mix. Slight modifications in the concentration of the premix

and primers were required depending on the target (Goberna et al. 2010a). Thermocycling was initiated with a denaturation at 95°C for 5 min, followed by 40 cycles comprising 20 s at 95°C, 20 s at an annealing temperature specific for each target (Table 1) and 20 s at 72°C. Runs were completed with a melting analysis (65°C–95°C, ramp 0.5°C min^{-1}) to check for product specificity and primer dimer formation. This was further checked on 2% agarose gels run at 80 V for 60 min.

Prior to the quantification assays, spiking experiments were performed to test for PCR efficiency reduction due to the presence of inhibitors in the sludge matrix. For each target, three standard curves were constructed: 1) the conventional standard curve, with each reaction mix containing 16 μL reaction premix (as above), 2 μL standard DNA and 2 μL distilled water, 2) a standard curve spiked with sludge DNA with negligible levels of the target (16 μL premix, 2 μL standard DNA and 2 μL sludge DNA) and 3) a standard curve spiked with purified sludge DNA. Five standard solutions were used to build each curve. All standards and samples were run in duplicate.

2.3 Combining the AnaeroChip and Real-Time PCR. Experiments in Pilot Anaerobic Reactors

Two experiments were performed using four continuously stirred tank reactors (CSTRs) located in the Unit of Environmental Engineering at the University of Innsbruck (Fig. 4). The volume of biogas produced was measured using a gas meter located on top of each reactor. Gas samples were collected daily in gas-tight bags and the biogas composition (CH_4, CO_2 and H_2) measured using a Biogas Monitor BM 2000 (Geotechnical Instruments, UK). Sludge samples were collected daily from the tap located at the bottom of the reactor. Reactors were ran for 35 days.

Experiment 1. Four start-up strategies were tested in order to define the best conditions for microbial adaptation and reactor performance in a BIO4GAS® full-scale reactor (Wett et al. 2007; Wett and Insam 2010). Two reactors were completely filled with cattle manure and progressively heated from 20°C up to 37°C at a slow (4 wk; reactor manure_slow) and a fast rate (2 wk; reactor manure_fast). Feeding with cattle manure started on day 28 with feed flow rates of 6 and 24 L d^{-1}, respectively. The other two reactors were seeded with sludge from a stably operating biogas plant and progressively loaded with increasing amounts of cattle manure at a low (from 0.75 to 6 L d^{-1}; reactor seed_low) and high feed flow rate (from 0.75 to 24 L d^{-1}; reactor seed_high) to simulate both usual and overload conditions (Gadermaier 2009; Schoen et al. 2009).

Experiment 2. The adaptation of the methanogenic communities to the codigestion of cattle manure and olive mill wastes (OMW) was investigated (Goberna et al. 2010a, 2010b). We ran one reactor with diluted cattle manure (3:1 manure:-water; reactor MAN), one with diluted OMW (3:1 water:OMW; reactor OL), and a third reactor with a mixture of both materials (3:1 manure:OMW; reactor

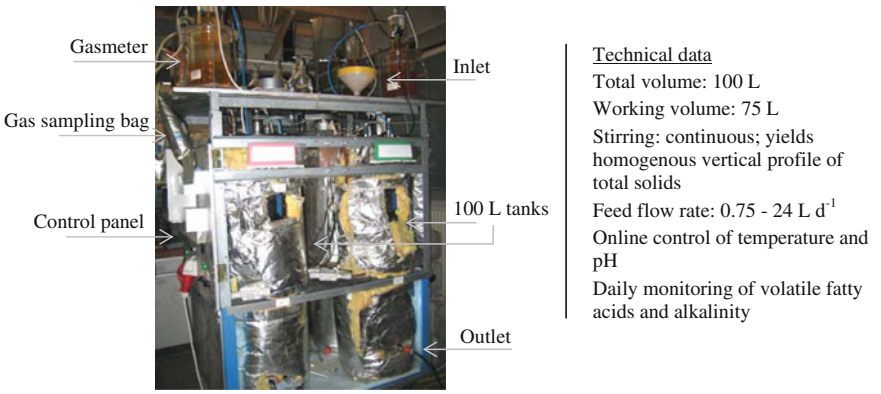

Fig. 4 100 L CSTRs in the Institute of Infrastructure (University of Innsbruck)

MAN_OL). All reactors were initially charged with 75 L manure and progressively heated up to 37°C. Reactors were fed daily with 3.5 L d^{-1} of cattle manure and an equivalent amount of effluent sludge was removed, until a stable and similar methane production was reached at (mean ± SD) 14.0 ± 3.1 L CH_4 d^{-1}. This steady state was maintained for three days prior to the start of the experiment. On day 4, the feeding with specific solutions was initiated.

3 Results and Discussion

The ANAEROCHIP targets prokaryotes involved in the anaerobic digestion of wastes. The first version of the chip includes most lineages of methanogenic archaea known to be important in anaerobic digestion under mesophilic and thermophilic conditions (Franke-Whittle et al. 2009b). A new version including several bacterial guilds (hydrolytic-fermentative bacteria, acetogens, acidogens, hydrogenogens, etc.) is currently under development.

The prototype holds 103 probes, each printed in triplicate: a universal probe for archaea; 98 probes for methanogens (2 probes at family level, 4 probes targeting multiple genera, 79 probes at genus level and 13 probes at species level); two negative controls (bacterial probes) and three hybridisation controls (a positive control, a non-binding control and a blank). All the probes designed had a length between 17 and 24 base pairs (plus the T-spacer), a melting temperature between 56.3 and 61.4°C, a relatively low potential of formation of secondary structures (hairpins, loops and dimers) and were specific to their targets after analysis with the ARB software. Still, in order to account for the possible incomplete specificity in vitro, multiple probes were designed to target the same taxon (Wagner et al. 2007). Target genera and species, as well as the number of probes for the parallel detection of each taxon are given in Table 2.

Table 2 Methanogenic genera and species targeted by the ANAEROCHIP

Phylogenetic affiliation		Target	Presence[1]	No. of probes
Order	Family			
Methanobacteriales	Methanobacteriaceae	*Methanobacterium* sp.		6
		M. formicicum		2
		Methanobrevibacter sp.		4
		M. smithii		3
		Methanothermobacter sp.		4
		M. thermoautotrophicus		2
		Methanosphaera sp.		6
		M. stadtmanae		3
Methanomicrobiales	Methanocorpusculaceae	*Methanocorpusculum* sp.		4
	Methanomicrobiaceae	*Methanoculleus* sp.		5
		Methanogenium sp.		4
		Methanomicrobium sp.		4
	Methanospirillaceae	*Methanospirillum* sp.		3
		M. hungatei		3
	Unclassified	*Methanocalculus* sp.		6
Methanosarcinales	Methanosarcinaceae	*Methanosarcina* sp.		3
		Methanococcoides sp.		6
		Methanohalobium sp.		5
		Methanolobus sp.		4
	Methanosetaceae	*Methanosaeta* sp.		4
Methanococcales	Methanocaldococcaceae	*Methanocaldococcus* sp.		4
Uncertain		Uncultured clone 2–10		7

[1] Grey colour indicates detection in sludge after the digestion of cattle manure and/or olive-mill wastes

The optimisation of the ANAEROCHIP involved three steps. In a first phase, we hybridised it with a battery of pure cultures to: 1) discard unspecific probes, and 2) set the hybridisation conditions yielding the highest fluorescent signals and the lowest number of false positive and false negative signals. Only two probes were discarded and hybridisation at 55°C for 4 h gave the best results. The chip was submitted to hybridisation with: 1) PCR products obtained from decreasing amounts of DNA 2) PCR products of decreasing concentration, to define its sensitivity threshold. Detectable signals were obtained with 0.4 pg of DNA or 25 ng PCR products (Franke-Whittle et al. 2009b). In a second phase, the chip was hybridised with DNA extracted from anaerobic sludge collected from a full-scale CSTR in Voralberg, Austria. The same sample was analysed by constructing a 16S rRNA gene clone library with approximately 200 clones and sequencing the twelve ribotypes that were found. Only those ribotypes present at a frequency lower than 1.6% were not detected with the array (Franke-Whittle et al. 2009b).

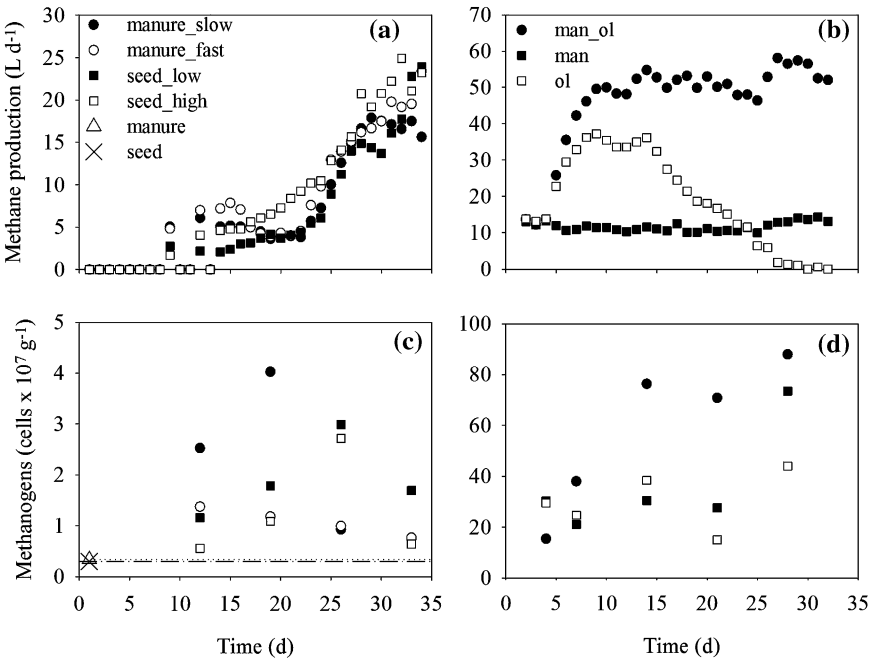

Fig. 5 Methane production (**a**,**b**), and methanogens (**c**,**d**) in the pilot anaerobic reactors

Finally, the ANAEROCHIP was evaluated by contrasting its results with those obtained by real-time quantitative PCR after analysing the sludge collected from pilot anaerobic reactors in two experiments. In Experiment 1, four reactor start-up strategies were assayed that resulted similarly successful in terms of reactor performance (Fig. 5a). The manure-based strategies produced biogas faster and were slightly more efficient in removing the waste pollutant load (Gadermaier 2009). Interestingly, the simulated overload conditions in the seed-based strategy operated at high flow rate did not induce reactor collapse, due to the sufficient inoculation of methanogenic biomass carried by the manure (Schoen et al. 2009). In Experiment 2, the codigestion of cattle manure and olive mill wastes was compared to the separate digestion of either residue. Reactor MAN_OL showed an increased methane production immediately after changing the load on day 4 from manure to manure:OMW (Fig. 5b). This stabilised on day 8 until the end of the experiment averaging 51.6 $\pm$ 3.3 L methane d^{-1}. Reactor MAN remained stable during the whole experiment with 11.7 $\pm$ 1.2 L d^{-1}. Reactor OL reacted rapidly to the initial change in feed until day 14 and then decreased rapidly most probably due to acidification of the reactor leading to methanogenesis inhibition through increased copper availability (Goberna et al. 2010b).

In both experiments, the ANAEROCHIP was used to screen the diversity of the methanogenic community. Twelve genera were detected in the sludge samples (Table 2), belonging to three out of the five taxonomic orders of described methanogens and displaying all known biochemical pathways for methane production,

i.e. from acetate, formate, H_2/CO_2 secondary alcohols and methyl compounds. Thus, the methanogenic communities were extremely rich in phylogenetic and metabolic terms. *Methanosarcina* probes showed by far the highest SNR values in all samples suggesting its generalized dominance. This has been previously reported in cattle manure digesting reactors (Demirel and Scherer 2008).

All methanogenic genera found to be more abundant using the ANAEROCHIP were quantified by real-time PCR. End-point PCR purified products from pure cultures or environmental clones were used as templates to build the standards for the quantification of the targets (Gadermaier 2009; Goberna et al. 2010a). The DNA concentration of the stocks ranged from 10^{10} to 10^{11} copies of the 16S rRNA gene per μL DNA. This concentration was found to remain stable for at least four months (maximum period tested) when stored at −20°C in low DNA binding tubes. The standard curves built for the target methanogenic genera were highly linear ($R^2 > 0.98$) for the range evaluated (1–10^9 copies of the gene per μL DNA).

Before conducting the quantification assays, the presence of inhibitors in the DNA extracts was tested. It is well reported that sludge DNA extracts may contain co-extracted substances that can inhibit the amplification reaction and quench fluorescence (Smith 2005) to an extent that undiluted DNA extracts may show no amplification of a certain target despite its presence in the sample. Testing several dilutions of the samples helps detecting inhibitors: the lesser the dilution, the more delayed the amplification (Smith 2005). Once the dilution is high enough so that the effect of the inhibitors is overcome, the increase in the Ct value should be proportional to the dilution factor (since Ct value is proportional to the initial DNA concentration). However, this is not the case for targets which are present in low numbers, such as methanogenic archaeal templates. In such cases, it is recommended to construct parallel curves with standards which are either unspiked or spiked with sludge DNA that contains a similar matrix to the sample DNA but negligible levels of the target (Strömbom & Kubista, pers. com.). We observed that spiking the standard curves with purified or non-purified sludge DNA did not change the amplification efficiencies compared to the unspiked standards (data not shown), indicating the negligible presence of inhibitors in our samples. Thus, neither dilution nor purification of the sludge DNA extracts was considered necessary.

In all cases, *Methanosarcina* represented more than 82% of all quantified methanogens, confirming the ANAEROCHIP results and indicating an uneven methanogenic community (Gadermaier 2009; Goberna et al. 2010a). After quantification of the number of gene copies per μL DNA in the samples, the results were converted to number of cells per gram sludge (wet weight). These calculations were based on two assumptions: 1) DNA extraction efficiency is equivalent among samples; 2) methanogens have an average of 2.5 copies of the 16S rRNA gene per cell (Klappenbach et al. 2001). During the start-up, methanogenic biomass in the manure and the seed sludge was sufficient to guarantee a smooth reactor performance, even under overload conditions (Figs. 5a, c). Methanogens peaked at days 19–26 and then decreased to levels slightly above those in the

initial materials. During the co-digestion of manure and OMW in reactor MAN_OL, a six-fold increase in the methanogenic biomass (mostly *Methanosarcina*) was detected that was correlated ($r_{Pearson} = 0.94$, $p = 0.02$) with the rise in biogas production (Figs. 5b, d). Reactors MAN and OL, which separately digested either cattle manure or OMW, showed fluctuating numbers of methanogens that did not reflect their constant or decreasing, respectively, methane production. Shifts in methanogenic activity, rather than biomass, most probably underlay such oscillations in biogas production.

4 Conclusions

We suggest a new approach for fingerprinting the prokaryotic communities involved in the biological treatment of organic wastes: the fast screening of the diversity using custom-made phylogenetic microarrays (COMPOCHIP and ANAEROCHIP) followed by the quantification of specific targets through real-time PCR. This might help us understand better the ecological needs of microbes in biodegradable residues, and eventually let us optimise their recycling.

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References

Demirel B, Scherer P (2008) The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion biomass to methane: a review. Rev Environ Sci Biotechnol 7:173–190

Felske A, Akkermans ADL, De Vos WM (1998) Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. Appl Environ Microbiol 64:4581–4587

Franke-Whittle IH, Knapp BA, Fuchs J, Kaufmannd R, Insam H (2009a) Application of COMPOCHIP microarray to investigate the bacterial communities of different composts. Microb Ecol 57:510–521

Franke-Whittle IH, Goberna M, Pfister V, Insam H (2009b) Design and development of the ANAEROCHIP microarray for investigation of methanogenic communities. J Microbiol Meth 79:279–288

Franke-Whittle IH, Goberna M, Insam H (2009c) Design and testing of real-time PCR primers for the quantification of *Methanoculleus*, *Methanosarcina*, *Methanothermobacter* and a group of uncultured methanogens. Can J Microbiol 55:611–616

Gadermaier M (2009). Monitoring prokaryotic communities during the start-up of anaerobic bioreactors. Institute of Microbiology, Diploma Thesis. University of Innsbruck, Innsbruck, Austria

Goberna M, Insam H, Franke-Whittle IH (2009) Effect of biowaste sludge maturation on the diversity of thermophilic bacteria and archaea in an anaerobic reactor. Appl Environ Microbiol 75:2566–2572

Goberna M, Gadermaier M, García C, Wett B, Insam H (2010a) Adaptation of methanogenic communities to the cofermentation of cattle excreta and olive mill wastes at 37°C and 55°C. Appl Environ Microbiol 76:6564–6571

Goberna M, Schoen MA, Sperl D, Wett B, Insam H (2010b) Mesophilic and thermophilic co-fermentation of cattle excreta and olive mill wastes in pilot anaerobic digesters. Biomass Bioenerg 34:340–346

Grosskopf R, Janssen PH, Liesack W (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl Environ Microbiol 64:960–969

Günther S, Groth I, Grabley S, Munder T (2006) Design and evaluation of an oligonucleotide-microarray for the detection of different species of the genus *Kitasatospora*. J Microbiol Meth 65:226–236

He Z, Gentry TJ, Schadt CW, Wu L, Liebich J, Chong SC, Huang Z, Wu W, Gu B, Jardine P, Criddle C, Zhou J (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. ISME J 1:67–77

Insam H, Franke-Whittle IH, Goberna M (2010) Microbes in aerobic and anaerobic waste treatment. In: Insam H, Franke-Whittle IH, Goberna M (eds) Microbes at work. From wastes to resources. Springer-Verlag, Heidelberg, Germany, pp 1–34

Klappenbach JA, Saxman PR, Cole JR, Schmidt TM (2001) rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res 29:181–184

Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, Schleifer K, Wagner M (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl Environ Microbiol 68:5064–5081

Ludwig W, Strunk O, Westram R et al (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–1371

Neufeld JD, Mohn WW, de Lorenzo V (2006) Composition of microbial communities in hexachlorocyclohexane (HCH) contaminated soils from Spain revealed with a habitat-specific microarray. Environ Microbiol 8:126–140

Sanguin H, Herrera A, Oger-Desfeux C, Dechesne A, Simonet P, Navarro E, Vogel M, Moënne-Loccoz Y, Nesme X, Grundmann G (2006) Development and validation of a prototype 16S rRNA-based taxonomic microarray for Alphaproteobacteria. Environ Microbiol 8:289–307

Schadt CW, Zhou J (2006) Advances in microarray-based technologies for soil microbial community analyses. In: Nannipieri P, Smalla K (eds) Nucleic acids and proteins in soil. Springer-Verlag, Heidelberg, Germany, pp 189–203

Schoen MA, Sperl D, Gadermaier M, Goberna M, Franke-Whittle I, Insam H, Ablinger J, Wett B (2009) Population dynamics at digester overload conditions. Bioresour Technol 100:5648–5655

Shigematsu T, Tang Y, Kawaguchi H, Ninomiya K, Kijima J, Kobayashi T, Morimura S, Kida K (2003) Effect of dilution rate on structure of a mesophilic acetate-degrading methanogenic community during continuous cultivation. J Biosci Bioeng 96:547–558

Skillman LC, Evans PN, Naylor GE, Morvan B, Jarvis GN, Joblin KN (2004) 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. Anaerobe 10:277–285

Smith CJ (2005) Quantitative real-time PCR. In: Osborn AM, Smith CJ (eds) Molecular microbial ecology. Taylor & Francis, Oxford, UK, pp 151–165

Stralis-Pavese N, Sessitsch A, Weilharter A, Reichenauer T, Riesing J, Csontos J, Murrel J, Bodrossy L (2004) Optimisation of diagnostic microarray for application in analysing landfill methanotroph communities under different plant covers. Environ Microbiol 6:347–363

Talbot G, Topp E, Palin MF, Massé DI (2008) Evaluations of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic reactors. Water Res 42:513–537

Wagner M, Smidt H, Loy A, Zhou J (2007) Unravelling microbial communities with DNA-microarrays: challenges and future directions. Microb Ecol 53:498–506

Wett B, Insam H (2010) Biogas technology–controlled gas flow for enhanced mixing, heating and desulfurization. In: Insam H, Franke-Whittle IH, Goberna M (eds) Microbes at work. From wastes to resources. Springer-Verlag, Heidelberg, Germany, pp 79–91

Wett B, Schoen MA, Phothilangka P, Wackerle F, Insam H (2007) Model-based design of an agricultural biogas plant: application of anaerobic digestion model no.1 for an improved four chamber scheme. Water Sci Technol 55:21–28

Hydrolases Activities of Extracted Humic Substances During Vermicomposting of Damaged Tomatoes Wastes Using a Continuous-Supplying System

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Abstract The behaviour of extracelullar hydrolytic enzyme activities in a continuous-supply vermicomposting system has never been studied previously. The aim of this study was to assess under such system the extracellular enzyme activities glucosidase, urease, acid phosphatase, and protease, which were extracted following the pyrophosphate-extraction method at pH 7.1. Vermicomposting was carried out using a medium-sized rectangular vermireactor continually supplied with damaged tomatoes (10 kg week^{-1}) during a 5-month period on a layer of mature sheep manure. The earthworms were then removed, and the vermicomposted organic material was matured over a 2-month period. Total earthworm biomass reached its greatest value after 3 months, coinciding with peak levels for pyrophosphate-extractable carbon content and extracellular urease, acid phosphatase and protease activities. Extracellular β-glucosidase activity peaked in the 4th month, when microbial activity was also at its greatest level. At the end of the vermicomposting period, phosphatase and protease activity decreased to levels similar to those recorded at the beginning of the vermicomposting process. By contrast, β-glucosidase and urease activity values were grater than those recorded at the start of the vermicomposting process. Humic-enzyme complexes generated during the vermicomposting period were unable to resist denaturation, inactivation, and degradation caused by the air-drying during the maturation phase. Although the mature vermicompost obtained showed higher content in humus-enzyme complexes than the initial mature sheep manure, the assayed continuous-supply vermicomposting system was unefficient for enhancing the formation of stabilized humus-enzymes complexes.

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1 Introduction

In Spain, approximately 66,100 ha are dedicated to greenhouse horticultural crops, most of which is located in Andalusia (Southern Spain). This intensive agricultural system generates large amounts of different types of waste every year, including vegetable wastes, which have become the greatest problem associated with this type of agriculture by exacerbating environmental problems associated with this crop. Damaged fruit waste consists of unmarketable fruits due to bruising, inappropriate size, rotting, and insect attack as well as marketable fruits that are discarded to raise market prices. In the Motril region alone (Granada, Southern Spain), about 15,000 tonnes of fruit waste were rejected in 2007, representing 24% of all greenhouse waste generation. Particularly, tomato fruit accounts for 80% of all damaged greenhouse fruit waste generates in this region.

Vermicomposting is an effective low-cost method of transforming organic waste into a stable end-product called vermicompost, which is characterized by relatively high levels of humus-like substances, active microorganisms and enzymes. This biological process can be carried out on a small, medium, or large scale by using a windrow, bed, or bin system and flow-through reactors. The type of system adopted—non-continuous batch-supply mode or continuous-flow supply mode—depends on the nature of the waste input recycled (Edwards 1995). Previous works have shown that non-continuous vermicomposting systems are capable of producing organic soil amendments and biofertilizers from agricultural, agroindustrial, and urban waste (Elvira et al. 1998; Nogales et al. 2005; Melgar et al. 2009). However, to date, a continuous-supply system has never been used to transform fruit wastes into stabilized organic soil amendment.

Formation of complexes between humic substances and extracellular enzymes is a mechanism to stabilize and protect enzymes in soil, avoiding its denaturation, inactivation and degradation (Burns 1982; Nannipieri et al. 1996). In non-continuous vermicomposting processes, the activity of some extracellular hydrolytic enzymes extracted with pyrophosphate increased or remained constant, thus suggesting that the humus enzyme complexes resisted microbial and earthworm attack (Benítez et al. 2000, 2005). This is particularly relevant from an ecological point of view, as stabilized and active humus-enzyme complexes in soil environments can reactivate soil C, N, and P-cycles (Pascual et al. 2002). In addition, the active humus-enzyme complexes in the vermicomposts have been found to be particularly useful for soil biochemical remediation when they are used as soil amendments (Benítez et al. 2004; Romero el al. 2005)

As the behavior of extracellular hydrolytic enzyme activity in continuous-supply vermicomposting systems has never been studied, the objective of this study was to analyze the evolution of extracellular enzyme activities during a continuous vermicomposting in order to monitor the process. The relationship between extracellular enzyme activities and humic-like substances was also studied as a possible tool for characterizing the degree of stability of the vermicompost obtained as well as its potential biochemical contributions to soil quality

and fertility. To do this, β-glucosidase, acid phosphatase, protease, and urease extracellular enzyme activities were determined in the pyrophosphate extract during a continuous-supply vermicomposting process using damaged tomato-fruit.

2 Materials and Methods

A medium-sized rectangular metallic vermireactor (0.6 × 0.9 × 0.2 m) was designed, and 0.1 cm mesh was placed at the bottom of the vermireactor. A 5 cm layer containing 15 kg dry weight of sheep manure, whose chemical characteristics are described in Table 1, was placed on the mesh to provide an appropriate habitat for earthworms. A total of 500 g of clitellated and non-clitellated earthworms (*Eisenia fetida*) were inoculated in this layer. The worms came from a culture stock in the Estación Experimental del Zaidín (CSIC) in Granada (Spain). Fifteen days after earthworm inoculation, the vermireactor was filled with a liquid-paste from damaged tomatoes (moisture: 92%, pH: 3.9, EC: 1.8 dS m^{-1}, TOC: 459 g kg^{-1}, TKN: 23 g kg^{-1} and C/N: 20) at a loading rate of 10 kg $week^{-1}$. The damaged tomatoes were obtained from greenhouse crops in the Motril region (Granada, Southern Spain). Vermicomposting was carried out under controlled temperature conditions (25°C), and no water was added other than that already present in the semiliquid tomato paste. After 5 months, the earthworms were removed by hand, and the vermicomposted organic substrate was left in a pile for a 2-month maturation period without water addition. During the vermicomposting process, the organic substrate was sampled monthly by means of five cylindrical cores (600 cm^3), which were evenly placed in the vermireactor. In each core, earthworm biomass was recorded, and the worms were replaced in the vermireactor. A fraction of the organic substrate (~100 g) contained in each core was taken, homogenized and stored in plastic vials at −20°C for analysis. In addition, five cores were also taken from the initial layer of sheep manure and the vermicompost obtained after the maturation period for analysis. Chemical and enzyme activities analysis of organic substrate contained in each core was carried out in triplicate.

The pH, electrical conductivity (EC), total organic carbon (TOC) and total Kjeldahl nitrogen (TKN) were determined according to validated methods (MAPA 1986). The ammonium–N concentration (NH_4^+–N) was determined after extraction with 2 M KCl using a modified salicylate-nitroprusside colorimetric method (Kandeler and Gerber 1988). Water-soluble carbon (WSC) was extracted with distilled water (1:10 w/v) by mechanical shaking at 60°C for 1 h. WSC was analyzed in the supernatant after centrifugation 8,000 *g*.

The pyrophosphate extractable carbon (PEC) was extracted with $Na_2P_4O_7$ (0.1 M, pH 7.1) in a 1:10 (w:v) ratio by mechanical shaking at 37°C for 24 h. The suspension was centrifuged at 8,000 *g*; the supernatant was filtered through a 0.45 μm Millipore membrane, which was then dialyzed for 7 days against distilled water, which was changed daily, in order to generate the pyrophosphate

Table 1 Chemical analyses in the sheep manure (S), at initial (V0) and after the vermicomposting period (V5), and in mature vermicompost (MV)

	pH	EC dS m^{-1}	NH_4^+–N mg kg^{-1}	TOC g kg^{-1}	TKN g kg^{-1}	WSC g kg^{-1}	C/N	Moisture %
S	8.6 ± 0.3^c	1.8 ± 0.1^b	438 ± 32^c	138 ± 6^b	9.6 ± 0.2^c	12 ± 0.5^c	14 ± 0.1^a	7 ± 0.2^c
V0	8.3 ± 0.5^c	1.7 ± 0.1^b	271 ± 42^b	140 ± 3^b	12 ± 1.2^b	18 ± 1.5^b	11 ± 0.6^b	55 ± 1^a
V5	9.5 ± 0.5^b	4.5 ± 0.1^a	$1{,}992 \pm 285^a$	280 ± 12^a	24 ± 0.9^a	25 ± 3^a	12 ± 0.5^b	57 ± 3^a
MV	10.4 ± 0.3^a	4.4 ± 0.03^a	$1{,}761 \pm 71^a$	148 ± 6^b	13 ± 0.4^b	17 ± 0.2^b	11 ± 0.1^b	25 ± 0.8^b

In each parameter, different letters indicate significant differences ($P < 0.05$)

extractable carbon (PEC) solution. Humic acid-like compounds (HAL) were extracted from 10 ml of a PEC solution by means of acidification with 97% H_2SO_4 (pH 1.0) and then centrifuged. The precipitate was dissolved in 10 mL of 0.5 M NaOH to obtain the HAL solution. To determine the C content of the WSC, PEC, and HAL solutions, 1 mL of each solution was digested with 1 mL of 1 N $K_2Cr_2O_7$ and 2 mL of 97% H_2SO_4 at 160°C for 30 min. Spectrophotometry ($\lambda = 590$ nm) was used to quantify the Cr^{3+} produced by the reduction of Cr^{6+} (Sims and Haby 1971).

Dehydrogenase activity was determined incubating 0.5 of solid organic sample at 25°C for 20 h with 0.2 mL of 0.4% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium chloride (INT). Controls without INT were also incubated. The iodonitrotetrazolium formazan (INTF) produced by the reduction of INT was extracted with acetone:tetrachloroethene (1.5:1) and measured in a spectrophotometer at 490 nm (García et al. 1997). To determine the absolute extracellular β-glucosidase and the absolute extracellular acid phosphatase activity, 0.5 mL of PEC solution was incubated at 37°C for 2 h with 0.5 mL of each enzyme-substrate solution and 2 mL of 0.1 M maleate buffer at pH 6.5. An enzyme-substrate solution with 0.05 M 4-nitrophenyl-β-D-glucanopyranoside (*p*-NG) or 0.115 M *p*-nitrophenyl phosphate (*p*-NPP) was used as enzyme-substrate for β-glucosidase or acid phosphatase activity, respectively. Controls were run as an enzyme test, but the enzyme-substrate was added at the end of incubation, before the determination of enzyme-product. The *p*-nitrophenol (*p*-NP) produced in the enzyme reaction was extracted and determined spectrophotometrically at 398 nm as described by Tabatabai and Bremner (1969). Absolute extracellular urease and protease activities were determined using 0.5 mL of PEC solution, 2 mL of 6.4% urea or 0.5 mL N-α-benzoyl-L-argininamide respectively as substrates, and 2 mL of 0.1 M phosphate buffer at pH 7.0. Controls were run as an enzyme test, but distilled waster was added instead of the enzyme-substrate. Enzyme tests and controls were incubated at 37°C for 2 h and straight afterwards the reaction was stopped cooling down at 2°C for 15 min as described by Nannipieri et al. (1980). The NH_4^+ released into the solution from the hydrolytic reaction was measured using an ammonium-selective electrode (ORION, mod. 95–12). The specific extracellular enzyme activity was calculated from the absolute extracellular enzyme activity values divided by the C content of the PEC solution.

The evolution of all parameters determined during the whole process was statistically tested using repeated analysis of variance (ANOVA) measures. The least significant difference test (LSD) was used for post-hoc mean separation. A paired-sample *t*-test was performed to assess the differences in chemical parameters between initial sheep manure and mature vermicompost. All statistical analyses were conducted using SPSS® Windows Version 13.0 (Chicago, Illinois, USA).

3 Results and Discussion

The weekly application of damaged tomatoes did not significantly increase total earthworm biomass during the first two months of vermicomposting (Fig. 1). Nevertheless, a pronounced increase was observed in the 3rd month, when the percentage of total earthworm biomass was 3.3-fold higher than that at the beginning of the process. However, earthworm biomass decreased sharply in the 4th month, in coincidence with the peak in the microbial activity, as indicated by dehidrogenase activity (Fig. 1), which is used to assess overall microbial activity during vermicomposting (Benítez et al. 1999). From the 4th month until the end of the vermicomposting process, total earthworm biomass decreased slightly, though not significantly. Conversely, a significant fall in dehydrogenase activity could suggest a microbial turnover in the vermireactor microbiota. The reduction in earthworm biomass could be due to the following factors (shown in Table 1): (i) an increase in ammonium concentration in the substrate as a consequence of the mineralization of proteins from the tomato-fruit waste and manure layer, (ii) an increase in pH value due to the decomposition of abundant organic anions in tomatoes, as reported for other plant residues (Xu et al. 2006), and (iii) an increase in electrical conductivity (EC) recorded in the vermireactor substrate due to the weekly salt intake by the tomatoes waste added to the vermireactor. These increases in the organic substrates are well known to negatively affect normal earthworm development during the vermicomposting process (Nogales et al. 2008). The increases in ammonium, pH and EC could be explained by: (i) excessive fresh organic matter in the vermireactor at the 3rd month as consequence of continuous additions of tomato-fruit waste and (ii) the high level of earthworm activity in the 3rd month which greatly stimulated microbial activity. These two factors triggered microbial growth and activity in the vermireactor substrate, leading to imminent system instability. To optimize the benefits of this continuous-supply system in subsequent trials, the organic loading rate should therefore be reduced, the earthworm population halved in the 3rd month, and another vermireactor constructed with an additional manure layer.

The pyrophosphate extractable carbon (PEC) and the humic-acid like substances (HAL) contents (Fig. 2) peaked in the 3rd month as a result of large amounts of organic matter from additions of tomato-fruit waste. The increase in both PEC and HAL may indicate that all the fresh substrate added was efficiently

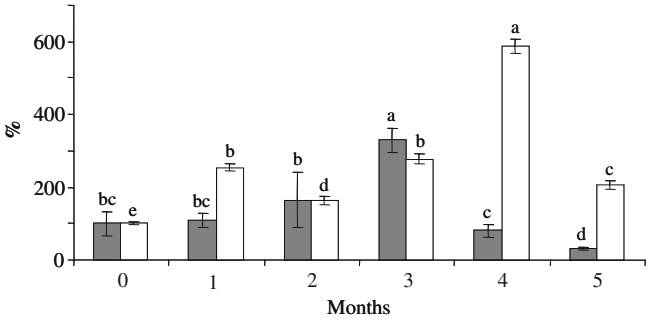

Fig. 1 Percentage changes in total earthworm biomass (■) and dehydrogenase activity (□) during the vermicomposting period. *Bars* represent standards errors. In each parameter, columns with different letters indicate significant differences ($P < 0.05$)

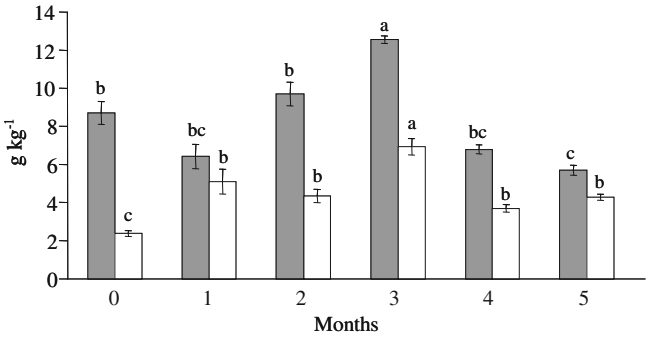

Fig. 2 PEC (■) and HAL (□) during the vermicomposting process. *Bars* represent standards errors. In each parameter, columns with different letters indicate significant differences ($P < 0.05$)

transformed by earthworms. However, from the 4th month until the end of the vermicomposting process, while more tomato-fruit waste was added, PEC and HAL content fell sharply. This could be attributed to a fall in earthworm biomass together with an increase in microbial activity. The PEC solution contains both humic-like and more recalcitrant compounds (Benítez et al. 2000) which were possibly not degraded by earthworms. Both types of compound could have subsequently been degraded by the proliferation of microorganisms in the 4th month. From the 4th month onwards, PEC and HAL content remained more or less stable.

Absolute extracellular β-glucosidase activity significantly increased and reached peak levels in the 4th month (Fig. 3a). Although this extracellular enzyme activity later decreased in the 5th month, it remained above the level recorded at the beginning of the vermicomposting process. The maximum level recorded by extracellular β-glucosidase activity coincided with the peak in microbial activity

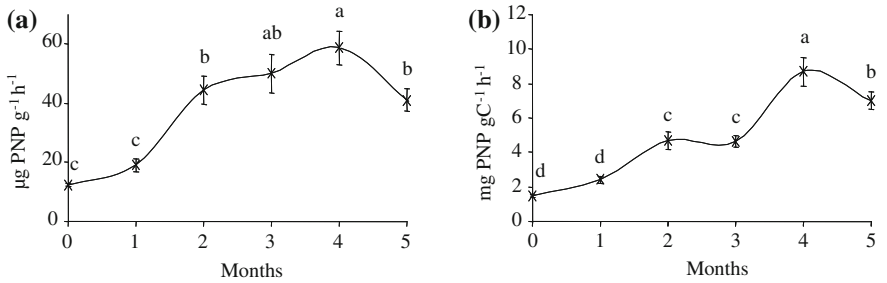

Fig. 3 Absolute (**a**) and specific (**b**) extracellular β-glucosidase activity during the vermicomposting process. Values are means of fifteen replicates. *Bars* represent standards errors. Different letters indicate significant differences ($P < 0.05$)

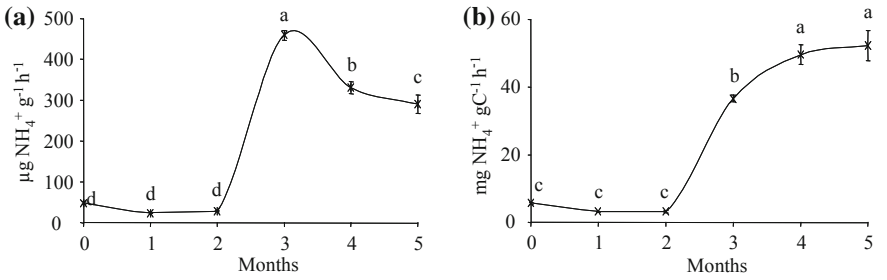

Fig. 4 Absolute (**a**) and specific (**b**) extracellular urease activity during the vermicomposting process. Values are means of fifteen replicates. *Bars* represent standards errors. Different letters indicate significant differences ($P < 0.05$)

(Fig. 1). This suggests that extracellular β-glucosidase activity greatly depends on microbes which are responsible for enzyme synthesis, whereas that the reduction in earthworm biomass, PEC, and HAL observed from the 4th month onwards did not affect this extracellular enzyme.

In contrast with absolute extracellular β-glucosidase activity, absolute extracellular urease activity (Fig. 4a) behaved differently, recording a sharper increase in the 3rd month, coinciding with growth in earthworm biomass (Fig. 1), and then fell significantly at the end of the vermicomposting process. This pattern of enzyme growth suggests that urease activity in organic extracts was strongly influenced by the earthworm population. On the other hand, despite the reduction in PEC and HAL recorded in the 5th month, the level of absolute extracellular urease activity was higher than that recorded at the beginning of the process. This suggests that a fraction of this extracellular enzyme may bind to humic matter during the vermicomposting process, thus able to remain active in the 5th month despite the high NH_4^+–N concentrations recorded in the substrate (Table 1), which inhibit extracellular enzyme activity (McCarty et al. 1992).

The absolute extracellular activity of acid phosphatase and protease showed a similar trend during the vermicomposting process (Figs. 5a and 6a). In both cases,

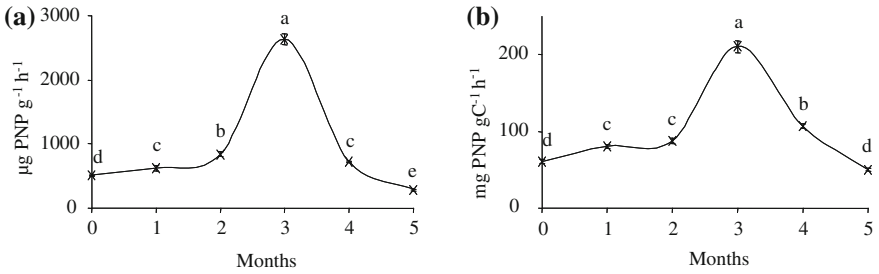

Fig. 5 Absolute (**a**) and specific (**b**) extracellular acid phosphatase activity during the vermicomposting process. *Bars* represent standards errors. Different letters indicate significant differences ($P < 0.05$)

enzyme activity increased significantly and reached a peak in the 3rd month, coinciding with maximum earthworm biomass and PEC content levels, and then decreased sharply to below the values recorded at the start of the process. As with the pattern for absolute extracellular urease activity, the highest level of acid phospatase and protease activity was recorded in the 3rd month, suggesting that the large earthworm population transformed the biological composition of the fresh organic matter through enrichment with microorganisms and extracellular enzymes. In line with these findings, Parthasarathi and Ranganathan (1999, 2000) reported that, compared to initial wastes, the casts freshly produced by earthworms have higher microbial, phosphatase and protease activity as well as larger microbial populations. The decrease in both types of absolute extracellular enzyme activities from the 3rd month onward could be explained by the reduction in earthworm biomass and the environmental constraints of adverse physico-chemical conditions (increased pH) and product inhibition (NH_4^+–N) observed in the vermireactor (Fig. 1 and Table 1).

The specific extracellular activity (activity per unit of extracted carbon) of theses enzymes showed a similar pattern to absolute extracellular activity (Figs. 3b, 5b and 6b), except in the case of specific extracellular urease activity (Fig. 4b), which unlike its absolute extracellular activity (Fig. 4a), continued to increase from the 3rd month up to the end of vermicomposting period. Benítez et al. (2000) concluded that the fact that both absolute and specific extracellular enzyme activity increase during vermicomposting gave rise to several assumptions: 1) the association of enzyme with humic substances did not affect activity enzyme sites, 2) the humic-enzyme complex is capable of resisting the microbial or earthworm attack as well as extracellular proteases and others inhibitory and degrading effects, and that 3) extracellular enzymes in vermicompost are more dependent on the type of humic compound than the quantity of C in the extract (García et al. 1993).

Slight increases in PEC and HAL was observed in mature vermicompost (MV) as compared with those recorded in fresh vermicompost (V5) (Table 2). In comparative terms, PEC and HAL content in the mature vermicompost (MV) was

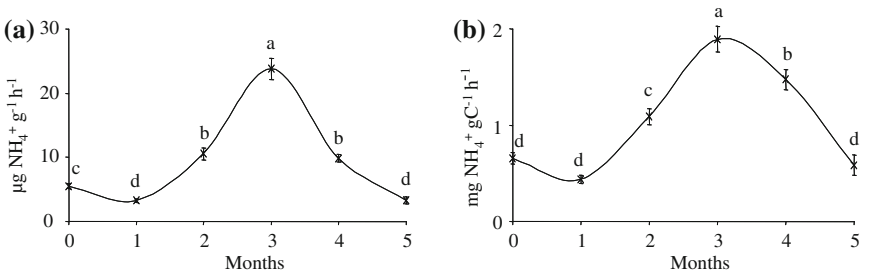

Fig. 6 Absolute (**a**) and specific (**b**) extracellular protease activity during the vermicomposting process. *Bars* represent standards errors. Different letters indicate significant differences ($P < 0.05$)

Table 2 PEC, HAL, absolute and specific extracellular hidrolases in the sheep manure (S), after the vermicomposting period (V5), and in the mature vermicompost (MV)

		S	V5	MV
PEC g kg^{-1}		2.1 ± 0.1^{c}	5.7 ± 0.3^{b}	6.3 ± 0.1^{a}
HAL g kg^{-1}		0.6 ± 1^{b}	4.3 ± 0.2^{a}	4.4 ± 0.03^{a}
β-glucosidase	Absolute (μg PNP g^{-1} h^{-1})	1.7 ± 0.2^{c}	41 ± 4^{a}	11 ± 0.4^{b}
	Specific (mg PNPg C^{-1} h^{-1})	0.8 ± 0.1^{c}	7 ± 0.5^{a}	1.8 ± 0.1^{b}
Acid phosphatase	Absolute (μg PNP g^{-1} h^{-1})	7.1 ± 1.5^{c}	287 ± 14^{a}	211 ± 4^{b}
	Specific (mg PNP gC^{-1} h^{-1})	2.9 ± 0.7^{c}	50 ± 1.3^{a}	34 ± 0.7^{b}
Protease	Absolute (μg NH_4^+ g^{-1} h^{-1})	14 ± 0.9^{a}	3.2 ± 0.5^{b}	0.7 ± 0.1^{c}
	Specific (mg NH_4^+ g C^{-1} h^{-1})	7.2 ± 0.4^{a}	0.6 ± 0.1^{b}	0.11 ± 0.01^{c}
Urease	Absolute (μg NH_4^+ g^{-1} h^{-1})	$4.1 \pm 0.^{9}c$	291 ± 23^{a}	29 ± 1.6^{b}
	Specific (mg NH_4^+ g C^{-1} h^{-1})	2.0 ± 0.4^{c}	52 ± 4^{a}	4.6 ± 0.2^{b}

In each parameter, different letters indicate significant differences ($P < 0.05$)

3- and 7.7-fold higher, respectively, than the levels recorded in sheep manure (S). However, the absolute and specific extracellular enzyme activities were significantly lower in MV than those recorded in V5 (Table 2). It is important to note that most of the humic-enzyme complexes generated by the vermicomposting process were unable to resist denaturation, inactivation, and degradation caused by the air-drying of the vermicompost during the maturation phase (Table 1). Burns (1982) reported that the formation of humic complexes stabilizes enzymes and ensures their persistence, which would otherwise be impossible under adverse extracellular environmental conditions. On the other hand, it is well known that air-drying leads to inactivation of extracellular enzymes (Dick 1994). Since extracellular enzyme activity depends mainly on free enzymes in the vermicompost solution and a smaller fraction linked to humic substances, the drying of the vermicompost caused inactivation of free extracellular enzymes in the organic extract, while stabilized enzymes were protected against the adverse effects of low water content. Absolute and specific extracellular enzyme activities measured in air-dried mature vermicompost thus resembles the activity of enzymes closely linked to humus colloids. Thus, it was

found that the levels of absolute and specific extracellular β-glucosidase, urease, and acid phosphatase activity were significantly higher in the mature vermicompost than in the initial sheep manure used (Table 2). However, extracellular protease enzyme complexes produced during the vermicomposting process were not sufficiently stabilized and were thus strongly degraded by drying during the maturation phase, as suggested by the lower extracellular enzyme activity recorded in the mature vermicompost as compared to the initial sheep manure.

4 Conclusions

Extracellular hydrolytic enzyme activities measured in the pyrophosphate extract during the continuous-supplying vermicomposting process showed a pattern that has not previously been reported. Extracellular β-glucosidase activity was related to the microbial activity, while extracellular urease, acid phosphatase, and protease activities were influenced by the earthworm biomass observed during the vermicomposting period. After the maturation period, all extracellular enzyme activity measured in the pyrophosphate extract eventually decreased due to air-drying as the free extracellular enzymes and enzymes weakly linked to humus colloids were denatured or inactivated. Therofore, the assayed continuous-supply vermicomposting system was unefficient for enhancing the formation of stabilized humus-enzymes complexes. Despite this fact, the mature vermicompost obtained showed greater extracelular enzyme activities (β-glucosidae, acid-phosphatase and urease) as compared with those recorded in the sheep manure used as an initial layer for tomato-fruit waste bioconversion.

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References

Benítez E, Nogales R, Elvira C, Masciandaro G, Ceccanti B (1999) Enzymes activities as indicators of the stabilization of sewage sludges composting by *Eisenia andrei*. Bioresour Technol 67:297–303

Benítez E, Nogales R, Masciandaro G, Ceccanti B (2000) Isolation by IEF of humic–urease complexes from earthworms processed sewage sludges. Biol Fertil Soils 31:489–493

Benítez E, Melgar R, Nogales R (2004) Estimating soil resilience to toxic waste by measuring enzyme activities. Soil Biol Biochem 36:1615–1621

Benítez E, Sainz H, Nogales R (2005) Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. Bioresour Technol 96:785–790

Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423–427

Dick RP (1994) Soil enzyme activities as indicators of soil quality. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (eds) Defining soil quality for a sustainable environment. American Society of Agronomy-Soil Science Society of America, Special Publication No. 35, Madison,WI, pp 107–124

Edwards CA (1995) Historical overview of vermicomposting. BioCycle 36:56–58

Elvira C, Sampedro L, Benítez E, Nogales R (1998) Vermicomposting of sludges from paper mill and dairy industries with *Eisenia andrei*: a pilot scale study. Bioresour Technol 6:205–211

García C, Henández T, Costa F, Ceccanti C, Ganni A (1993) Hydrolases in the organic matter fractions of sewage sludge: changes with composting. Bioresour Technol 45:47–52

García C, Hernández T, Costa F (1997) Potencial use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun Soil Sci Plan 28:123–134

Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72

MAPA (1986) Métodos Oficiales de Análisis. Vol III Plantas, Productos Orgánicos Fertilizantes, Suelos, Agua, Productos Fitosanitarios y Fertilizantes Inorgánicos. Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain

McCarty GW, Shogren DR, Bremner JM (1992) Regulation of urease production in soil by microbial assimilation of nitrogen. Biol Fertil Soils 12:261–264

Melgar R, Benítez E, Nogales R (2009) Bioconversion of wastes from olive oil industries using the epigeic earthworm *Eisenia Andrei*. J Environ Sci Health–Part B 44:488–495

Nannipieri P, Ceccanti B, Cervelli S, Matarese E (1980) Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016

Nannipieri P, Sequi P, Fusi P (1996) Humus and Enzyme Activity. In: Piccolo A (ed) Humic substances in terrestrial ecosystems. Elsevier Science, Amsterdam, The Netherlands, pp 293–328

Nogales R, Cifuentes C, Benítez E (2005) Vermicomposting of winery wastes: A laboratory study. J Environ Sci Health–Part B 49:659–673

Nogales R, Domínguez J, Mato S (2008) Vermicompostaje. In: Moreno J, Moral R (eds) Compostaje. Editorial Mundi-Prensa, Madrid, Spain pp 187–2008

Parthasarathi K, Ranganathan LS (1999) Longevity of microbial and enzyme activity and their influence on NPK content in pressmud vermicasts. Eur J Soil Biol 35:107–113

Parthasarathi K, Ranganathan LS (2000) Aging effect on enzyme activities in pressmud vermicasts of Lampito mauritii (Kinberg) and Eudrilus eugeniae (Kinberg). Biol Fertil Soils 30:347–350

Pascual JA, Moreno JL, Hernández T, García C (2002) Persistence of immobilised and total urease and phosphatase activities in a soil amended with organic wastes. Bioresour Technol 82:73–78

Romero E, Benítez E, Nogales R (2005) Suitability of wastes from olive-oil industry for initial reclamation of a Pb/Zn mine tailing. Water Air Soil Pollut 165:153–165

Sims JR, Haby VA (1971) Simplified colorimetric determination of soil organic matter. Soil Sci 112:137–141

Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenylphosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Xu JM, Tang C, Chen ZL (2006) The role of plant residues in pH change of acid soils differing in initial pH. Soil Biol Biochem 38:709–719

L-glutaminase Activity of Organic Amendments

Juana Isabel López, Milagros Navarro, Salvador González and Carlos García

Abstract High contents of organic matter are present in composts of urban wastes, part of which are easily hydrolysable and mineralisable by soil microorganisms. The metabolic use of this organic fraction depends on the activity of hydrolytic enzymes produced by soil microbial communities, especially those related with N cycle, in which L-glutaminase plays an important role. In this work, L-glutaminase was extracted from compost of the organic fraction of urban solid wastes using pyrophosphate solutions at different molarities, different pHs and under different operational conditions. Optimal conditions for L-glutaminase extraction were 0.2 M sodium pyrophosphate at pH 11, a compost-solution ratio 1:10 (w:v) and 90 min of end-over-end shaking at 4°C. Under these conditions, an important increase in the extracted activity was found compared to the initial L-glutaminase in the compost (81.6 ± 6.9 μg NH_4^+–N g^{-1} compost 3 h^{-1}). Crude extract was fractionated by micro- and ultrafiltration into three molecular fractions: UF_1 (MW < 10 kDa), which accumulated soluble low MW-compounds, UF_2 (MW > 10 kDa, $\phi < 0.45$ μm), with desalted humus-like materials and extracellular proteins and UF_3 ($\phi > 0.45$ μm), with cell-associated enzymes. All of the molecular fractions displayed L-glutaminase activity, but the extraction using 0.2 M at pH 11, displayed the highest enzymatic activity in the UF_1 fraction probably as a consequence of both, cell-lysis and the dissociation of the quaternary structure of the enzyme.

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1 Introduction

In Mediterranean areas, the lack of organic matter affects negatively the fertility and productivity of soils facilitating intense degradative processes such as soil erosion (Albaladejo and Díaz 1990; García et al. 2000). The incorporation of exogenous organic matter to these soils is a form to remediate this problem because it favours soil microbial activity and biogeochemical cycles (Pascual et al. 1997). The use of this type of amendment, in which composts from different urban wastes are included, supposes also a fertiliser contribution to soils, particularly nitrogen (Ayuso et al. 1996). Important amounts of this nitrogen are found in organic forms and it would be of great interest to know which is the rate of their mineralization. Organic nitrogen in soils consists of a variety of compounds including amino acids, present as proteins and peptides, and amino sugars (Stevenson 1994). Nitrogen mineralization is influenced by the interaction between both, substrates of different biochemical composition and the enzymes synthesized by the microbial communities (Sinsabaugh 1994). Enzymatic activities related to soil N cycle increase with the organic matter contribution, existing a positive correlation between organic C and soil N content (Frankenberger and Tabatabai 1991a).

An aminohydrolitic enzyme that plays a very important role in N mineralization processes is L-glutaminase (Nourbakhsh and Alinejadian 2009). This enzyme is responsible for catalysing the hydrolitic degradation of the L-glutamine amide group to produce glutamic acid and NH_3. L-glutaminase is widely spread in nature and was firstly detected in soils by Galstyan and Saakyan (1973). Plants and microbes are probable sources of L-glutaminase activity in soils, but the main source is believed to be microbial (Imada et al. 1973; Frankenberger and Tabatabai 1991b); L-glutaminase activity plays an important role in maintaining soil quality and is shown to be a responsive indicator to organic fertilization (Deng et al. 2006; Hojjati and Nourbakhsh 2007) or cropping systems (Dodor and Tabatabai 2007).

In soils, an important part of the extracellular enzyme activity could be associated to the humic fraction, making up what we know as humus-enzyme complexes (Nannipieri et al. 1990). Different humus-enzyme complexes have been extracted from soils using saline solutions of different nature and concentration: a mixture of 0.2 M phosphate and 0.2 M EDTA at pH 8 was used by to extract cellulase, phosphatase, protease and β-glucosidase form soil (Batistic et al. 1980); 0.14 M sodium pyrophosphate solution at pH 7.1 to extract phosphatase, urease and protease (Nannipieri et al. 1980); 0.01 M sodium pyrophosphate solution at neutral pH values for β-glucosidase (Busto and Pérez-Mateos 1995); and 0.05 M sodium pyrophosphate at pH 9 to extract proteases from the compost of an urban solid waste (Rad et al. 1995).

The aim of this work was to optimise the extraction procedure of L-glutaminase activity from a compost obtained from the organic fraction of an urban solid waste, using pyrophosphate as extractant solution ($Na_2H_2P_2O_7$). Different variables were tested: the concentration of the extractant, the extraction pH, the contact time between compost and extractant, the extractant:compost ratio, and

the use of a sequential extraction procedure using both, sodium and potassium pyrophosphate.

2 Materials and Methods

2.1 Urban Organic Waste Compost

The material used in this experience was a compost obtained from the biodegradable fraction of an urban solid waste generated in the city of Burgos (Spain) after a bio-oxidative process of four weeks and a maturation process of 6 months. The sample was taken from different points of the compost heap, sieved (< 2 mm) and carried out to the laboratory where it was stored in polyethylene bags, at ambient temperature until its use. The fraction smaller than 2 mm was crushed in an agate mill balls 50 agate kept at −20°C until its analysis. The compost was characterised in its physical and chemical properties according to MAPA (1986)and are shown in Table 1.

2.2 Extraction of Enzymatic Complex

The extraction was carried out using 0.2 M $Na_2H_2P_2O_7$ at a pH ranging from 2 to 11. The solid:liquid ratio was 1:20 (w:v) which was considered suitable for the extraction of compost humic substances (Masciandaro et al. 2000). Optimal extraction was carried out at 4°C by end-over-end shaking at 250 rpm for 30 min. Thereafter, the mixture was centrifuged at 21,000 g for 30 min at 4°C and filtered (Whatman, No. 4).

For method optimization, different concentrations of pyrophosphate: 0.05, 0.1 and 0.2 M, all of them with the same pH rank (2–11), solid:extractant ratios: 1:3, 1:5, 1:10, 1:20 and 1:40 (w:v), increasing extraction times: from 5 to 180 min, and a sequential extraction involving sodium and potassium pyrophosphate were assayed.

2.3 Ultrafiltration of Extracts

The initial extract was fractionated by ultrafiltration (Minitan System, Millipore) in three molecular fractions following the method defined by Rad et al. (1995). Firstly, 50 mL of extract was diafiltered through a 0.45 μm cut-off membrane (Millipore, type HVLP) with the continuous addition of 200 mL of distilled water. The retentate was fitted to 50 mL with distilled water to preserve the initial volume

Table 1 Characteristics of the compost used

Parameter	Mean ± error
Dry matter[#]	86.1 ± 4.3
Ash[#]	77.7 ± 3.1
Bulk density[++]	2.53 ± 0.21
pH (1/5 distilled water)	7.7 ± 0.3
Organic matter[#]	19.15 ± 1.12
Total N[#]	0.57 ± 0.11
Total P[#]	0.37 ± 0.08
Fe[‡]	20,725 ± 1,053
Al[‡]	17,405 ± 1,142
Mn[‡]	343 ± 21
Cr[‡]	207 ± 16
Zn[‡]	711 ± 23
Ni[‡]	87.8 ± 6.2
Pb[‡]	626 ± 78
Cd[‡]	8.7 ± 1.8
Cu[‡]	350 ± 41
L-glutaminase[##]	81.6 ± 6.9

[#] in %; [++] in kg L^{-1} ; [‡] in mg kg^{-1} ; [##] in μg NH_4^+ –N g^{-1} compost 3 h^{-1}

of extract and constituted the UF_3 fraction, containing mainly cell-bound enzymes. The filtered solution was then passed through another membrane of 10 kDa MWCO (Millipore, Type PTGC). It was initially concentrated to 50 mL and thereafter diafiltered with 300 mL of distilled water. Fifty mL of retentate were finally obtained containing the exocellular extracted molecules in a salt-free UF_2 fraction. Finally, 50 mL of the UF_1 fraction, containing salts and compounds with MW $<$ 10 kDa, was obtained by concentrating the last filtrate in a rotary evaporator at 40°C.

2.4 Measurement of L-glutaminase Activity

L-glutaminase was assayed using D, L-glutamine as substrate and the colorimetric determination of the NH_3 liberated as a consequence of amidohydrolysis reaction. Briefly, 1 g of compost was mixed with 2 mL of 0.1 M phosphate buffer at pH 8 and 1 mL of substrate, L-glutamine 3% (w/v) in water and incubated for 3 h at 37°C. The enzymatic reaction was stopped by the addition of 7 mL of KCl–Ag_2SO_4 solution, filtered and the ammonium released determined colorimetrically according Kandeler and Gerber (1988); controls of enzyme activity were also performed adding the substrate after the enzymatic reaction was stopped. For liquid samples, 1 mL of crude or ultra filtered extracts and 1 mL of phosphate buffer were used with the same protocol.

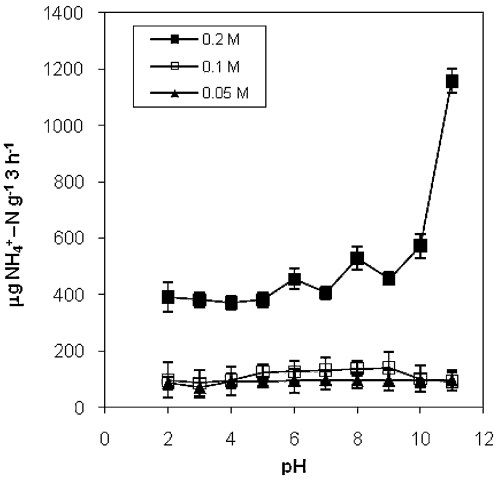

Fig. 1 Influence of the molarity of pyrophosphate and pH of extraction in the recovery of L-glutaminase from compost

2.5 Statistical Analysis

A one-way ANOVA was carried out with the different variables to assess differences between treatments using Stat graphics. The comparison of means was made according the LSD multiple range test procedure calculated at $P < 0.05$ level.

3 Results and Discussion

3.1 Influence of Molarity and pH on the Extraction of L-glutaminase in Compost by Pyrophosphate

Several hydrolases, such as phosphatases, β-glucosidases or ureases, has been detected in stabilised composts or vermicomposts of organic wastes from very different origin: sludges, crop residues or manures (García et al. 1995; Benítez et al. 1999; Vuorinen 1999). However, there is a lack of knowledge of the presence and the activity of other hydrolytic enzymes related to N cycle. In this work, the L-glutaminase activity determined in compost samples was 81.62 ± 6.9 µg NH_4^+–N g^{-1} compost 3 h^{-1}. This fact means that aminohydrolase synthesis is not only an active biochemical pathway during the active phase of composting processes, it is also an evidence that stabilization mechanisms would be present during the curing phase of compost which would be responsible for maintaining the enzyme activity in the stabilised final product.

The combined effect of pyrophosphate concentration and extraction pH is reported in Fig. 1. Optimal extraction conditions were found using 0.2 M $Na_2H_2P_2O_7$ at strongly basic pH values (pH 11). Statistical analysis of these results

Table 2 Statistical significance of ANOVA (F-value) of the optimization procedure for L-glutaminase extraction from compost

Molarity and pH		Molecular fractionation		Sequential extraction	
[Pyr]	$13{,}898^{***}$	UF	$16{,}494^{***}$	Seq	$4{,}023^{***}$
pH	461^{***}	pH	$24{,}690^{***}$	pH	739^{***}
[Pyr]*pH	477^{***}	UF*pH	$7{,}260^{***}$	Seq*pH	112^{***}

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns non-significant

(Table 2) revealed significant influences of the tested variables and their interaction, concentration of pyrophosphate and pH of the extracting solution, in the amount of extracted L-glutaminase.

The most diluted solutions of pyrophosphate (0.05 and 0.1 M) displayed a flat pH-extraction profile with a level of enzymatic activity similar to that found in the original compost sample; however, the use of more concentrated pyrophosphate solutions (0.2 M) increased notably the amount of L-glutaminase in the crude extracts; this activity reached up to 13 times the activity initially pretend in the compost sample at the optimum pH of extraction (1157 μg $N–NH_4^+$ g^{-1} compost 3 h^{-1} at pH 11). Sodium pyrophosphate solutions at strongly basic pH values has been traditionally used to extract organic matter from soil by means of two important effects: under alkaline conditions, functional groups of organic colloids became deprotonised and a net negatively charge appears at the colloidal surface which favours the solubilisation of these humic fractions; at the same time, the anion pyrophosphate could act as a ligand of di- and trivalent cations, disrupting clay-organic complexes (Kononova and Belchikova 1970). Although organic and inorganic components in compost could be compositionally very different to those present in soils, both mechamisms, solubilisation and complexation, of will be present in matured compost samples and they will be responsible for the extraction of important amounts of humus-like materials with associated enzymatic activities. In this way, using sodium pyrophosphate at slightly basic pH values, Rad et al. (1999) co-extracted phosphatases, cellulases and proteases from a compost of municipal solid wastes and (Benitez et al. 2005) extracted humus-enzyme complexes with β-glucosidase, phosphatase and urease activities from a vermicompost of olive wastes.

In general, extraction of soil enzymes strongly changes their state and activity; in some cases, important amounts of enzyme activity was lost as a consequence of operational procedures, mainly if the enzyme has an intracellular location. But in many other cases, increases in the initial enzyme activity were also reported (Nannipieri et al. 1980; Bonmatí et al. 1998). In general, enzymes acting upon macromolecular substrates, such as cellulases and proteases, would increase substrate accessibility and consequently important increases in enzyme activity were achieved (Nannipieri et al. 1982; McClaugherty and Linkins 1988; Rad et al. 1999).

Slightly alkaline or neutral pHs have been used as optimum extraction pHs for several enzymes in different organic wastes (García et al. 1993; Rad et al. 1999;

Masciandaro et al. 2000); in contrast, in our work, optimum pH for L-glutaminase extraction from compost was found at strongly basic pH conditions. Also, it is stablished that the maximum solubility and stability of an enzyme is reached around its optimal pH of the enzyme activity (Palmer 1997). In soils, Frankenberger and Tabatabai (1991a) reported an optimum activity peak at pH 10 for L-glutaminase and that could be one of the reasons of its high solubility and stability at high pH values of extraction. At the same time, strong alkaline conditions, cellular lysis or alterations in the permeability of the cellular membrane of microbial cells will take place and as a consequence, L-glutaminase would be liberated to the exocellular medium or an increase in substrate accessibility would be produced. With dried sewage sludges, Lerch et al. (1993b) observed an increase of extraction of small peptides and a decrease in the extraction of macromolecules using strong alkaline solutions as a consequence of an increase of protein hydrolysis.

3.2 Molecular Fractionation of Extracted L-glutaminase Activity

Tangential flow ultra- and microfiltration are pre-purification techniques that achieve the separation of biomolecules according their molecular weight by interposing membrane filters of controlled MWCO. Their main advantage are that the tangential flow makes more difficult membrane clogging with less operational times for ultrafiltration procedures, but at the same time, the use of a pressurised flow makes more difficult to achieve the accuracy of other molecular separation techniques such as gel filtration, applied to protein purification. Gel filtration or ultrafiltration processes has been widely used in humus-enzyme purification steps (Nannipieri et al. 1982; Tabatabai and Fu 1992).

In Fig. 2 appears the distribution of extracted L-glutaminase, using 0.2 M pyrophosphate at different extraction pHs, in three main molecular fractions: UF_1 (MW $<$ 10 kDa), UF_2 (MW $>$ 10 kDa, $\phi < 0.45$ μm) and UF_3 ($\phi > 0.45$ μm), with cell-associated enzymes. Statistical analysis of these results (Table 2) revealed significant infuences of the variables and their interaction, molecular size of the fraction and pH of the extracting solution, in the amount of extracted L-glutaminase.

The pH-activity profile confirmed the positive effect of the strong alkaline conditions in the extraction of L-glutaminase for all of the molecular fractions. However, some differences could be observed: UF_3 fraction displayed a very variable extracted activity at pH < 9; more alkaline values increased the activity of this fraction. The same behaviour was displayed by the UF_2 fraction, but in this case, only at an extraction pH of 11, the activity was increased. The UF_1 fraction showed a more constant increase in activity from pH 6 to 11; this fraction

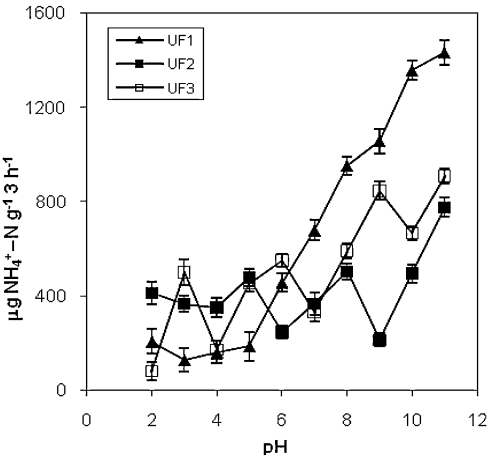

Fig. 2 Molecular fractionation by ultrafiltration of L-glutaminase activity extracted with 0.2 M pyrophosphate from compost

displayed the highest activity of the three molecular fractions at pH values higher than 9. The total of extracted activity in the three fractions at pH 11 reached 2.5 times the activity of the crude extract.

L-glutaminase produced different microorganims have been purified and characterised in its molecular weight and kinetic properties. Hartman and Stochaj (1973) after the purification of a L-glutaminase produced by *Eschirichia coli* determined that the quaternary structure of the enzyme was a tetramer with subunits of 28 kDa of MW; similar results were obtained by Davidson et al. (1977) with L-glutaminase obtained from a culture of *Pseudomonas acidovorans*, which had 156 kDa of MW and with four subunits of 39 kDa.

A possible dissociation of the enzyme depending on the pH of extraction could be responsible for both, the presence of enzymatic activity in UF_1 and the total increase of activity in the sum of fractions. Ultrafiltration at 10 kDa imply the use of a pressurised flow of 1.8 10^5 Pa, that could modify the structure of globular proteins, forcing their pass through the membrane. In the rest of the work we chosen an extraction pH of 7 as a compromise between the amount of enzyme extracted and a lesser alteration of its quaternary structure.

3.3 Influence of Compost: Extractant Ratio

The yield of enzyme extraction was increased according with the increase of the compost: extractant ratio (Fig. 3). This increase was linear until the use of a compost: extractant ratio of 10 (w:v); for higher ratios this proportionality was progresively lost, probably by understimation of the activity in very diluted extracts. Similar results were obtained by Criquet et al. (2002) with protein extraction from the litter of forest soils in which the accuracy in protein quantitation limited the optimal ratio that must be used in the extraction procedure.

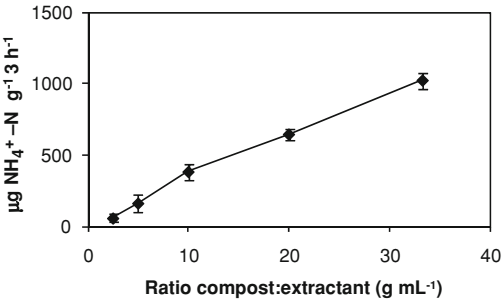

Fig. 3 Influence of the ratio extractant: compost in the extraction of L-glutaminase activity with 0.2 M pyrophosphate at pH 7

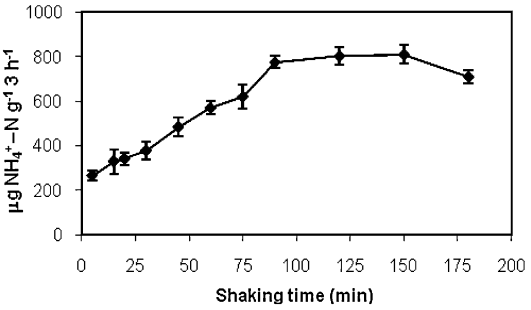

Fig. 4 Influence of the shaking time for the extraction of L-glutaminase activity

3.4 Influence of the Shaking Time

The amount of extracted L-glutaminase activity increased with the agitation time form 5 to 90 min reaching a maximum activity higher than 800 µg NH_4^+–N g^{-1} compost 3 h^{-1}. Shakinkg times higher than 150 min carried out a decrease in the amount of extracted enzyme besides the temperature during extraction and centrifugation was maintained at 4°C (Fig. 4).

As important constituents of organic N in soils and other organic residues, the extraction of proteins has been an important tool for their quantification (Schulten and Schnitzer 1998). However, the feasibility of their extraction varies depending the origin and the association mechanisms involved in protein immobilization in soils. In other organic samples such as litter samples of forest soils, the maximum amount of protein extraction was achieved after six hours of agitation using several saline solutions which include pyrophosphate (Criquet et al. 2002); in different dried samples of sludge, Lerch et al. (1993a) achieved optimal aqueous extraction after 16 h, time that was reduced to 12 h using alkaline solutions of 1 M NaOH, and to 6 h with the introduction of detergents such as Triton X-100. Using this compost, 3 h of shaking time was enough to reach the maximum of protein extraction (López-Fernández 2003); however long shaking periods could cause enzyme denaturalization or proteolytic clevage that produced the loss of its

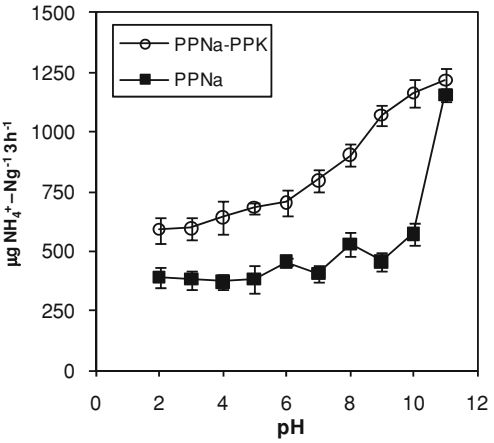

Fig. 5 Evaluation of the sequential procedure (sodium pyrophosphate + potassium pyrophosphate) against one-step procedure for the extraction of L-glutaminase from compost

activity. In soils, stable humus-enzyme complexes have been isolated after 18 h of agitation, this means that more strong immobilization mechanisms would be present between extracellular enzymes and humic substances in soil than in compost, were less condensed structures of humus-like materials are presents.

3.5 Efficacy of a Sequential Procedure

The use a sequential process of involving a the consequitive use of sodium and potasium pyrophosphate (0.2 M at pH 9), has been succesfully used in the extraction of metals associated to protein fracctions in sewage sludges (Navarro González 2002). Therefore, we have applied this sequential procedure to the extraction of L-glutaminase activity at range of pH that have been assayed (2–11). As it is shown in Fig. 5, the sequential extraction procedure significatively increased the amount of extracted enzyme in respect to that with a single extraction step and also depending with the increase of pH (Table 2); however, at pH 11 no statistically significant differences were obtained between both procedures.

Several sequential extraction procedures have been used to extract humus enzyme complexes; Busto and Pérez-Mateos (1995) using four consecutive extractions of 0.1 M sodium pyrophosphate at pH 7 observed that only one-seventh of β-glucosidase activity extracted in the first step was achieved in the second step and minimal amounts of activity in third and fourth steps. In this work, to consecutive steps increased notably the yield of extraction at acid or neutral pH values; however, at pH 11 other extraction mechanisms would be involved different to organic matter solubilization by pyrophosphate. At this pH, cellular lysis and the modification of quaternary structure of the enzyme would have an important contribution to enzyme extraction, as it has been discussed before.

4 Conclusions

- An important enzyme in soil N-cycle, such as L-glutaminase activity, is present in stabilised samples of compost of urban wastes and could be extracted in both forms: free or associated to humus-like organic compounds.
- The extraction process of the glutaminase activity in compost is optimum when it is carried out with 0.2 M sodium pyrophosphate at alkaline pH, a ratio compost: extractant of 1:10 (g mL^{-1}) and a shaking time of 90 min at 4°C.
- Molecular fractionation by micro- and ultrafiltration supposes the isolation of different soluble enzymatic fractions. Under strong alkaline conditions (pH 11) important amounts of enzyme were extracted, mainly in UF_1 fraction (MW < 10 kDa) as a consequence of both, cell-lysis or the dissociation of quaternary structure of the enzyme, which strongly increases the total extracted activity.

References

Albaladejo J, Díaz E (1990) Degradación y regeneración del suelo en el litoral mediterráneo español: experiencias en el proyecto LUCDEME. In: Albaladejo J, Stocking MA, Díaz E (eds) Degradación y Regeneración del Suelo en Condiciones Ambientales Mediterráneas. CSIC, Murcia, Spain, pp 191–212

Ayuso M, Hernández T, García C, Pascual JA (1996) A comparative study of the effect on barley growth of humic substances extracted from municipal wastes and from traditional organic materials. J Sci Food Agric 59:313–319

Batistic L, Sarkar JM, Mayaudon J (1980) Extraction, purification and properties of soil hydrolases. Soil Biol Biochem 12:59–63

Benítez E, Nogales R, Elvira C, Masciandro G, Ceccanti B (1999) Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. Bioresour Technol 67:297–303

Benítez E, Sainz H, Nogales R (2005) Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. Bioresour Technol 96:785–790

Bonmatí M, Ceccanti B, Nannipieri P (1998) Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biol Biochem 20:2113–2125

Busto MD, Pérez-Mateos M (1995) Extraction of humic β-glucosidase fractions from soil. Biol Fertil Soils 20:77–82

Criquet S, Farnet AM, Ferre E (2002) Protein measurement in forest litter. Biol Fertil Soils 35:307–313

Davidson L, Brear DR, Wingard P, Hawkins J, Kitto GB (1977) Purification and properties of L-glutaminase-L-asparaginase from *Pseudomonas acidovorans*. J Bacteriol 129:1379–1386

Deng SP, Parham JA, Hattey JA, Babu D (2006) Animal manure and anhydrous ammonia amendment alter microbial carbon use efficiency, microbial biomass, and activities of dehydrogenase and amidohydrolases in semiarid agroecosystems. Appl Soil Ecol 33:258–268

Dodor DE, Tabatabai MA (2007) Amidohydrolales in soils as affected by cropping systems. Appl Soil Ecol 24:73–90

Frankenberger WT, Tabatabai MA (1991a) L-glutaminase activity of soils. Soil Biol Biochem 23:869–874

Frankenberger WT, Tabatabai MA (1991b) Factors affecting L-glutaminase activity in soils. Soil Biol Biochem 23:875–879

Galstyan ASh, Saakyan EG (1973) Determining the activity of soil glutamine. Sov Soil 5:335–337

García C, Hernández T, Costa F, Ceccanti B, Masciandaro G, Calcinai M (1993) Evaluation of the organic matter composition of raw and composted municipal wastes. Soil Sci Plant Nutr 39:99–108

García C, Ceccanti B, Masciandaro G, Hernandez T (1995) Phosphatase and glucosidase activities in humic substances from animal wastes. Bioresour Technol 53:79–87

García C, Hernández T, Roldán A, Albaladejo J, Castillo V (2000) Organic amendment and mycorrhizal inoculation as a practice in afforestation of soils with *Pinus halepensis* Miller: effect on their microbial activity. Soil Biol Biochem 32:1173–1181

Hartman SC, Stochaj EM (1973) Glutaminase A of *Escherichia coli*. Subunit structure and cooperative behavior. J Biol Chem 248:8511–8517

Hojjati S, Nourbakhsh F (2007) Effects of cow manure and sewage sludge on the activity and kinetics of L-glutaminase in soil. Biol Fertil Soils 43:491–494

Imada A, Igarasi S, Nakahama K, Isono M (1973) Asparaginase and glutaminase activies of microorganisms. J Gen Microbiol 76:85–89

Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72

Kononova MM, Belchikova NP (1970) Use of sodium pyrophosphate to separate and characterize organo-iron and organo-aluminun compounds in soil. Sov Soil Sci 2:351–365

Lerch RN, Azari P, Barbarick KA, Sommers LE, Westfall DG (1993a) Sewage sludge proteins: II. Extract characterization. J Environ Qual 22:625–629

Lerch RN, Barbarick KA, Azari P, Sommers LE, Westfall DG (1993b) Sewage sludge proteins: I. Extraction methodology. J Environ Qual 22:620–624

López-Fernández JI (2003) Revalorización "No Energética" de residuos orgánicos fermentados. Materia orgánica residual como fuente de enzimas proteolíticos y amidohidrolíticos. Unpublished Doctoral Thesis, Universidad de Burgos, Burgos, Spain

MAPA (1986) Métodos Oficiales de Análisis. Tomo III. Plantas, Productos Orgánicos Fertilizantes, Suelos, Aguas, Productos Fitosanitarios, Fertilizantes Inorgánicos. Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain

Masciandaro G, Ceccanti B, García C (2000) "*In situ*" vermicomposting of biological sludges and impacts on soil quality. Soil Biol Biochem 32:1015–1024

McClaugherty CA, Linkins AE (1988) Extractability of cellulases in forest litter and soil. Biol Fertil Soils 6:322–327

Nannipieri P, Ceccanti B, Ceverlli S, Matarese E (1980) Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016

Nannipieri P, Ceccanti B, Conti C, Bianchi D (1982) Hydrolases extracted from soil: Their properties and activities. Soil Biol Biochem 14:257–263

Nannipieri P, Grego S, Ceccanti B (1990) Ecological significance of the biological activity in Soils. In: Bollag JM, Stotzky G (eds) Soil biochemistry, vol 6. Marcel Dekker, New York, pp 293–355

Navarro González M (2002) Uso agrícola de iodos de EDAR: Nuevos tratamientos de descontaminación química como aproximación a los criterios actuales de salud del suelo. Unpublished Doctoral Thesis, Universidad de Burgos, Burgos, Spain

Nourbakhsh F, Alinejadian A (2009) Arginine ammonification and L-glutaminase assays as rapid indices of corn nitrogen availability. J Plant Nutr Soil Sci 172:127–133

Palmer T (1997) Undestanding enzymes, 4th edn. Ellis Harwood, New York

Pascual JA, García C, Hernández T, Ayuso M (1997) Changes in the microbial activity of an arid soil amended with urban organic wastes. Biol Fertil Soils 24:429–434

Rad JC, Navarro González M, González Carcedo S (1995) Characterization of proteases extracted from a compost of municipal solid wastes. Geomicrobiol J 13:45–56

Rad JC, Navarro-González M, González-Carcedo S (1999) Use of pyrophosphate to extract extra- and intracellular enzymes from a compost of municipal solid wastes. In: Berthelin J, Huang P-M, Bollag J-M, Andreux F (eds) Effect of mineral-organic-microorganism interactions on soil and freshwater environments. Plenum Press, The Hague, pp 349–359

Schulten HR, Schnitzer M (1998) The chemistry of soil organic nitrogen: a review. Biol Fertil Soils 26:1–15

Sinsabaugh RL (1994) Enzymatic analysis of microbial pattern and process. Biol Fertil Soils 17:69–74

Stevenson FJ (1994) Humus chemistry: genesis, composition, reactions, 2nd edn. Wiley, New York

Tabatabai MA, Fu M (1992) Extraction of enzymes from soils. In: Stotzky G, Bollag JM (eds) Soil biochemistry, vol VI. Marcel Dekker, New York, pp 197–227

Vuorinen AH (1999) Phosphatases in horse and chicken manure composts. Compost Sci Util 7:47–54

Effect of DOR Incubated with Saprobe Fungi on Hydrolytic Enzymes Activities and Chemical Properties of Rhizospheric Soil of Lettuce

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Abstract The dry olive residue (DOR), a by-product of the olive mill industry, is produce in large quantities in Mediterranean countries. The phytotoxic and antimicrobial properties of this residue adversely impact on soil qualities. The objective of this study was to investigate the evolution of soil enzymes activities (acid phosphatase, β-glucosidase, protease and urease) and chemical properties (pH, phenols, total organic carbon (TOC) and soluble carbohydrates) after the agronomic application on lettuce of either un-treated DOR or DOR incubated with inmobilized *Panus tigrinus* or *Fusarium lateritium*. A decrease of total phenols and phytotoxicity on lettuce plants in presence of treated DOR related to the un-treated residue was detected. We observed a decrease of all hydrolytic enzymes, except protease, after the soil incubation with un-treated DOR for 15 days. It appears that the high concentration of phenolics compounds inhibited the activities of these enzymes. However, the microbial activity was stimulated by the addition of DOR incubated with the saprobe fungi as indicated by the increase of soil enzyme activities detected. The protease activity was always higher than the control soil irrespective of the type of amendant. The TOC and phenols of rhizospheric soil of lettuce increased after 15 and 30 days of agronomic application of untreated or treated residue and a general decrease of the soluble carbohydrates contents was found after 30 days of soil incubation. The DOR detoxified by saprobe fungi has been seen to have a positive effect to restore the loss rhizospheric soil functionality detected after the addition of un-treated residue.

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1 Introduction

The industry of olive oil generates large quantities of residues in short periods of time. The two phase centrifugation system generates 4 million tons of dry olive residues (DOR) per year. Several studies have indicated toxic and antimicrobial effects of this residue (Sampedro et al. 2009c). These properties of olive residue adversely impact both soil and water qualities. The application of DOR onto soil might lead to negative effects, including stimulation of widespread plant pathogens and phytotoxicity (Bonanomi et al. 2006). To date several studies reported chemical and physical processes for the DOR conversion in order to decrease the environmental impact. An alternative for sustainable recycling of DOR is through composting with other agricultural by-products (Alburquerque et al. 2006). The preponderance of plant cell wall macromolecules, low amount of nitrogen and the presence of antimicrobial compounds make its composting slow. However it has been described that the phytotoxicity of DOR can be reduced by incubating it with some inmobilized saprophytic fungi for 4 weeks (Sampedro et al. 2009b).

A common characteristic of Mediterranean soils is their low organic matter content. Maintenance of soil organic matter is important for the long-term productivity of agroecosystems. For this reason, the application of materials with high organic matter content to semiarid soils has become a common environmental practice for soil restoration, maintaining soil organic matter, reclaiming degraded soils, and supplying plant nutrients (Tejada et al. 2007). The addition of agricultural residues to soil, may lead the benefits, because it stimulates soil microbial growth and activity (Randhawa et al. 2005) and increases soil fertility and quality (Mondini and Sequi 2008). For this reason, their use in the restoration of degraded zones is promising. However, the effect of these residues on soil properties depends on its principal and dominant component (Chaves et al. 2004). An altered biological activity of soil may result when residues containing toxic organic compounds are incorporated. Several studies have shown the occurrence of adverse effects of this practice on soil properties, which include the application of phytotoxic compounds such as polyphenols to soil (Cayuela et al. 2008).

Soil enzymes are involved as biological catalysts in specific reactions of P, C, N cycles and act in the depolymerisation of different constituents of organic wastes (Cayuela et al. 2008). The enzymes phosphatase, β-glucosidase are involved in P and C cycles, respectively and protease and urease related to the N cycles. Among hydrolytic enzymes, acid phosphatase and β-glucosidase activities have been frequently used as indicators of changes in quantity and quality of soil organic matter (SOM) (Gil-Sotres et al. 2005). Bending et al. (2002) reported that the activities of most enzymes increase as native SOM content increases, reflecting larger microbial communities and stabilization of enzymes on humic materials. Since the enzymes may react to changes in soil management more quickly than other variables they can be used as early indicators of biological changes (Masciandaro et al. 2004).

The DOR might be a possible candidate to the addition of organic matter to soil (Roig et al. 2006). The incubation of DOR with saprobe fungi is expected to represent

Table 1 Chemical characteristics of soil

pH	8.6
CIC (meq 100 g^{-1})	8.261
TN (%)	0.136
P (mg kg^{-1})	88
K (mg kg^{-1})	115
Mg (meq 100 g^{-1})	1.69
Na (meq 100 g^{-1})	0.07
$CO_3^=$ (%)	18.5
Clay (%)	16.65
Sand (%)	30.73
Silt (%)	52.62

a suitable treatment for detoxification and organic matter stabilization, in order to obtain an adequate soil amendment able to exert beneficial rather than adverse effects on soil properties. The study of the impact of un-treated DOR and DOR incubated with saprobe fungi in microbial ecology and the biological and chemical properties of soil is almost inexistent. Dynamics of enzymatic activities and chemical properties of soil are expected to give information about soil functionality.

The aim of this work was to investigate the evolution of chemical and biological properties of rhizospheric soil of lettuce after the addition of DOR transformed by saprobe fungi. The soil, amended with DOR, was periodically analysed for functionally related properties such as soil enzyme activities involved in the P, C and N cycle. Furthermore, different physical and chemical properties such as pH, TOC, phenols, etc. were also monitored.

2 Materials and Methods

2.1 Materials

The soil employed in this study was obtained from the field of the Estación Experimental del Zaidín (Granada, Spain). Its main properties were summarized in Table 1.

DOR was collected from an "orujo" manufacturer (Sierra Sur S.A., Granada, Spain). The main characteristics of DOR determined by Sampedro et al. (2008) were summarized in Table 2.

2.2 Organisms and Inoculum Preparation

Panus tigrinus (CBS 577.79) and *Fusarium lateritium* (BACF Cult. N° 2317) were maintained at 4°C and routinely subcultured every month on potato dextrose agar slants. Inoculum preparation and incubation conditions were as previously

Table 2 Chemical characteristics of DOR

pH	5.13
TOC (g kg^{-1})	519 ± 3.0
TEC (g kg^{-1})	203 ± 16.4
Total phenols (g kg^{-1})	42.1 ± 1.9
Lignin (%)	24.7 ± 1.3
Cellulose (%)	17.1 ± 0.7
Hemicellulose (%)	12.8 ± 0.4
Total N (g kg^{-1})	187 ± 13
Total phosphorus (%)	0.21 ± 0.03

reported (Sampedro et al. 2009b). Polyurethane sponge (PS) cubes, 0.5 cm width each, were rinsed with water in a 1:20 (w/v) ratio and autoclaved (121°C for 20 min) twice prior to their use. Five milliliters of the inoculum (ca. 50 mg of dw) was aseptically added to 50 g of sterilized supports and incubated at 28°C for 7 days.

2.3 DOR Pretreatment

Deionized water was added to DOR to adjust the moisture content to 25% (w/w) prior to sterilization (3 cycles in autoclave at 120°C for 20 min). Then, colonized solid supports (0.24 g of PS), were covered with 17.5 g of DOR. Solid-state cultures on DOR were carried out at 28°C in the dark under stationary conditions for 30 days. Non-inoculated and sterilized DOR samples were prepared and incubated as above. All the amendments used in this experiment were previously sterilized.

2.4 Soil Incubation Procedure

The experiments were carried out in 0.5 L pots containing non sterilized soil mixed with sand 1:1 by volume. Lettuce (*Lactuca sativa* L.) was used as the test plant. Seeds were sterilised, pregerminated and selected for uniformity prior to transplanting. Plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cold-white lamps, 400 nmol m^{-2} s^{-1} at 400–700 nm, with a 16/8 h day night cycle at 25/19°C and 50% relative humidity. DOR and DOR incubated with *P. tigrinus* and *F. lateritium* was applied to the 0.5 L soil pots at concentrations of 0 and 35 g kg^{-1} soil.

The rhizospheric soil of lettuce was collected after 15 and 30 days, sieved (2 mm), homogenate and subsequently stored at 4°C. Plants were harvested after 30 days and their dry mass was determined.

2.5 Chemical and Enzymatic Analyses

pH was determined in 1:10 (w/v) water soluble extract (Cayuela et al. 2008). The total organic carbon (TOC) of soil was determined by the wet oxidation method (Mingorance et al. 2007). The reaction is carried out with 3 mL $K_2Cr_2O_7$ and 6 mL de H_2SO_4 and Cr^{3+} resulting from organic C oxidation and determined by spectrophotometry. Soluble carbohydrates in soil were analyzed according to Brink et al. (1960). To extract phenols, 1 g DOR was incubated for 24 h in 20 mL distilled water/acetone mixture (50:50 v:v) under orbital shaking (200 rpm).Total phenolic contents (TP) of extracts were determined according to Sampedro et al. (2009a), using tanin acid as the standard and expressed as mg g^{-1} of DOR.

The activities of acid phosphatase and β-glucosidase were determined according to Eivazi and Tabatabai (1988) and (1977) respectively. Urease was analyzed with the procedure developed by Kandeler and Gerber (1988) and the protease activity was determined according to the method of Ladd and Butler (1972).

2.6 Statistical Treatment of Data

The data were subjected to one-way ANOVA. The mean values of 4 replicate pots were compared using the LSD test ($P = 0.05$). Percentage data were subjected to arcsine transformation before analysis.

3 Results and Discussion

3.1 DOR Treatment

A general decrease of total phenols of DOR was observed after 4 weeks of incubation of this residue with the saprobe fungi *P. tigrinus* and *F. lateritium* immobilized in PS (Fig. 1). The decrease of phenols content by *F. lateritium* was lower than that of *P. tigrinus*. These results can be attributing to differences in the enzymatic machinery implicated in the degradation of phenolic compounds. Previous studies reported the removal of DOR phenolic compounds after the incubation of this residue with some saprobe fungi during 20 weeks (Sampedro et al. 2005). It has also described that the addition of immobilized fungal inocula can reduce the time necessary for DOR detoxification, obtaining high removals of phenols (Sampedro et al. 2009b).

No significant changes in pH were obtained with the incubation of DOR with *P. tigrinus*, however a increase of this parameter was detected after *F. lateritium* incubation. This increase has been previously reported for DOR biotreated with

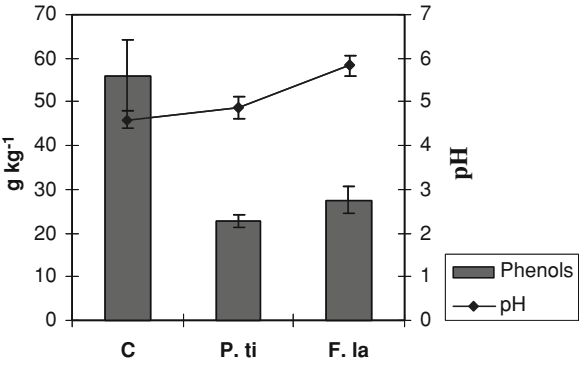

Fig. 1 Total phenols (g kg^{-1}) and pH of untreated DOR (*C*) and DOR incubated with *P. tigrinus* (*P.ti*) and *F. lateritium* (*F.la*). Error bars represent the standar error of means

some saprobe fungi (Sampedro et al. 2004) and during the composting of DOR (Alburquerque et al. 2006) and can be explained as an intrinsic outcome of the residue mineralization. Furthermore, the microbial decomposition of abundant organic anions present in DOR could lead to an increase in the pH during the most active phase of the composting process (Arienzo and Capasso 2000). However, other studies indicated that the pH of DOR treated with other ligninolytic fungi such *Coriolopsis rigida* and *Phanerochaete chrysosporium* was similar to the untreated residue (Sampedro et al. 2004).

3.2 Phytotoxicity of DOR

The application of DOR at the dose of 35 g kg^{-1} reduce the shoot dry weight related to that of plants grown in absence of this residue in non sterilized soil (Fig. 2). This residue exerts both phytotoxic and anti-microbial activity when applied to soil (Baeta-Hall et al. 2005; Bonanomi et al. 2006).

The phytotoxic effect of DOR incubated with both saprobe fungi was lower than that of un-treated DOR. Therefore the incubation of this residue with *P. tigrinus* and *F. lateritium* reduce it phytotoxicity in lettuce plant. The phytotoxic effects of olive mill residue in higher plants has been tested in several studies and the removal of phytotoxicity of this residue in plants such tomato (*Lycopersicum esculetum*, L.) and alfalfa (*Medicago sativa* L.) was observed after their incubation with saprobe fungi (Casa et al. 2003; Aranda et al. 2009; Sampedro et al. 2009c).

3.3 Evolution of Soil Enzyme Activity

In the present study, we analyze the evolution of the four hydrolytic enzymes activities in soil growth in presence of lettuce plants after the agronomic application of un-treated DOR and DOR incubated with saprobe fungi in order to

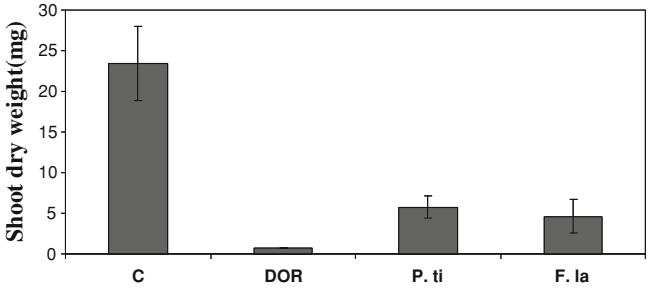

Fig. 2 Shoot dry weight of lettuce (*Lactuca sativa* L.) grown in non sterilized soil (*C*) in presence of untreated DOR (*DOR*) and DOR incubated with *P. tigrinus* (*P.ti*) and *F. lateritium* (*F.la*). Error bars represent the standar error of means

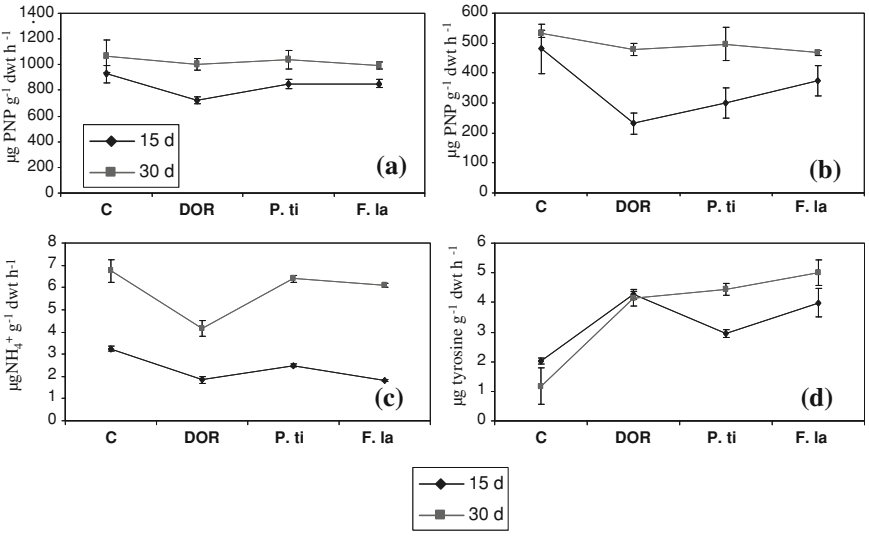

Fig. 3 Dynamics of soil enzymes activity in the rhizospheric of lettuce (*C*) in response of incubation with DOR untreated (*DOR*) or incubated with *P. tigrinus* (*P.ti*) and *F. lateritium* (*F.la*). **a** *β*-Glucosidase; **b** Acid phosphatase; **c** Urease; **d** Protease

determine the impact of these amendants on the soil functionality. These types of enzymes are related to the mineralization of important nutrient elements as C, N and P and provide an early indication of changes in soil fertility (Ceccanti et al. 1994).

β-Glucosidase activity shows the state of the organic matter and the processes occurring therein (Garcia et al. 1994). We observed a reduction of *β*-glucosidase activity in the agronomic application of DOR to rhizospheric soil of lettuce after 15 days (Fig. 3a). It is interesting to note that the *β*-glucosidase activity is reduced by interaction with lignin or carbohydrate-lignin complexes (Berlin et al. 2006). However, an increase in this enzymatic activity was observed in soil after the treatment with DOR incubated with both saprobe fungi. Similar results were also

reported in DOR composting with sheep manure and grape stalks (Cayuela et al. 2008). The higher level of β-glucosidase activity in soil added by DOR incubated with the saprobe fungi related to the untreated DOR may be due to an increase in microbial biomass after addition of organic amendants (Tejada et al. 2006). The application of DOR treated with fungi was reported to results in an increase effect of ß-glucosidase activity ascribed to the available substrates involved in the C cycle. At the end of the experiment, we detected similar values of β-glucosidase activity with all the amendants tested, respect to the control soil. Under these conditions, we can conclude that the incubation of rhizospheric soil of lettuce with treated DOR during 15 days have a positive effect to restore the loss soil functionality detected after the addition of un-treated residue.

Similar results were obtained for the acid phosphatase activity (Fig. 3b). The levels of acid phosphatase activity in rhizospheric soil of lettuce amended with DOR untreated or treated with *P. tigrinus* after 15 days where lower than the control soil. However, the incubation of the residue with *F. lateritium* was able to maintain the same level of acid phosphatase activity than the control. In fact, the application of DOR to Petri dishes containing soil and incubated during 15 days stimulated the acid phosphatase activity (Sampedro et al. 2009a). In the present stuy, no significant changes were observed in the acid phosphatase activity after 30 days of plant growth. Similar effects were also observed upon addition of OMW to soil where no significant differences in this activity measured after 28 days between OMW treated and control soil were obtained (Piotrowska et al. 2006). This data suggests that microorganisms had consumed the main P-cycle metabolizable substrates, indicating a relationship between the increase in microbial activity and the accumulation of phosphatase enzyme in the humic molecule.

Urease catalyses the hydrolysis of urea to carbon dioxide and ammonium, and it is widely distributed in microorganisms, plants and animals (Nannipieri et al. 2002). The urease activity of rhizospheric soil of lettuce decreases after the amendment with untreated DOR for 15 and 30 days (Fig. 3c). This can be attributed to the low level of N and available substrates of the residue during the mineralization process. In the treatment with un-treated DOR, the low activity indicated no stabilization of organic matter without the formation of complexes with humic-like substances. Some authors found the same result when unprocessed dry olive cakes were used as mulches (Benítez et al. 2000). The incubation of DOR with *P. tigrinus* can increase urease activity after 15 and 30 days of incubation. This fact could be attributed to the formation of complexes with humic-like substances witch protect the extracellular enzymes. DOR treated with *F. lateritium* only increase urease activity after 30 days.

It is important to note that levels of protease in rhizospheric soils of lettuce amended with DOR were always higher than the control soil irrespective of both, the type of amendant added (untreated or treated) and the incubation time of soil with the amendant (Fig. 3d). We observed that despite the presence of phenols in soil added with DOR, the levels of this enzymatic activity were significantly higher than those found in the control soil. This effect was also observed in other

Table 3 Chemical properties of rhizospheric soil of lettuce after 15 and 30 days of agronomic application of DOR un-treated and incubated with *P. tigrinus* and *F. lateritium*

Treatments	Incubation time	pH	TOC (g kg^{-1})	Carbohydrates (mg kg^{-1})	Phenols (g kg^{-1})
C	15d	8.01 ± 0.04	14.28 ± 1.18	93.97 ± 6.02	1.09 ± 0.05
	30d	7.88 ± 0.09	15.80 ± 0.63	55.98 ± 8.07	1.14 ± 0.02
DOR	15d	7.84 ± 0.02	20.42 ± 0.30	192.05 ± 3.24	2.84 ± 0.01
	30d	8 ± 0.1	21.45 ± 1.65	54.90 ± 4.95	1.93 ± 0.01
P. tigrinus	15d	7.88 ± 0.03	17.76 ± 1.63	158.97 ± 11.91	1.47 ± 0.02
	30d	7.96 ± 0.04	21.93 ± 0.42	55.49 ± 3.82	1.34 ± 0.04
F. lateritium	15d	7.75 ± 0.05	27.26 ± 0.12	175.88 ± 11.18	1.78 ± 0.03
	30d	7.88 ± 0.07	21.42 ± 0.11	53.52 ± 1.19	1.56 ± 0.02

enzymatic activities (dehidrogenase and fluorescein diacetate hydrolase) upon the addition of DOR to soil (Sampedro et al. 2009a). Again, other studies indicated that the application of OMW supposes a shift in the soil's microbial community with a dominance of copiotrophic bacteria and the subsequent increase of enzymatic activities (Kotsou et al. 2004). The protease activity of rhizospheric soil of lettuce amended with DOR treated with *F. lateritium* was always higher than the soil incubated with untreated ADOR. However, the incubation of DOR with *P. tigrinus* didn't alter the protease activity of soil amended. Protease activity was one of the most sensible parameter to indicate changes in soil fertility and the differences found in the experiment between both fungi can be explain for the different behaviour of the fungus tested. The increase of soil protease is in agreement with other studies where different organic amendment were added to soil, indicating that this type of organic matter had a beneficial effect on the activity of these types of enzymes (Renella et al. 2005; Tejada et al. 2006). In addition, organic matter material may contain these enzymes and may stimulate microbial activity in the soil (Pascual et al. 1998).

3.4 Effects of DOR Application in the Chemical Properties of Rhizospheric Soil of Lettuce

Table 3 shows four biochemical indices for the evaluation of the DOR impact in rhizospheric soil of lettuce. All treatments showed a slight decrease in pH of rhizospheric soil incubated with the residue during 15 days. This fact was also observed in the 1st stages of composting process of DOR, as a result of the release of organic acids from the decomposition of the most labile organic fractions (Cayuela et al. 2008). Similar values of pH in rhizospheric soil control and incubated 30 days with either DOR untreated or treated with the saprobe fungi may indicated the initial stage for the soil stabilisation. No significant changes of soil pH were also observed upon the addition of low amount of OMW (Piotrowska et al. 2006). The increase of incubation time of soil with this residue for more than

30 days may cause the stabilization of soil and the increase of pH values therein. Several studies showed that pH increased when composting mass achieved a high degree of stability, probably due to the decarboxilation of organic anions during the aerobic decomposition (Cayuela et al. 2004).

Total organic carbon (TOC) in soil has an impact on ecosystem sustainability, affecting chemical, physical and biological characteristics of soil (Reeves 1997). The addition of untreated or treated DOR to rhizospheric soil of lettuce increased significatively the TOC values. The higher levels of TOC were obtained with the DOR incubated with *F. lateritium* after 15 days treatment. Throughout the incubation time, the TOC values of all amendants soils remain higher than the control soil. In fact, the addition of other olives residues such as OMW to soil also supposes an increase of TOC (Piotrowska et al. 2006).

The carbohydrates values were increased 112%, 73% and 95%, in rhizospheric soil of lettuce incubated during 15 days with DOR untreated and treated with *P. tigrinus* and *F. lateritium*, respectively, respect to the non-amended soil (Table 3). This result are in agreement with those of Carrasco et al. (2009) who showed a increase of the carbohydrates values with other organic amendments as sugar beet residue (125% in the amended soil with respect to the non-amended soil). The lower increase of carbohydrates content in rhizospheric soil incubated with treated DOR respect to the un-treated DOR could be attributed to the previous incubation process of DOR before the addition to soil. A general decrease of soluble carbohydrate contents was found in the rhizospheric soil either with or without amendement at the end of the experiment. It is knows that the effect of mineral fertilizers plus DOR on the productivity and quality of maize crop reported a soluble carbohydrate contents decrease gradually along the maize grow cycle (Tejada and Gonzalez 2004). This could be explain for the transfers of soluble carbohydrates from leaves to grains as has been described by Rajcan et al. (1999).

The soil use in the present study has 1.09 mg g^{-1} of extractable phenols (Table 3). The phenols contents of rhizospheric soil of lettuce were increased 160, 34 and 63% after the incubation during 15 days with DOR untreated and treated with *P. tigrinus* and *F. lateritium*, respectively. Previous studies reported the high content of phenolic compounds in DOR and the possibility to remove these toxic compounds after the incubation of this residue with saprobe fungi (Sampedro et al. 2005). A decrease of phenols concentrations were detected in rhizospheric soil added with DOR and DOR incubated with saprobe fungi during 30 days (Table 3). We suppose that this fact can be due to the presence in soil the allochthonous microorganisms capable of degrade phenolic compounds.

4 Conclusions

In conclusion, these results seem to confirm that the impact of DOR on rhizospheric soil of lettuce properties depend on the relative amounts of toxic

compounds added (overcoat phenolic compounds). In fact, β-glucosidase, phosphatase and urease activity activities were different on the rhizospheric soil after application of DOR untreated or incubated with saprobe fungi at the beginning of the experiment. However, β-glucosidase and phosphatase activity at the end of the experimental period were similar for all treatments. A stabilization of enzymes related to the C and P cycles was found in the rhizospheric soil either with or without amendement at the end of the experiment. In other hand, the improvement of hydrolase activities related to the N cycle such protease in the soils suggests that the DOR amendments increased soil microbial growth counteracts any inhibitory effect of the toxic compounds. Soil microorganisms degrade organic matter through the production of diverse extracellular enzymes and for this reason after the application of the treated residue to soil, soil enzymatic activities increased. The addition of these DOR amendments to soil supposes an increase of organic matter, associated with an increase of either TOC or phenols. DOR detoxify by saprobe fungi has been seen to have a positive effect to restore the loss soil functionality detected after the addition of untreated residue.

References

Alburquerque JA, González J, García D, Cegarra J (2006) Effects of bulking agent on the composting of "alpeorujo", the solid by-product of the two-phase centrifugation method for olive oil extraction. Bioresour Technol 91:195–200

Aranda E, Sampedro I, Díaz R, García-Sánchez M, García-López JA, Ocampo JA, García-Romera I (2009) Dry matter and root colonization of plants by arbuscular mycorrhizal fungi in presence of physical fractions of dry olive mill residue inoculated with saprobe fungi. Symbiosis 47:133–140

Arienzo M, Capasso R (2000) Analysis of metal cations and inorganic anions in olive oil mill wastewaters by atomic absorption spectroscopy and ion chromatography Detection of metals bound mainly to the organic polymeric fraction. J Agric Food Chem 48:1405–1410

Baeta-Hall L, Sàágua MC, Bartolomeu ML, Anselmo AM, Rosa MF (2005) Bio-degradation of olive oil husks in composting aerated piles. Bioresour Technol 96:69–78

Bending GD, Turner MK, Jones JE (2002) Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities. Soil Biol Biochem 34:1073–1082

Benítez E, Melgar R, Sainz H, Gómez M, Nogales R (2000) Enzyme activities in the rhizosphere of pepper (capsicum annum L.) grown with olive cake mulches. Soil Biol Biochem 32: 1829–1835

Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J (2006) Inhibition of cellulase xylanase and β-glucosidase activities by softwood lignin preparations. J Biotechnol 125:198–209

Bonanomi G, Giorgi V, Del Sorbo G, Neri D, Scala F (2006) Olive mill residues affect saprophytic growth and disease incidence of foliar and soilborne plant fungal pathogens. Agric Ecosyst Environ 115:194–200

Brink RH, Dubach JRP, Lynch DL (1960) Measurement of carbohydrates in soil hydrolyzates with anthrone. Soil Sci 89:157–166

Carrasco L, Caravaca F, Azcón R, Kohler J, Roldan A (2009) Addition of microbially-treated sugar beet residue and a native bacterium increases structural stability in heavy metal-contaminated mediterranean soils. Sci Total Environ 407:5448–5554

Casa R, D'Annibale A, Pieruccetti F, Stazi SR, Giovannozzi Sermanni G, Locascio B (2003) Reduction of the phenolic components in olive-mill wastewater by an enzymatic treatment and its impact on durum wheat (*Triticum durum* Desf.) germinability. Chemosphere 50:959–966

Cayuela ML, Bernal MP, Roig A (2004) Composting olive mill waste and sheep manure for orchard use. Compost Sci Util 12:130–136

Cayuela ML, Mondini C, Sánchez Monedero MA, Roig A (2008) Chemical properties and hydrotlytic enzume activities for the characterisation of two-phase olive mill wastes composting. Bioresour Technol 99:4255–4262

Ceccanti B, Pezzarosa B, Gallardo-Lancho FJ, Masciandaro G (1994) Bio-tests as markers of soil utilization and fertility. Geomicrobiol J 11:309–316

Chaves B, De Neve S, Hofman G, Boeckx P, Van Cleemput O (2004) Nitrogen mineralization of vegetable root residues and green manures as related to their (bio)chemical composition. Eur J Agron 21:161–170

Eivazi F, Tabatabai MA (1977) Phosphatases in soils. Soil Biol Biochem 9:167–172

Eivazi F, Tabatabai MA (1988) Glucosidases and galactosidases in soils. Soil Biol Biochem 20:601–606

García C, Hernández T, Costa F, Ceccanti B (1994) Biochemical parameters in soils regenerated by the addition of organic wastes. Waste Manage Res 12:457–466

Gil-Sotres F, Trasar-Cepeda C, Leirós MC, Seoane S (2005) Different approaches to evaluating soil quality using biochemical properties. Soil Biol Biochem 37:877–887

Kandeler E, Gerber H (1988) Short-term assay os soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72

Kotsou M, Mari I, Lasaridi K, Chatzipavlidis I, Balis C, Kyriacou A (2004) The effect of olive oil mill wastewater (OMW) on soil microbial communities and suppressiveness against Rhizoctonia solani. Appl Soil Ecol 26:113–121

Ladd JM, Butler JHA (1972) Short-term assays of soil proteolytic enzyme activity using proteins and dipeptide derivates as substrates. Soil Biol Biochem 4:19–30

Masciandaro G, Ceccanti B, Benedicto S, Lee HC, Cook F (2004) Enzyme activity and C and N pools in soil following application of mulches. Can J Soil Sci 84:19–30

Mingorance MD, Barahona E, Fernández-Gálvez J (2007) Guidelines for improving organic carbon recovery by the wet oxidation method. Chemosphere 68:409–413

Mondini C, Sequi P (2008) Implication of soil C sequestration on sustainable agriculture and environment. Waste Manage 28:678–684

Nannipieri P, Kandeler E, Ruggiero P (2002) Enzyme activities and microbiological and biochemical processes in soil. In: Burns RG, Dick RP (eds) Enzymes in the environment: activity ecology and applications. Marcel Dekker, New York, pp 1–33

Pascual JA, Hernandez T, Garcia C, Ayuso M (1998) Enzymatic activities in an arid soil amended with urban organic wastes: laboratory experiment. Bioresour Technol 64:131–138

Piotrowska A, Iamarino G, Rao MA, Gianfreda L (2006) Short-term effects of olive mill waste water (OMW) on chemical and biochemical properties of a semiarid mediterranean soil. Soil Biol Biochem 38:600–610

Rajcan I, Dwyer LM, Tollenaar M (1999) Note on relationship between leaf soluble carbohydrate and chlorophyll concentrations in maize during leaf senescence. Field Crops Res 63:13–17

Randhawa PS, Condron LM, Di HJ, Sinaj S, McLenaghen RD (2005) Effect of green manure addition on soil organic phosphorus mineralisation. Nutr Cycl Agroecosyst 73:181–189

Reeves DW (1997) The role of soil organic matter in maintaining soil quality in continuous cropping systems. Soil Till Res 43:131–167

Renella G, Mench M, Gelsomino A, Landi L, Nannipieri P (2005) Functional activity and microbial community structure in soils amended with bimetallic sludges. Soil Biol Biochem 37:1498–1506

Roig A, Cayuela ML, Sánchez-Monedero MA (2006) An overview on olive mill wastes and their valorisation methods. Waste Manage 26:960–969

Sampedro I, Romero C, Ocampo JA, Brenes M, García-Romera I (2004) Removal of monomeric phenols in dry mill olive residue by saprobic fungi. J Agric Food Chem 52:4487–4492

Sampedro I, D'Annibale A, Ocampo JA, Stazi SR, García-Romera I (2005) Bioconversion of olive-mill dry residue by *Fusarium lateritum* and subsequent impact on its phytotoxicity. Chemosphere 40:1393–1400

Sampedro I, Aranda E, Díaz R, García-Sánchez M, Ocampo JA, García-Romera I (2008) Saprobe fungi decreased the sensitivity to the toxic efect of dry olive mill residue on arbuscular mycorrhizal plants. Chemosphere 70:1383–1389

Sampedro I, Giubilei MA, Cajthaml T, Federici E, Federici F, Petruccioli M, D'Annibale A (2009a) Short-term impact of dry olive mill residue addition to soil on the resident microbiota. Bioresour Technol 100:6098–6106

Sampedro I, Marinari S, Grego S, Petruccioli M, Federici F, D'Annibale A (2009b) Immobilized inocula of white-rot fungi accelerate both detoxification and organic matter transformation in two-phase dry olive-mill residue. J Agric Food Chem 57:5452–5460

Sampedro I, Cajthaml T, Marinari S, Petruccioli M, Grego S, D'Annibale A (2009c) Organic matter transformation and detoxification in dry olive mill residue by the saprophytic fungus paecilomyces farinosus. Process Biochem 44:216–225

Tejada M, González JL (2004) Effects of application of a by-product of the two-step olive oil mill process on maize yiels. Agron J 96:692–699

Tejada M, García C, González JL, Hernández MT (2006) Organic amendment based on fresh and composted beet vinasse: influence on physical, chemical and biological properties and wheat yield. Soil Sci Soc Am J 70:900–908

Tejada M, Moreno JL, Hernández MT, García C (2007) Application of two beet vinasse forms on soil restoration: effects on soil properties in an arid environment in southern spain. Agric Ecosyst Environ 119:289–298

Soil Microbial Population Changes in Soil Biodisinfection Process

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Abstract Soil biodisinfection process is based on the biodegradation of incorporated organic amendments to the soil. Soil microorganisms are directly responsible for this decomposition, so the soil biodisinfection process stimulates the soil microbial population activity. These microbial populations increase also produces increased levels of exoenzymes in the soil. We used plant by-products from strawberry crop as biofumigant in different application rates (10, 20 and 40 g of biofumigant kg^{-1} of agricultural soil). Microbial changes during the soil biodisinfection process were tested from two approaches, microbial catabolic abilities (Biolog® Eco) and microbial counts. We made counts of aerobic, anaerobic, *Pseudomonas* sp. and aminocyclopropane-1-carboxylate (ACC) degrading populations during the soil biodisinfection process (at 0, 7 and 20 d). The highest metabolic activity of soil microorganisms was found under 10 g kg^{-1} of soil treatment. Anaerobic microbial population increased significantly (7×10^6 CFU g^{-1} of agricultural soil) in the 40 g biofumigant kg^{-1} assay. Counts were decreased when the application rates were reduced, but these counts were higher than control assay values. Aerobic, *Pseudomonas* sp. and ACC degrading microbial populations had not significant changes by the addition of organic matter amendments. These data indicate that the activity of anaerobic microbial growth

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was stimulated by soil biodisinfection process. The soil biodisinfection technique is a viable and compatible method with other techniques for integrated crop management, as well as for biological control and plant growth stimulation in primary production.

1 Introduction

Biofumigation originally refers to the use of *Brassica* containing biocidal sulfur-compounds as green manure or rotation crops to control soil-borne pests and diseases (Angus et al. 1994). A lot of other plants have shown interesting activities against nematodes and fungi (Djian-Caporilano et al. 2005). Naturally occurring plant-derived volatiles that are fundamental flavour and fragrance constituents seem to possess antifungal activity. Zeringue et al. (1996) suggested that volatile compounds emitted from plants may be responsible for fungal inhibition. These volatile compounds have been studied for their potential as postharvest inhibitors of several fruits (Vaughn et al. 1993; Archbold et al. 1997; Neri et al. 2006). In this study strawberry by-products were used as biofumigant due to its potential fungicide effect. During the process of biodecomposition of organic matter, which produces volatile substances with biocide action (Sarwar and Kirkegaard 1998; Bello et al. 2003), the microbial activity of the soil is stimulated (Piedrabuena et al. 2006). When organic matter is added, a sequence of microbiological changes occurs with a growth of microorganisms that depends on the amount and properties of organic matter incorporation. The principal effect is the temporal increase in the quantities of nutrients that have an impact on the living portion of the soil. A residual population of beneficial organisms can survive (rhizobacteria, protozoans or fungi) and they are biocontrol organisms (Baker 1988; Kloepper et al. 1988). It has been demonstrated that soil biodisinfection process stimulates the biological activity of soils, increasing the populations of antagonistic organisms of pathogens such as *Pseudomonas fluorescens* and *Bacillus* sp., as well as the populations of saprophagous and bacteriophagous nematodes, and decreasing parasitic forms of fungus and nematodes (Piedrabuena et al. 2006). However, other authors had suggested that competition among rhizospheric organisms is the cause of the effect of biofumigation (Baker and Cook 1974).

Crop residues are usually considered as 'waste' materials without any value and even as contamination sources. However, since it is known that incorporating crop residues into the soil increases microbial activity (Piedrabuena et al. 2006), the soil biodisinfection process can be efficiently introduced in the agricultural production system, at competitive prices, by the use of agro-industrial residues (Bello et al. 2003).

The objective of this work was to analyze microbial changes during the soil biodisinfection process from two approaches: microbial catabolic abilities (Biolog® Eco substrate utilization profiles) and changes in anaerobes, aerobes, *Pseudomonas* sp. and ACC degrading microorganisms (microbial counts).

Table 1 Chemical composition of organic matter amendment (strawberry crop by-products) used in the assays. Data are given in fresh basis

Parameter	Value
pH	4.3
Moisture content	95.3%
Total nitrogen	2.7%
Total organic carbon	54%
Total organic matter	93%
C/N	20

2 Materials and Methods

2.1 Plant Material Used in the Soil Biosisinfection Process Assay

For the development of the soil biodisinfection process assays, strawberry (*Fragaria* × *ananassa*) by-products were used as biofumigant at different application rates (10, 20 and 40 g of biofumigant kg^{-1} of agricultural soil). One agricultural soil was taken from Pampliega (Burgos, Spain). The physical and chemical characteristics of this soil were: pH (1/2.5), 6.5; clay, 4.6%; silt, 18.4%; sand, 77% and organic matter, 11 g kg^{-1}. The different assays were named F2.5, F5 and F10 in relation to the application rates of 10, 20 and 40 g kg^{-1}, respectively (each tray was 250 g of agricultural soil). To study the effect of organic matter amendments, this set of trays was mixed with organic wastes from *Fragaria* × *ananassa*, nursery plants, and then they were introduced in plastic bags and maintaned at 30°C for 20 days (Bello et al. 2003). Other tray was used as control, without biofumigant, named T assay. The chemical composition of the strawberry by-products is showed in Table 1. The methodology used for the determination of the strawberry by-products and soil parameters were carried out according to those described in Ministerio de Agricultura, Pesca y Alimentación, MAPA (1994).

2.2 Community Catabolic Profiles. Biolog® Plates

Bacterial communities were characterized by their metabolic fingerprints, using Biolog Eco® micro-plates (BIOLOG Inc., Hayward, CA). Two grams of agricultural soil of each treatment and sampling time (0 and 20 days) were homogenised in 20 ml of distilled water with an Omnimixer at 16.000 rpm for 1 min. Three replicates per treatment were performed. After centrifugation of soil suspension (750 g, 10 min), the supernatant was filtered through glass wool. Suspensions were diluted 100-fold in sterile water and sown in Biolog Eco plates. Plates were incubated in dark at 25°C. Absorbance values at 595 nm were measured each 24 h until 192 h with an Asys High Tech Expert 96 spectrophotometer and Microwin 2000 analysis software. Absorbance values of the wells were

blanked against the control well (corrected absorbance values). All negative values were set to zero. The average well colour development (AWCD) was calculated as the mean of the 31 blanked absorbance values. Then, the absorbance value of each well was divided by the AWCD in order to minimise the influence of inoculum density differences between plates (Baudoin et al. 2001). Kinetics of AWCD were represented in order to determine the speed and the level of development of the bacterial communities using the 31 substrates provided. Two-way ANOVA were performed for kinetics of AWCD values.

With corrected absorbance values at 120 h of incubation of the Biolog plates, the percentage of intensely utilized substrates compounds (those which have an absorbance >AWCD) within each of the substrate categories was calculated for each plate and was named substrate richness (Baudoin et al. 2002). Also, metabolic diversity using Shannon's diversity index ($H = -\Sigma[n_i/N * \text{Log } n_i/N]$) was measured. Where n_i was the corrected absorbance values of each substrate and N the sum of all corrected absorbance values of all substrates of Biolog Eco plates. One-way ANOVA was carried out for metabolic diversity results.

2.3 *Microbiological Changes of Total Anaerobes, Aerobes,* **Pseudomonas** *sp. and ACC Degrading Microorganisms*

In order to determine the microbiological changes of anaerobes, aerobes, *Pseudomonas* sp. and aminocyclopropane-1-carboxylate (ACC) degrading populations during the organic matter incorporation process, different selective media were used.

Ten-fold dilutions of substrates from each tray (F2.5, F5, F10 and T assays) at 0, 7 and 20 days were prepared in peptone water (Difco Laboratories), and later spread-plated on PCA medium (Oxoid, UK) for aerobes, KING A medium (Merck) for *Pseudomonas*, ACC medium for detecting ACC degrading bacteria, and pour-plated in PCA medium (Oxoid) for anaerobic bacteria. All the plates were grown (PCA in anaerobic conditions) at 30°C for 72 h and each 24 h bacterial number were counted. The control assay (T) was performed under the same conditions without organic matter amendments incorporation. One-way ANOVA was carried out for microbiological changes results.

2.4 *Statistical Analysis*

One-way ANOVA and two-way ANOVA were carried out depending on the cases as indicated above. When differences were significant, the LSD post-hoc test was also performed (Sokal and Rohlf 1980). These analyses were carried out with STATGRAPHICS Plus 5.0 software.

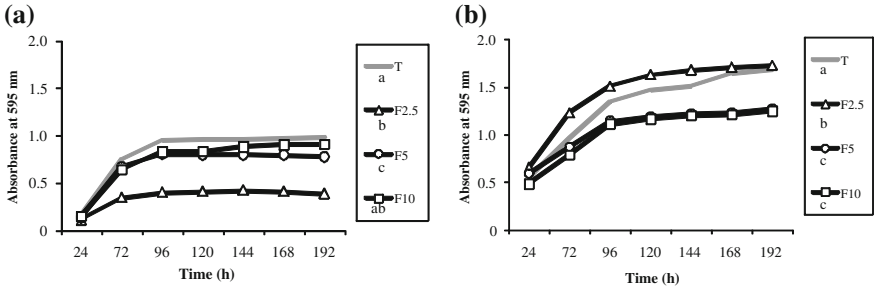

Fig. 1 Kinetics of AWCD (average well colour development) mean average values, (n = 3) of: **a** Biolog Eco plates inoculated with bacterial suspension from day 0 organic matter incorporation process; **b** Biolog Eco plates inoculated with bacterial suspension from day 20 organic matter incorporation process. Different letters indicate significant differences in curves trends between treatments. Two-way ANOVA, $P \leq 0.05$

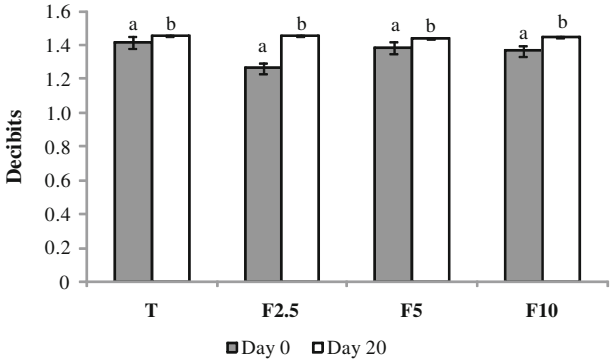

Fig. 2 Metabolic diversity calculated using Shannon's diversity index with Biolog data (at 120 h of incubation) of bacterial community (Biolog Eco plates) from day 0 and day 20 from organic matter incorporation process. Different letters indicate significant differences. One-way ANOVA, $P \leq 0.05$

3 Results

3.1 Bacterial Community Catabolic Profiles: Biolog® Eco Plates

Organic matter incorporation process produced relevant changes in bacterial communities' catabolic profiles. First, kinetics of AWCD were represented (Fig. 1) in order to determine the speed and the level of development of the bacterial communities using the 31 substrates provided. Bacterial community, the day 0 of organic matter incorporation process presented significantly lower values in F2.5 treatments than in the others (Fig. 1a). However, in the day 20 of organic matter incorporation process, the highest values were found for F2.5 treatment

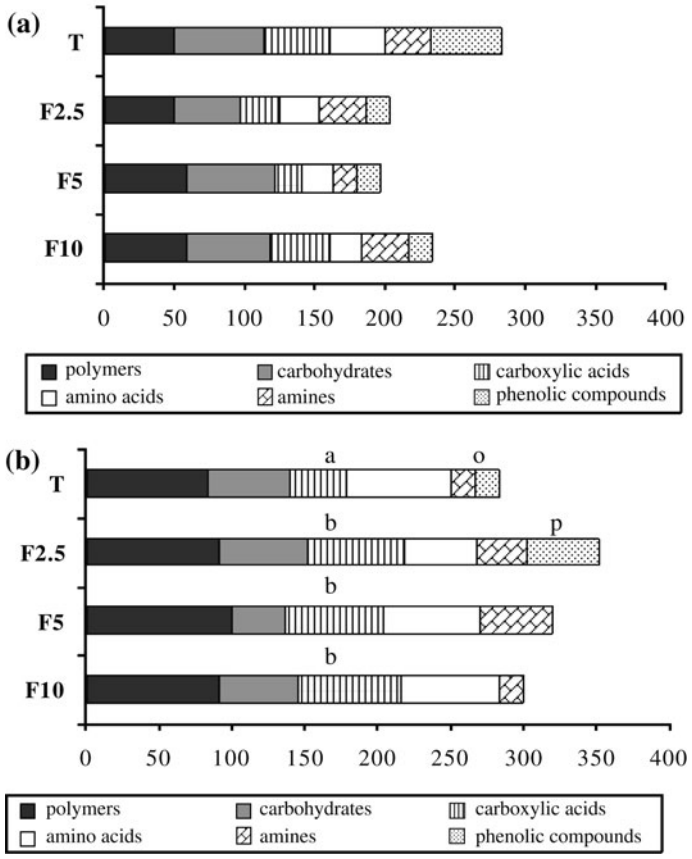

Fig. 3 Percentage of intensely utilized substrates (those which have an absorption > Average Well Colour Development) within each group of compounds: **a** Biolog Eco day 0, **b** Biolog Eco day 20. Day 0 and day 20 correspond to sampling days in organic matter incorporation process. One-way ANOVA. Different letters indicate significant differences ($P \leq 0.05$) between treatments for each categories of compounds: polymers, carbohydrates, etc

which showed significant differences respect to the others (Fig. 1b). Opposite to this, bacterial communities from F5 and F10 treatments had very high values of AWCD at day 0 (at 192 h of incubation) (Fig. 1a) and the lowest values compared to the other treatments at day 20 of organic matter incorporation process (Fig. 1b).

With regard to metabolic diversity of bacterial communities, values were under 1.4 at day 0 (Fig. 2a), and over 1.4 at day 20 of organic matter incorporation process (Fig. 2b). This increase of metabolic diversity after the 20 days of incubation with organic matter was significant (data not shown) but there were not significant differences between treatments in each moment (day 0 and day 20). Bacterial community metabolic diversity seems to be more similar among treatments at day 20, after organic amendment incorporation process (Fig. 2b).

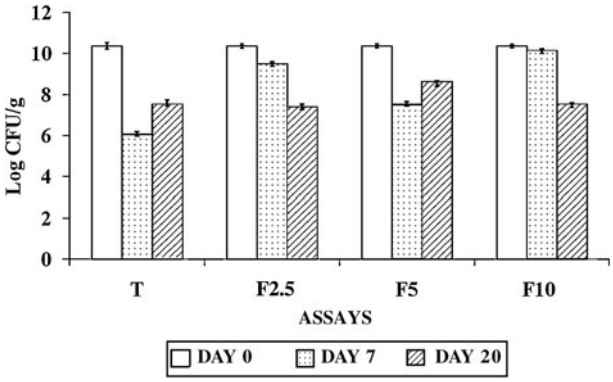

Fig. 4 Anaerobic microorganisms population changes at 0, 7 y 20 d of the soil biodisinfection process using three different application rates of biofumigant (F10, F5 y F2.5). T (control assay)

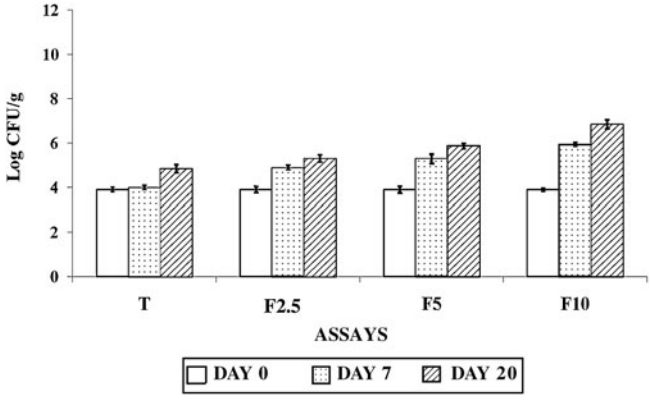

Fig. 5 Aerobic microorganisms population changes at 0, 7 y 20 d of the soil biodisinfection process using three different application rates of biofumigant (F10, F5 y F2.5). T (control assay)

Substrate richness graphs indicate which substrates were used intensively by bacterial communities (Figs. 3a, b). For bacteria, no significant differences were found for the utilization of each group of compounds under the different treatments the day 0 of the incubation period (Fig. 3a) and some differences appeared for carboxylic acids and phenolic compounds on the day 20 (Fig. 3b). At day 20 of organic amendment incorporation process, phenolic compounds were not used as carbon source by bacterial communities under F10 and F5 treatments. The highest carbon utilization was found for bacterial communities under T treatment at day 0 (Fig. 3a) and F2.5 at day 20 (Fig. 3b). Amino acids were used more at day 20 than at day 0.

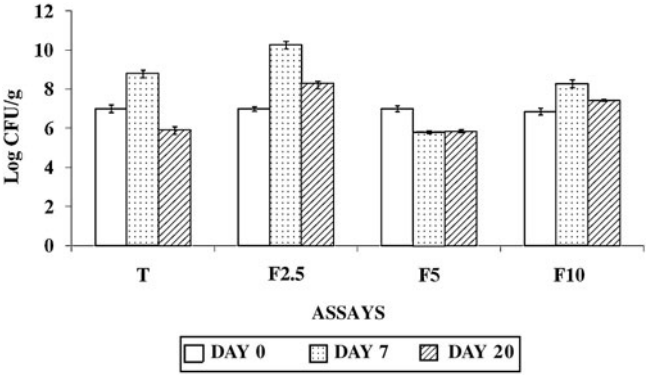

Fig. 6 *Pseudomonas* sp. population changes at 0, 7 y 20 d of the soil biodisinfection process using three different application rates of biofumigant (F10, F5 y F2.5). T (control assay)

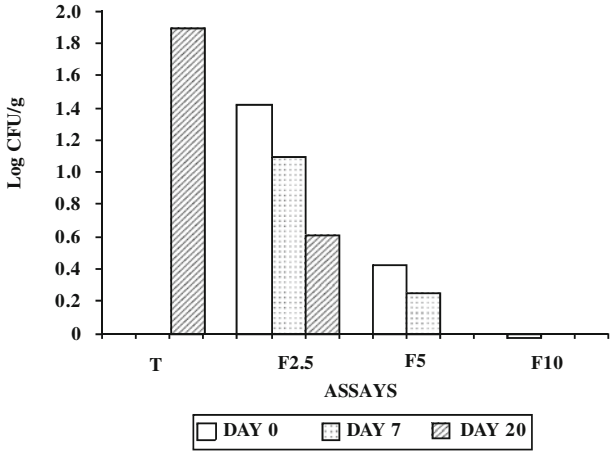

Fig. 7 Changes in ACC degrading population counts during organic matter incorporation process. Samples were sown in ACC and MSACC media. Data showed are only the ACC population

3.2 Microbiological Changes of Total Anaerobes, Aerobes, **Pseudomonas** *sp. and ACC Degrading Microorganisms*

At 20 d of soil biodisinfection process, F10 assay anaerobic counts increased significantly (7×10^6 cfu g^{-1} of agricultural soil), while the F5 and F2.5 assays counts reached values of 8×10^5 and 2×10^5 cfu g^{-1} respectively, and the T assay values were 7×10^4 cfu g^{-1} (Fig. 4). The aerobic and *Pseudomonas* sp.

populations did not change in the F2.5, F10 and F5 assays regard to the T assay (Figs. 5 and 6).

Organic matter amendments incorporation modified the ACC degrading population; when the biofumigant application rate used was higher, the ACC population was lower. Under T treatment there was not ACC degrading population at day 0 but at day 20 it appeared (Fig. 7).

4 Discussion

Microbial changes during the incubation with the organic matter were tested from two approaches, microbial catabolic abilities and microbial counts. As for the first, Biolog Eco plates have been used to study catabolic profiles and functional diversity of bacterial communities during the organic matter incubation process. The use of Biolog Eco plates can be consider as a quantitative and qualitative study of catabolism of culturable soil bacterial community. Over the years, increased understanding of the Biolog assay has demonstrated the reproducibility of Biolog profiles and supported the theory that shifts in Biolog metabolic diversity patterns are related to shifts in community composition (Schutter and Dick 2001; Crecchio et al. 2004). Biolog technique does provide a rapid and convenient assay for culturable heterotrophic organisms, and give sensitive indicators of changes in soil microbial functional diversity due to different disturbances (Pietikäinen et al. 2000; Pankhurst et al. 2001; Rogers and Tate 2001). In this way, Huang et al. (2008) detected changes in behaviour of soil microorganisms, growing in Biolog plates, obtained of a soil treated with plant residues (mulch treatment) from control soil (non-mulch treatment).

In this way, kinetics of AWCD (average colour development) of Biolog Eco plate throughout plate incubation, showed that F2.5 treatment produced an increase of bacterial metabolic speed after the 20 days of treatment, since it presented significantly lower values than the other treatments in day 0 (Fig. 1a) and significantly higher to the others in day 20 (Fig. 1b). Opposite to this, bacterial communities from F5 and F10 treatments decreased its metabolic speed during the treatment. Since shifts in Biolog catabolic patterns have been related to shifts in community composition (Schutter and Dick 2001; Crecchio et al. 2004) it could be noted from our results that after the 20 days of incubation with the organic matter, bacterial communities are overall more metabolically active in view of metabolic values of both days (Figs. 1a, b). Moreover, F2.5 application rate seems to stimulate metabolic speed of bacteria more than the other treatments.

However, no significant differences between treatments with regard to metabolic diversity value for bacterial communities were found. For bacteria, metabolic diversity values were significantly higher after the 20-days incubation period (data not shown), indicating that organic matter added possibly increased genetic diversity related to bacterial metabolism.

With regard to substrate richness, calculated as the percentage of highly used substrates within each substrate categories, F2.5 treatment increases the utilization of substrates. However the F5 and F10 treatments decrease the value of the wealth of the substrate. All the differences found between treatments and between day 0 and day 20 (Fig. 3) could be indicating changes in bacterial metabolic abilities since microbial capacity to use the different Biolog plate compounds is a reflection of metabolic capacities of these bacteria at the moment of the sampling (Schutter and Dick 2001; Crecchio et al. 2004).

Microbial populations typically fluctuate and vary regularly in response to the addition of organic amendments (Van Bruggen and Semenov 2000). Organic matter degradation leads to the modification of the atmosphere of the soil, increasing the content of carbon dioxide, reducing the oxygen and producing anaerobiosis conditions. Organic matter incorporation process helps the growth and activity of anaerobic microorganisms (Blok et al. 2000; Bello et al. 2003). We determined the highest increase of anaerobic counts when the highest application rate of the biofumigant was used (Fig. 4). In the present work, aerobic population decreased due to the oxygen decrease in the organic matter incorporation process (Fig. 5). Blok et al. (2000) have studied anaerobic soil conditions (concentration of oxygen) that occur both in the biofumigation as in biofumigation complemented with the application of plastic covers techniques. The combination of both control methods achieves to control the growth of pathogenic fungi. This stimulation of anaerobic microorganisms can contribute to the control of plant pathogens, since it is well known that some genera such as *Bacillus* and *Clostridium* produce antimicrobial substances.

Pseudomonas sp. counts, under F2.5, F5 and F10 treatments, decreased notably at day 20 (Fig. 6). Organic matter incorporation process did not stimulate the ACC degrading population (Fig. 7). In fact, the counts decreased at the final day in F2.5, F5 and F10 treatments progressively, with the highest decrease occurring under F10 treatment (Fig. 7).

The control treatment (T) increased the number of bacteria able to degrade ACC, and between the treatments with organic matter, F2.5 was the one which showed an increased presence of ACC degrading bacteria (Fig. 7), coinciding with the one with the greatest stimulation of metabolic speed.

The microbial reduction occurred in the aerobes. *Pseudomonas* sp. and ACC degrading populations, can be caused by other environmental factors, such as the mechanisms of competition for space and/or nutrients and physicochemical changes in the soil. This could be pointing out to a high activity, biomass and microbial diversity, which, in natural and agricultural soils, have been associated with suppression of plant diseases (Mäder et al. 2002; Mulder et al. 2003). It has been well-documented that survival of many plant pathogens is strongly reduced under anaerobic soil conditions. The exact mechanism is not clear, but lack of oxygen, the accumulation of toxic products resulting from anaerobic decomposition processes, and biocontrol by anaerobic microorganisms have been implicated as critical factors (Cook and Baker 1983; Strandberg 1987).

5 Conclusions

In conclusion, during the soil biodisinfection process, it was under F2.5 (10 g kg^{-1} of soil) treatment when metabolic activity of soil microorganisms was more increased. Aerobic, *Pseudomonas* sp. and ACC degrading populations decreased while anaerobes populations increased with organic matter treatments. All these microorganisms shifts could be indicating a joint effect of volatile anti-fungal compounds and microorganism protective effect of organic matter, and further studies would be required for a deeper understanding of the involved microorganisms.

References

Angus JF, Gardner PA, Kirkegaard JA, Desmarchelier JM (1994) Biofumigation: isothiocyanates released from *Brassica* roots inhibit the growth of take-all fungus. Plant Soil 162:107–112

Archbold DD, Hamilton-Kemp TR, Barth MM, Langlois BE (1997) Identifying natural volatile compounds that control gray mold (*Botrytis cinerea*) during postharvest storage of strawberry, blackberry, and grape. J Agric Food Chem 45:4032–4037

Baker R (1988) *Trichoderma* spp as plant growth stimulants. CRC Crit Rev Biotechnol 7:97

Baker KF, Cook RJ (1974) Biological control of plant pathogens. WH Freeman & Co, San Francisco CA

Baudoin E, Benizri E, Guckert A (2001) Metabolic fingerprint of microbial communities from distinct maize rhizosphere compartments. Eur J Soil Biol 37:85–93

Baudoin E, Benizri E, Guckert A (2002) Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. Appl Soil Ecolo 19:135–145

Bello A, López-Pérez JA, García Álvarez A (2003) Biofumigación en Agricultura Extensiva de Regadío. Producción Integrada de Hortícolas. Editorial Mundi-Prensa, Madrid, Spain

Blok WJ, Lamers JG, Termorshuizen AJ, Bollen GJ (2000) Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology 90:253–259

Cook RJ, Baker KF (1983) The nature and practice of biological control of plant pathogens the american phytopathological society. St. Paul, MN

Crecchio C, Gelsomino A, Ambrosoli R, Minati JL, Ruggiero P (2004) Functional and molecular responses of soil microbial communities under differing soil management practices. Soil Biol Biochem 36:1873–1883

Djian-Caporilano C, Bourdy G, Cayrol JC (2005) Nematicidal and nematode-resistant plants. In: Regnault-Roger C, Philogène BJR, Vincent C (eds) Biopesticids of plant origin. Lavoisier and Intercept, Paris, France, pp 174–224

Huang Z, Xu Z, Chen C (2008) Effect of mulching on labile soil organic matter pools, microbial community functional diversity and nitrogen transformations in two hardwood plantations of subtropical Australia. Appl Soil Ecol 40:229–239

Kloepper JW, Lifshitz R, Schroth MN 1988 *Pseudomonas* inoculants to benefit plant production. ISI Atlas of Science-Animal & Plant Sciences 60–65

Mäder P, Flieβbach A, Dubois D, Gunst L, Fried P, Niggli U (2002) Soil fertility and biodiversity in organic farming. Science 296:1694–1697

MAPA (1994) Métodos Oficiales de Análisis. Tomo III. Plantas, Productos Orgánicos Fertilizantes, Suelos, Aguas, Productos Fitosanitarios, Fertilizantes Inorgánicos. Publicaciones del Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain

Mulder C, de Zwart D, van Wijnen HJ, Schouten AJ, Breure AM (2003) Observational and simulated evidence of ecological shifts within the soil nematode community of agroecosystems under conventional and organic farming. Funct Ecol 17:516–525

Neri F, Mari M, Brigati S (2006) Control de *penicillium expansum* by plant volatile compounds. Plant Pathol 55:100–105

Pankhurst CE, Yu S, Hawke BG, Harch BD (2001) Capacity of fatty acid profiles and substrate utilization patterns to describe differences in soil microbial communities associated with increased salinity or alkalinity at three locations in south australia. Biol Fertil Soils 33:204–217

Piedrabuena A, García-Álvarez A, Díez-Rojo MA, Bello A (2006) Use of crop residues for the control of *meloidogyne incognita* under laboratory conditions. Pest Manag Sci 62:919–926

Pietikäinen J, Hiukka R, Fritze H (2000) Does short-term heating of forest humus change its properties as a substrate for microbes. Soil Biol Biochem 32:277–288

Rogers BF, Tate RL (2001) Temporal analysis of the soil microbial community along a toposequence in pineland soils. Soil Biol Biochem 33:1389–1401

Sarwar M, Kirkegaard JA (1998) Biofumigation potential of brassicas. Plant Soil 201:91–101

Schutter M, Dick R (2001) Shifts in substrate utilization potential and structure of soil microbial communities in response to carbon substrates. Soil Biol Biochem 33:1481–1491

Sokal RR, Rohlf FJ (1980) Introducción a la Bioestadística. Editorial Reverté, Barcelona, Spain

Strandberg JO (1987) The effect of flooding on plant pathogen populations. In: Snyder GH (ed) Agricultural flooding of organic soils. Bulletin 870 agricultural experiment station institute of food and agricultural sciences, university of florida. Dolphin, Gainesville, FL, pp 41–56

Van Bruggen AHC, Semenov AM (2000) In search of biological indicators for soil health and disease suppression. Appl Soil Ecol 15:13–24

Vaughn SF, Spencer GF, Shasha BS (1993) Volatile compounds from raspberry and strawberry fruit inhibit postharvest decay fungi. J Food Sci 58:793–796

Zeringue HJ, Brown RL, Neucere JN, Cleveland TE (1996) Relationship between C6–C12 alkanal and alkenal volatile contents and resistance of maize genotypes to aspergillus flavus and aflatoxin production. J Agric Food Chem 44:403–407