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Emerging Organic Contaminants in Sludges

Analysis, Fate and Biological Treatment

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The Handbook of Environmental Chemistry

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Emerging Organic Contaminants in Sludges

Analysis, Fate and Biological Treatment

Volume Editors: Teresa Vicent · Glòria Caminal · Ethel Eljarrat · Damià Barceló

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Aims and Scope

Since 1980, *The Handbook of Environmental Chemistry* has provided sound and solid knowledge about environmental topics from a chemical perspective. Presenting a wide spectrum of viewpoints and approaches, the series now covers topics such as local and global changes of natural environment and climate; anthropogenic impact on the environment; water, air and soil pollution; remediation and waste characterization; environmental contaminants; biogeochemistry; geoecology; chemical reactions and processes; chemical and biological transformations as well as physical transport of chemicals in the environment; or environmental modeling. A particular focus of the series lies on methodological advances in environmental analytical chemistry.

Series Preface

With remarkable vision, Prof. Otto Hutzinger initiated *The Handbook of Environmental Chemistry* in 1980 and became the founding Editor-in-Chief. At that time, environmental chemistry was an emerging field, aiming at a complete description of the Earth's environment, encompassing the physical, chemical, biological, and geological transformations of chemical substances occurring on a local as well as a global scale. Environmental chemistry was intended to provide an account of the impact of man's activities on the natural environment by describing observed changes.

While a considerable amount of knowledge has been accumulated over the last three decades, as reflected in the more than 70 volumes of *The Handbook of Environmental Chemistry,* there are still many scientific and policy challenges ahead due to the complexity and interdisciplinary nature of the field. The series will therefore continue to provide compilations of current knowledge. Contributions are written by leading experts with practical experience in their fields. *The Handbook of Environmental Chemistry* grows with the increases in our scientific understanding, and provides a valuable source not only for scientists but also for environmental managers and decision-makers. Today, the series covers a broad range of environmental topics from a chemical perspective, including methodological advances in environmental analytical chemistry.

In recent years, there has been a growing tendency to include subject matter of societal relevance in the broad view of environmental chemistry. Topics include life cycle analysis, environmental management, sustainable development, and socio-economic, legal and even political problems, among others. While these topics are of great importance for the development and acceptance of *The Handbook of Environmental Chemistry,* the publisher and Editors-in-Chief have decided to keep the handbook essentially a source of information on "hard sciences" with a particular emphasis on chemistry, but also covering biology, geology, hydrology and engineering as applied to environmental sciences.

The volumes of the series are written at an advanced level, addressing the needs of both researchers and graduate students, as well as of people outside the field of "pure" chemistry, including those in industry, business, government, research establishments, and public interest groups. It would be very satisfying to see these volumes used as a basis for graduate courses in environmental chemistry. With its high standards of scientific quality and clarity, *The Handbook of*

Environmental Chemistry provides a solid basis from which scientists can share their knowledge on the different aspects of environmental problems, presenting a wide spectrum of viewpoints and approaches.

The Handbook of Environmental Chemistry is available both in print and online via www.springerlink.com/content/110354/. Articles are published online as soon as they have been approved for publication. Authors, Volume Editors and Editors-in-Chief are rewarded by the broad acceptance of *The Handbook of Environmental Chemistry* by the scientific community, from whom suggestions for new topics to the Editors-in-Chief are always very welcome.

Damià Barceló
Andrey G. Kostianoy
Editors-in-Chief

Volume Preface

This book on *Emerging Organic Contaminants in Sludges: Analysis, Fate and Biological Treatment* provides information on the current state of the art with regard to emerging organic contaminants (ECs) with specially focus on their occurrence in sludge. Much scientific work describes the presence of ECs in rivers, urban wastewater, industrial effluents, etc. Some of these ECs are characterized by their hydrophobicity although their tendency to accumulate in sludge is scarcely reported.

This book aims to review and compile the main developments and knowledge acquired in this field, and tries to remedy this lack of information. The book is structured in nine different chapters, covering the impact of ECs on biosolids applications, advanced chemical analytical methods, the occurrence and fate of ECs in sludge, and biological degradation techniques, with special focus on treatment by fungi. Finally, the last chapter discusses the main conclusions and possible future trends, these being the starting point to be taken into account in future studies in the field.

Due to the demand for sustainable technologies, bioremediation is regarded as one of the best method for pollutant degradation because of its cost, efficiency and environmental friendliness. Among all the microorganisms that can be used in bioremediation, fungi play an important role. The considerable research effort on fungi capacities demonstrates that their biochemical and physiological characteristics can be important for a wide range of biotechnological applications to be used for degrading organic contaminants. As a result of their great promise, this book includes four chapters on fungal degradation, starting with sludge treatments and going on to the degradation of three selected EC families, pharmaceuticals, UV filters and PBDEs, as case studies. These specific studies allow the analysis and development of two important aspects related to degradation processes – metabolite identification and toxicity.

The large number of figures, tables and case studies, together with the double disciplinary approach, environmental analysis and engineering, make *Emerging Organic Contaminants in Sludges: Analysis, Fate and Biological Treatment* a relevant and interesting book for a broad audience of scientific researchers such as environmental chemists, environmental engineers, biotechnologists, ecotoxicologists and professionals responsible for waste and water management. We hope that

the information in this book can also be useful to extend the results obtained for the selected families to new ECs.

Finally we would like to thank all the contributing authors of this book for their time and effort in preparing this comprehensive compilation of research papers.

Barcelona, Spain

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Contents

Introduction to Organic Contaminants in Soil: Concepts and Risks

L. Valentín, A. Nousiainen, and A. Mikkonen

Abstract This chapter introduces an overview of the main aspects of soil contamination: the types of contaminants, the threats to soil biota and quality, the effects on humans and animals and legislation concerning contaminants, with a focus on European laws and regulations. Although soil is a non-renewable natural resource, humans have increasingly used it as a contaminant sink ever since the Industrial Revolution. Pollution endangers natural soil mechanisms that allow the soil to keep itself in balance and compromises the supply of soil-based goods and services. Detrimental effects of contaminants on soil microbiota may be directly related to loss of biodiversity and functions such as the recycling of nutrients. However, this direct negative effect is still debated among microbial ecologists because microbial communities may be surprisingly resilient (i.e. able to recover from contamination effects) and/or functionally redundant. Likewise, the potential toxicity of soil contamination to humans is controversial as investigations on the mix of different contaminants are very limited. Due to accumulated evidence of the potential toxicity of single contaminants on human health and animals, mostly based on in vitro or in vivo assays, international guidelines, such as the Stockholm Convention on Persistent Organic Pollutants, have established a list of priority contaminants. Despite worldwide concern about soil contamination, Europe still lacks a common soil legislative tool because the proposed Soil Directive Framework is currently under negotiation. The lack of a European directive is nevertheless compensated by national policies at the member state level.

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Contents

1 Soil as a System and Resource

The quality of water and air is of immediate concern for most people because we all consume these natural resources on a daily basis. The importance of soil, the generally thin layer of unconsolidated material on bedrock, is more difficult to grasp for an average citizen or politician. Nonetheless, soil is the "the biogeochemical engine of Earth's life support system" [1]. It provides us with food, fodder, fibre and fuel. In addition to these readily rateable agriculture and forestry goods, soils deliver ecosystem services that cannot be easily traded in markets. These life-supporting functions include, for example, recycling of carbon and essential nutrients of all living materials, filtering and storage of water, regulation of the atmosphere and biological control of pests [1, 2].

Continuing urbanisation, desertification intensified by global change and short-sighted agricultural practices threaten the natural soil capital [3]. In the next few decades, a decreasing land area is still expected to deliver soil-based goods and services to nine billion people [4]. The severity of this discrepancy is exacerbated by the fact that soils must be considered to be a largely non-renewable natural resource [3, 5]; the full functionality of land where the thin surface soil layer is lost by erosion or sandstorm, or severely degraded by persistent anthropogenic pollutants, will not be restored within one human generation. Although native people worldwide have traditionally valued and nurtured soil or "Mother Earth," the policy makers of the modern world have only rather recently identified the endangered status of many soils, much later than was the case for water and air resources. The EU Thematic Strategy for Soil Protection currently identifies erosion, organic matter decline, salinisation, compaction, landslides, contamination and sealing as the major threats to soils [6]. However, the long-awaited binding EU Soil Directive is still at the proposal level [5].

There is an ongoing debate in agricultural policy on whether intensified agriculture on restricted sites or wildlife-friendly farming on larger land areas would better secure delivery of soil-based goods and ecosystem services for the growing population [4]. The possibility of reverting to traditional agro-ecological or biological fertilisation strategies, such as the use of legumes with nitrogen-fixing symbiotic root nodule bacteria or symbiosis between crops and rock phosphate-leaching mycorrhizal fungi, is receiving increasing interest. Additionally, conventional (industrial) agriculture can only be sustainable if the soils are not over-exploited, meaning that the stripped natural soil capital, such as nutrients and carbon, must be replenished [1, 7]. In any case, more efficient recycling of nutrients from waste and by-products will be essential in the future [8]. The production of inorganic nitrogen fertilisers through the energy-intensive Haber–Bosch process will become more expensive due to the increasing price of energy. Moreover, readily utilisable phosphorus, available in a few geographically unevenly distributed and non-renewable phosphate rock deposits, may wane even faster than fossil fuel resources [8].

The protocols of conventional agriculture, especially tilling, deplete soil organic matter content, and climate change seems to intensify this negative development [3, 7]. Sufficient organic C correlates positively with essential soil physicochemical properties such as structure and water holding capacity [9]. Biologically, organic C is the common currency of soil, feeding the entire soil food chain, from microbial decomposers to higher organisms [7]. Accordingly, replenishing stocks of soil C by organic fertilisation in the form of manure or sludge is largely beneficial.

To understand, predict and manage the functioning of soil, it is necessary to stop perceiving it merely as dirt or an abiotic surface for the growth of vegetation. Soil is an intricate three-phase system composed of a variety of solids, water and dissolved substances, as well as gases in pore air [10]. The constituents are organised into three-dimensional structures that resemble fractals and change dynamically over time. Intriguingly, changes in soil structure are biologically mediated and take place in a coordinated manner [11]; soil could thus be called the most complex biomaterial on Earth [12]. In addition to the maintenance of soil structure, the majority of other essential soil functions are also biological by nature [13, 14].

Although the role of fauna such as earthworms cannot be neglected [15], the majority of soil properties can be traced to the activity of soil microbiota, especially bacteria, fungi and archaea [13, 16, 17]. It is estimated that approximately 90% of the energy flow in soil passes through microbial decomposers [18]. Moreover, the metabolic agility of soil microbes is not limited to the consumption of various carbonaceous compounds by heterotrophs. Many bacteria and archaea (i.e. prokaryotes) are able to grow autotrophically. Phototrophs such as soil surface cyanobacteria derive energy from light. Chemolithoautotrophs can literally consume rocks and simultaneously perform biogeochemical transformation processes traditionally considered to be within the field of soil chemistry [19]. This wide diversity of energy sources and living strategies is reflected in the phylogenetic diversity of the organisms: estimates of the number of different bacterial species in just one gram of soil are in the range of 10^4–10^7 [20]. The total number of prokaryotic cells in that one

gram may reach 10^{10}, but due to their micron-scale sizes, they only cover approximately the same percentage of soil particle surfaces, 10^{-6}%, as the percentage of the surface of the Earth covered by humans [12].

2 Soil Contamination as the Main Threat to Soil Quality

Humans have invented many means to make life more comfortable. Excavating fossil fuels for energy and the advent of agricultural chemicals and pharmaceuticals have facilitated the improvement of the quality of life for many people. Unfortunately, many of these inventions have a downside: chemicals needed for such improvements may have adverse health effects and impacts on the environment and humans. Careless manufacturing and application of industrial and agricultural products together with the lack of suitable waste disposal has caused chemicals to end up where they were not originally meant to be, causing contamination of soil, groundwater, sediments and the food chain itself. In this sense, a contaminated site may be defined as "a site with confirmed presence of dangerous substances caused by man to such level that they may pose a significant risk to a receptor in such a way that action is needed to manage the risk" [21]. Contaminated sites are not equally distributed. Many Western European countries and the United States experienced the early years of industrialisation, and therefore, historically contaminated point source sites, such as polychlorinated biphenyls (PCBs) and dioxin manufacturing facilities, are often found in these countries. Currently, many emerging economies seem willing to pay the price of environmental pollution for rapid development and an increase in the gross domestic product.

If soil contamination is classified according to the source of contamination, point sources are distinct from diffuse sources. A point source of contamination is frequently related to industrial discharges, improper waste disposal or accidental spills during the transportation or handling of hazardous substances. Domestic examples of point contamination are the careless use of septic tanks and leaking underground oil tanks. Diffuse contamination is associated with certain agriculture and forestry practices, transportation and improper waste and wastewater management. Another diffuse source of contamination is the atmospheric deposition of low-volatile compounds that may affect pristine areas at Arctic and Antarctic latitudes due to the "cold condensation effect" [22]. Soil contamination is often associated with groundwater contamination. Soil porewater moves vertically, at a rate largely determined by soil texture, when excess rain is absorbed into the deeper layers, thereby generating groundwater. By contrast, horizontal groundwater flow is driven by lakes and river systems. Vertical and horizontal groundwater flows are the means of transport for many water-soluble contaminants. Because groundwater pollution is very difficult and expensive to manage, it is imperative to prevent the leaching of contaminants into groundwater. On the other hand, non-water-soluble chemicals threat the function of primary producers living in the soil. Non-water-soluble chemicals also affect higher organisms because poorly biodegradable lipophilic substances tend to accumulate in the food chain.

Despite the difficulties of evaluating the true extent of contamination, the latest estimations in Europe in 2006 set a number of 3.5 million potentially contaminated sites [23]. Of these, approximately 0.5 million were expected to require urgent treatment. The National Priorities List of the 2012 US Environmental Protection Agency (EPA) identified 1,305 superfund sites, which are defined as abandoned hazardous waste sites [24].

2.1 Contaminants in Soil

Since the beginning of industrialisation, a great variety of anthropogenic chemical compounds have been synthesised for countless uses. Some chemical groups, such as organochlorides and nitroaromatic compounds, are purposefully synthesised, while the production and incineration of some other commodities, such as polyvinyl chloride (PVC) plastic, create undesired toxic by-products. After the chemical products have served their purpose, they often end up in the environment. The microbes responsible for recycling all matter are seldom well equipped to degrade the new types of molecules, which are therefore biodegraded slowly, if at all. The final destination of persistent contaminants is often the soil, or if they pass through a water treatment plant, either sewage sludge or sediment at the bottom of rivers, lakes or the sea, where they may accumulate, thereby rendering the environment hazardous to life.

The increase in environmental awareness and the acute effects of some toxic compounds have raised questions over the safety of using many chemicals invented for agricultural and industrial applications. A great deal of current research addresses the management and remediation of old contaminated sites. Recent concerns regard the safety of consumer chemicals, especially nanomaterials; the effect of pharmaceuticals on ecosystems; and the combined effect that chemical cocktails have on human and ecosystem health.

In the following sections, the origin and fate of different hazardous and recalcitrant chemicals are considered. Many of the compound groups have been synthesised for several decades around the world, and therefore, in many cases, comprehensive inventories of the production rates of these chemicals are limited. Emission inventories are collected for scientific and administrative purposes, with great differences in their reliability, as well as in their temporal and spatial coverage [25]. Attempts to collect detailed official emission databases have been undertaken by the United Nations under different conventions, such as the Convention on Long-Range Transboundary Air Pollution [26] with the additional Aarhus Protocol [27] and the Stockholm Convention on Persistent Organic Pollutants (POPs) [28]. Scientific studies often require data on other features beyond those deposited in the official databases, and therefore, many efforts have been undertaken to estimate source emission levels, environmental occurrence and final sinks [29–32]. To understand the current state of soil contaminated with hazardous, persistent chemicals, we have attempted to utilise both scientific and official sources, but the reader should bear in mind that these numbers are rough

estimates at best. Descriptions, source emissions and the types of emerging contaminants frequently found in sewage sludge are discussed in [33–39].

2.1.1 Hazardous Compounds Originating from Oil Products

Crude oils are heterogeneous mixtures of hydrocarbons formed underground under high pressure from the remains of organic material. They can be refined into a multitude of products. The main use of oil is as an energy source in traffic, heating and electricity production, as approximately 34% of the global energy demand is met by oil products [40]. Crude oil is also used as a raw material in many man-made materials, such as plastics, paints and solvents. Oil is produced globally in staggering quantities, as global oil demand is foreseen to be 92.9 megabarrels per day in 2015, with an increasing demand reaching 110 megabarrels in 2035 [40]. Crude oil is perhaps globally the most exploited non-renewable natural resource. Accordingly, substances deriving from crude oil are the most common polluters of the environment.

Fate of Fuel Oil

Most fuel oil ends up in its intended place of use, as an energy source in motor vehicles or heating. Burning oil products increases the global levels of atmospheric CO_2, the most important greenhouse gas. Impurities in the oil and incomplete combustion generate nitrous oxides, polycyclic aromatic hydrocarbons (PAH) and acid rain from sulphur dioxide because sulphur is a trace element found in many crude oils. Crude oil refining often includes steps in which oxygenated compounds, such as methyl tert-butyl ether (MTBE), are added for enhanced combustion. If the fuel oil ends up in soil, the water-soluble additives often leach into groundwater, rendering it unfit for drinking.

Crude oil is only found in a number of locations on Earth, and its uneven distribution requires a vast transportation network. Dramatic oil spills that are a consequence of oil transportation and events such as the explosion at the Deepwater Horizon oil rig attract much media attention because their effects on the environment are directly visible. The most common type of petroleum contamination in the environment, however, is from sources of smaller volumes. Leaking heating oil containers, gasoline station tanks and lines; improper handling of waste; and small accidental spills comprise most of the oil pollution in soil, waterways and groundwater. The information on the amount of petroleum products contaminating our soils is fragmented, but thorough estimates of oil contamination in the oceans have been attempted in several studies starting in the 1970s. Natural seeps are the largest contributor of oil in the sea, at 600 kilotons of oil each year. Oil release to the sea by human activities is nearly equivalent, at 480 kilotons each year [41]. As for soil, the European Environment Agency estimated in 2006 that crude oil was the most important pollutant of the investigated contaminated sites, at 33.7%, which was second only to heavy metal pollution (Table 1).

Table 1 Type and source of the most relevant group of contaminants in European soils

Contaminants	Example of compounds	Source of contamination[a]	Estimated percentage[b]	References
Heavy metals	Cu, Zn, Cd, Pb, Hg, Cr	Application of animal manure (D) Military facilities (P) Gasoline stations (P) Sawmills and wood preservation sites (P) Mining and metallurgical industry (P,D)	37.3	[21, 42]
Oil hydrocarbons	Alkanes, alkenes, cycloalkanes	Oil industry (P,D)	33.7	[23]
Chlorinated compounds	PCP, PCBs, PCDD/Fs	Manufacture of pesticide and herbicide (D) Wood preservation sites (P) Pulp and paper production (P) Municipal waste incineration (P,D) Plastics, fire-retardants manufacture (P,D)	Chlorinated phenols – 3.6 Chlorinated hydrocarbons – 2.4	[43, 44]
Monomeric aromatic hydrocarbons	Benzene, toluene, ethylbenzene, xylene (BTEX)	Oil industry (P,D) Gasoline stations (P) Manufactured gas plants (P,D)	6	[43]
PAHs	Benzo[a]pyrene, chrysene, fluoranthene	Oil industry (P,D) Gasoline stations (P) Manufactured gas plants (P,D) Wood preservation sites (P) Municipal waste incineration (P,D) Automobile exhaust (D)	13.3	[43, 45]
Nitroaromatics	TNT, nitrobenzene, nitrophenols, atrazine	Manufacture of aniline, dyes, drugs (P,D) Explosive industry, military facilities (P, D) Manufacture of pesticides and herbicides (D)	[c]	[46]

[a]P = point contamination; D = diffuse contamination

[b]According to the European Environmental Agency, the estimated percentage is based on the frequency with which a specific contaminant is reported to be the most important in the investigated site [23]

[c]Information not available

Fate of Plastic Products

Because plastic products are made of hydrocarbons, they can be burned. However, certain chlorine-rich plastic products, such as PVC, form dioxins when incinerated. Because oil-based plastics are not biodegradable, most man-made plastic that is not burned still exists today. Plastic waste is created in staggering numbers: by 1988 in the United States alone, 30 million tons of plastic were produced annually [47]. Plastic goods most often end up in municipal dumpsites and landfills. Plastic is an inert material and therefore not toxic, but the problem arises from the fact that it does not degrade [48]. Due to careless waste management, massive amounts of plastic are found floating on sea surfaces. Plastic litter comprises most worldwide marine litter, with fishing, merchant and recreational ships as the major sources of plastic litter [49]. Sea currents transport floating material to certain areas, where plastic litter converges as "plastic gyres" found in the Pacific Ocean [50] as well as the Caribbean Sea and the North Atlantic Ocean [51]. In these gyres, up to 580,000 plastic pieces per square kilometre have been reported. Plastic litter is harmful to marine organisms in several ways. For example, marine animals ingest plastic particles that can block their guts, and marine animals can become entangled in plastic debris. Floating plastic can also act as a vector for hazardous compounds or change the ecological balances in the oceans by transporting or providing a good breeding ground for invasive species [49].

Plastic products are known to eventually break down into smaller and smaller pieces (nanoparticles) until they are small enough to enter the cells of living organisms. Because the amount of discarded plastic is so substantial, nanoplastic particles pose an emerging environmental concern. The health effects of nanoplastics are not thoroughly understood, but polystyrene particles up to 240 nm in diameter have been proven to be transportable through placental cells [52].

Fate of BTEX Compounds

The monoaromatic compounds benzene, toluene, ethylbenzene and xylene, commonly found in crude oil, are often jointly called BTEX compounds. The most harmful of these compounds is benzene, which is a known carcinogen. BTEX compounds occur naturally near natural gas and petroleum deposits and are detected in the fumes of forest fires. Most of the highly volatile BTEX compounds released by human activity originate from fuel use and end up as pollutants in the air. Inhaling BTEX-polluted air is also the greatest hazard to humans by these compounds. BTEX compounds are water-soluble, and therefore, improper handling can also cause groundwater contamination.

Future of Petroleum Hydrocarbons

Although technologies for using alternate energy sources exist, most of the transportation infrastructure is still based on the consumption of refined crude oil.

It seems unlikely that alternate energy sources will surpass crude oil-based fuels in the near future. Therefore, effective methods for cleaning up oil-contaminated environments are required. Because of their high carbon concentration, oil products are often readily utilised by environmental microbes. Thus biological treatment methods are typically effective at oil-polluted sites, and many promising, large scale studies confirm that bioremediation is a feasible, cost-effective method for cleaning up oil contamination in situ. Such is the example of the clean-up approach applied to tackle the Deepwater Horizon oil spill in the USA. The oil was dispersed via the direct application of a dispersant that enhanced the natural biodegradation of the oil [53].

2.1.2 Polycyclic Aromatic Hydrocarbons

PAHs are not produced for commercial purposes. They are naturally formed during volcanic eruptions and forest fires. However, most PAHs originate from anthropogenic sources, such as the incomplete combustion of fossil fuels, wood and waste; automobile exhaust; and petroleum derivative spills [54]. Diffuse contamination of PAHs occurs via adsorption to airborne solid particles [55]. Wind transports the particles to distant locations where they are deposited directly onto the soil or indirectly to soil through vegetation. It is estimated that soil receives 0.7–1 mg/m^2 of PAHs by atmospheric emissions [56, 57]. In 2004, Zhang et al. estimated the total global atmospheric emission of the 16 PAHs listed by the US EPA to be 520 Gigagrams per year [58]. Due to their low solubility, PAHs tend to be deposited into sediments and soil. Once in the soil, PAHs may be degraded or transformed, which will determine their transport, distribution and concentration. PAHs bound to organic matter have reduced mobility but also have a higher resistance to biodegradation. The binding affinity of PAHs to soil organic matter is determined by the octanol–water partition coefficient (K_{ow}). PAHs with high molecular mass, and consequently high log K_{ow}, tend to have a higher affinity for soil organic matter.

2.1.3 Nitroaromatic Compounds

The production of nitroaromatic compounds is one of the largest chemical industries today. These compounds are used in explosives, as starting materials in the pesticide and pharmaceutical industries and in dyes, among many other applications. They are identified by one or more functional nitro groups attached to the aromatic ring structure. Some nitroaromatic compounds are formed naturally, with the reactions catalysed by sunlight or radicals. Certain compounds of this group, such as nitroaromatic antibiotics produced by members of the genus *Streptomyces*, are of biological origin; therefore, pathways for their degradation exist [59]. Despite this, many man-made nitroaromatic compounds have been identified as toxic, mutagenic, carcinogenic and persistent against degradation [60].

Aniline, which is used not only to synthesise drugs, pesticides and explosives but also as a building block for materials such as polyurethane foams, rubber, azo dyes, photographic chemicals and varnishes, is manufactured at a quantity of approximately three million tons each year [61]. The toxic effects of aniline include increased nitration of proteins in the spleen [62].

The most famous explosive is trinitrotoluene, or TNT, which was mainly used in warfare in both world wars, as well as in mining and building. TNT is still widely used and produced. Most of the current problems with TNT and nitroarene compounds are found in sites where ammunition was handled, stored or manufactured. TNT is a persistent contaminant, but its microbial degradation is possible both aerobically [63] and anaerobically [64].

Even though some nitroaromatic compounds are purposefully spread in the environment as pesticides, the majority of their environmental releases are accidental. For example, in the United States alone, 5.1 tons of nitrobenzene were released in soil in 2002 [65]. The greatest known industrial releases have occurred in China; in 2005, an explosion at a chemical factory resulted in the accidental release of 100 tons of benzene and nitrobenzene to the Songhua River [66].

2.1.4 Chlorinated Compounds

Chlorinated compounds have been and still are produced for many purposes. Many cleaning agents, pesticides and pharmaceutical products rely on the chemical properties of the chlorine atom attached to a hydrocarbon backbone or ring structure. Natural degradation pathways do exist for many of these compounds because chlorinated organic compounds are common in certain more complex and biologically active molecules. Some chlorinated compounds are more readily degraded than others, and their degrees of toxicity vary.

In 1994, US PVC manufacturers used 4.26 billion kg of chlorine to produce 4.95 billion kg of PVC [67]. PVC production is the most important reason for man-made organochlorides. The manufacture of the precursor for the plastic, vinyl chloride, is the main reason for dioxin pollution in the United States [68]. The US EPA lists accidental fires and burning of municipal waste containing PVC plastic as other major sources of dioxins [69]. Dioxins are slowly degradable, and they tend to accumulate in sediments. Another important source of organochloride production is the dry-cleaning industry, where the main compound used is tetrachloroethylene (TCE). Although the use of TCE has declined throughout the 1990s due to its classification as a hazardous compound by the US EPA, it is still used in dry-cleaning facilities. For example, in 2001, US plants produced approximately 148 million kg of TCE [70].

Of the 16 POPs listed in the 1998 Aarhus Protocol [27], 11 are organochloride pesticides, which have now been banned in several countries. Most concerns regarding these products relate to their toxicity, with health effects to humans ranging from lung damage and neurological problems to death. Many organochloride pesticides are lipophilic, and they accumulate in the adipose tissues.

Due to these properties and their biochemically stable molecular structures, they are not easily biodegraded in the environment. Organochlorides are transported globally by natural processes and have been observed to accumulate in the Arctic [71]. All pesticides are tested for human toxicity before they are given permission for use. They are often not acutely toxic, at least not in minor concentrations. However, due to their chemical properties, some pesticides are bioaccumulative and are enriched in the food chain to much larger concentrations than the manufacturer intended. The most famous example of this is dichlorodiphenyltrichloroethane (DDT), which was first synthesised in the 1940s. DDT has been banned in many countries, but totally phasing out its use is difficult because it is efficient in preventing malaria vectors, and it is still produced at 4–5 tons each year [72]. Organochlorides of low molecular mass are readily water-soluble and thus can easily contaminate groundwater. Due to the hydrophilic nature of the chlorine atom, organochlorides are efficient compounds in cleaning agents. TCE is used by 90% of dry-cleaners in the United States today. It is toxic to humans and a suspected carcinogen.

Perhaps the most problematic chlorinated compound group includes PCBs, which have been in use since the 1920s for a period of 70 years in hydraulic oils, insulating fluids in transformers and sealants, among other substances, until their toxicity and persistence against degradation were observed. Breivik et al. [30] estimated that the global historical production and consumption of 22 PCB congeners was 1.3 million tons between the years 1930 and 1993 [73]. PCB contamination seems to be unevenly distributed, as many of the highest contamination levels can be found in urban areas [74].

Dioxins are a large group of very toxic chemicals formed when organic matter is burned in the presence of chlorine. Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are among the most studied dioxins. Certain dioxins have in fact been coined as the most toxic compounds ever made by man. Dioxins found contaminating the environment are largely the result of human activity. The highest concentrations of dioxins are often found in soils and sediments near the facilities where they were produced or used. Today, the commercial production of many dioxins is banned, and point sources have been closed down. Currently, the largest source of dioxins is the incineration of chlorine-containing waste, which creates dioxin air pollution. Dioxins are lipophilic compounds that tend to accumulate in the adipose tissues of higher organisms. Human exposure to dioxins is mainly by consumption of dairy products, meat, fish and eggs. Dioxins are also found as impurities in PCB products or wood preservatives. A group of dioxins called chlorophenol herbicides, such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), are commercially produced in large quantities. Large areas of land were contaminated with this compound when it was sprayed in central Vietnam as a component of Agent Orange, a defoliant sprayed in quantities of hundreds of kilograms during the Vietnam War in the 1970s.

Chlorinated phenols have been traditionally applied in the production of wood preservatives, insecticides and disinfectants. Common chlorinated phenols found in wood preservatives are 2,3,4,6-tetrachlorophenol (TeCP), 2,4,6-trichlorophenol

(TCP), and pentachlorophenol (PCP), in order of abundance. Minor amounts of other trichlorophenols and dichlorophenols may also be present, as well as recalcitrant polychlorinated phenoxyphenols (PCPPs) and PCDD/Fs as impurities [75, 76]. In Finland, approximately 30,000 tons of CP products were used between 1934 and 1988, when they were banned because of their potential toxicity to humans and the environment [77, 78]. The careless manufacturing and application of wood preservatives together with the lack of suitable waste disposal caused massive contamination of river sediments and sawmill sites. For example, the river Kymijoki in southern Finland was identified as the largest source of dioxins accumulating in fish in the entire Baltic area. Similar products were used in other European countries, especially Nordic countries with a large forestry industry, such as Sweden [79].

2.2 *Effect of Organic Contaminants on Soil Biota and Quality*

Environmental contamination, either from diffuse or point sources, may compromise the ability of ecosystems to provide society with those goods and services that we require. Contamination is also linked to other widely recognised anthropogenic threats to sustainable functioning of our planet, such as the loss of biodiversity. As many soil properties and processes are conferred by soil biota, it is currently widely agreed that biological characteristics should be taken into account when evaluating the quality, or health, of soil [13, 16].

Microbial communities can respond to disturbances, such as contamination, in many different ways; any of these responses may result in perceived stability or the continuation of essential soil functions [80]. The key species may show resistance to perturbation, meaning that the pollutants have no negative (or positive) effect on them. If the initial reaction is negative but the key species are able to regain their numbers and functionality, the community is said to be resilient. If the key species are irreversibly affected but are replaced by other indigenous species that are able to perform the same task under the new conditions, we see redundancy. Only if all these backup strategies fail will the deleterious effects of contamination on soil functions be observed.

Some specific processes, such as the degradation of recalcitrant chlorinated compounds or nitrogen fixation in symbiosis with leguminous plants, can only be performed by a few specialised microbial genera. If these key populations are compromised, these soil functions may be completely lost. However, for the majority of general soil processes, there is functional redundancy, with a plethora of diverse microorganisms able to perform the same actions under slightly differing conditions [17]. For example, the decomposition of plant material is performed by both soil bacteria and fungi, with the former dominating in neutral or alkaline soils and the latter dominating under acidic conditions [81]. The perceived ecological relevance of biodiversity lies in this ability of other species to take over a task if the original agent is disturbed or eliminated (insurance theory). Biodiversity, generally referred to as richness (i.e. number of different species) from the point of view of conservation, is thus assumed to ensure ecosystem stability [16, 17].

The microbial diversity of soil is vast: up to ten million species with a collective prokaryotic genome ten thousand times the size of the human genome have been found in a mere handful of dirt. In addition to rapid microbial mechanisms of genetic reorganisation, and 3.7 billion years of evolution, this diversity is predominantly produced and maintained in soil by spatial isolation of dispersed cells and small colonies [82, 83]. Although the majority of soil bacteria or archaea will never interact, even with their neighbouring cells just a few millimetres away [10, 17], such a wide biodiversity can be expected to convey a very high level of functional redundancy in microbially mediated soil processes. The true relevance of the natural soil microbial species richness, especially because many species are found in very low abundance, is still a highly disputed topic among microbial ecologists [17]. For example, a high richness does not seem to be linked to higher rates of general soil processes such as degradation of organic C. On the other hand, high diversity has been connected to more efficient degradation of hydrocarbons [84] and a reduced persistence of invader species [85]. In addition, a severe decrease in microbial diversity seems to be associated with an increased susceptibility of soil processes to further perturbation [17].

Soil contamination with inorganic or organic pollutants commonly reduces the diversity or evenness (even distribution of species) of soil bacteria [16, 17, 85]. Generally, a combination of multiple stressors, such as different pollutants or contamination and drought, exerts especially high pressure on soil communities, and the combined negative effect may not be additive but rather synergistic [86]. In the case of soil microbes, this general ecological principle does not seem to hold; prior stress has been associated with both an increase in sensitivity and an increase in community resistance or resilience [16]. In the latter case, the explanation may lay either in similar physiological mechanisms of resistance to multiple stressors or in community adaptation through increased numbers of generally more resistant species. The production of persistent resting forms such as bacterial endospores under stressful conditions can result in increased resilience [16]. Dormancy in general, meaning minimal metabolic activity associated with minimal interaction with the environment, can deliver the same advantages and seems to be a common survival strategy for soil bacteria [83].

The apparent resistance of soil microbial communities to contaminants may not be caused by insensitivity of the exposed organisms but rather by the fact that the pollutants simply are not bioavailable in the specific environment to the organisms under observation on the time scale of the observation. This holds especially true for organic contaminants with high K_{ow} values [87]. Aromatic and halogenated compounds introduced into soil in sludge tend to remain adsorbed in sludge solids such as organic material or fine inorganic particles [88]. If the compounds are released due to degradation of the sludge-derived organic material, pollutant molecules may further be adsorbed on or absorbed in more recalcitrant soil organic matter [87]. Such sorption may be either reversible or irreversible, but in any case, the mobility of low-concentration organic contaminants with poor aqueous solubility in soil is very restricted. As soil microorganisms are often located in micropores or inside aggregates, their spatial separation from pollutants can efficiently reduce their exposure.

If an organic pollutant in soil is sufficiently bioavailable to exert toxic effects, it is generally also bioavailable to organisms able to degrade it. However, the opposite may not hold true because degradative bacteria often have specific mechanisms to improve access to and uptake of organic compounds [89]. The evolutionary rationale for acquiring such mechanisms is that the bacteria degrading organic pollutants are often able to use these as sources of energy, C and N [90]. Because the degradation capacity thus provides a combined competitive advantage in the form of both protection and cell building blocks, microbes have evolved pathways to catabolise seemingly any organic compound [91]. Such organisms are also widely spread. Hydrocarbon degraders can be detected in any environment contaminated with crude or refined oil [92], but degraders of many emerging contaminants [34] belong to bacterial taxa common in the soil environment [93]. Horizontal transfer of the degradation genes from a degrader to another bacterial species, genus or even family further increases the capacity of the microbial community to cope with contamination [16]. On the other hand, some of the newer compounds, especially halogenated aromatic molecules, may be thermodynamically and biochemically so challenging to catabolise that optimal degradation pathways are still under evolutionary development [94]. For such compounds, the degraders are not necessarily widely spread in nature yet. Moreover, the concentrations of such emerging contaminants in the environment may not be sufficient to support the development or maintenance of specialised degrader communities, whereas more abundant and readily utilisable pollutants such as oil typically cause a rapid increase in the number of degraders [95].

In addition to various persistent halogenated aromatics, the introduction of pharmaceutical compounds in the environment has received attention. The ecological effects of antibiotics have particularly been studied because they can disturb microbially delivered functions in various environments such as wastewater treatment plants or agricultural soils. Moreover, the risk of development and positive selection of antibiotic-resistant bacteria is a cause of wide concern. Such concerns may seem exaggerated for soil microbial ecology because any soil contains a diversity of microbes that can produce antibiotics or are resistant to them [85]. In fact, many antibiotics were originally found in soil-inhabiting Actinobacteria, and the search for new drugs from similar sources with modern technologies is ongoing [96]. However, if a significant concentration of an antibiotic could be introduced to soil via sewage sludge, the steep concentration gradient can cause an even more rapid development of antibiotic resistance in the heterogeneous soil environment than in more homogeneous aquatic habitats [97]. Another microbial risk related to sewage sludge is the potential introduction of human pathogens into soil. Diverse soil microbial communities can often outcompete invading species such as human or plant pathogens [14]. However, if the sludge contains both a contaminant that compromises the indigenous soil community and a pathogen already acclimatised to the contaminant, the risk of the pathogen becoming established and persisting in the soil environment increases sharply.

To conclude, a review of recent literature on the connections between soil contamination, microbial biodiversity and ecosystem functioning perhaps raises

more questions than answers. Although it is not evident how diversity (and what type of diversity) is related to soil ecosystem service efficiency and stability, a conservative attitude should be maintained because soil is a non-renewable natural resource. Moreover, soil clay and organic matter contents correlate positively with biodiversity and resilience [82] but negatively with pollutant bioavailability [87]. Thus, coarse-textured mineral soils that have the greatest need for improvement by organic fertilisers are also the ones most vulnerable to the negative effects of co-introduced contaminants.

Although the role of bacterial, archaeal and fungal communities in soil health is indisputable, macrobiota have their own important functions, even in intensively managed agricultural soil. The production of soil-based goods – food, fodder, fuel and fibre – obviously depends on plant growth, and primary production is the final driver of ecosystem services [7]. Unfortunately, plants may be, in general, more sensitive to soil contamination by organic pollutants than by bacteria. The exposure and response of plants to emerging contaminants thus deserves much more attention.

3 Health Effects of Organic Contaminants on Animals and Humans

The Stockholm Convention on POPs lists 22 priority chemical substances that pose potential risks of causing adverse effects to human health and the environment. Since the convention was enacted in 2004, evidence for the toxic effects of POPs such as DDT, dichlorodiphenyldichloroethylene (DDE), PCBs, PCDD/Fs and other halogenated compounds has been demonstrated for both humans and wildlife.

Due to their lipophilic nature, organic chemicals with high molecular mass tend to accumulate in the adipose tissues of living organisms. Thus, even though they are commonly present at low concentrations, adverse effects associated with chronic exposure are of a particular concern. Some POPs are capable of acting as endocrine disrupters that harm the reproductive system and development of wildlife [98–100]. For example, reproduction abnormalities, immune function impairments and thyroid deficiencies have been detected in Baltic grey seals and ringed seals due to PCB intake from food [98]. Exposure to PCBs has also caused immune dysfunction, reproductive failure, increased pup mortality, deformations and adult mortality in minks and ferrets. Other effects in mammals (e.g. seals, dolphins, porpoises and whales) linked to chlorinated compounds such as PCBs, hexachlorobenzene (HCB), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), toxaphene and DDT include immunotoxicity, dermal effects, carcinogenicity, vitamin and thyroid deficiencies and mass mortalities by infectious diseases [98, 101]. Water bodies contaminated by effluents from wastewater treatment plants and industries have reduced the viability of larvae of several species of fish; increased blood vitellogenin (i.e. a precursor of egg yolk protein formation) in male trout, carp, flounder and perch; and

caused thyroid damage in salmon species [98, 101]. In invertebrates, exposure to PCBs and tributyltin causes masculinisation in different species of female marine gastropods. In birds, among different reproductive effects, the most evident is DDE-induced egg-shell thinning. In reptiles, feminisation and increased developmental abnormalities of turtles and alteration of sex organ development and function in alligators have been associated with exposure to chlorinated compounds [98].

Although the effects of chronic exposure of humans to low levels of POPs are difficult to predict, some biological effects have been described. For example, exposure of children to PCBs and PCDD/Fs may be linked to an elevated risk for infectious diseases. Exposure of pregnant women to PCDD/Fs may cause lower fertility in their male offspring. The adverse effects to human health of acute and chronic exposure of high concentrations of POPs, especially among industrial workers exposed to daily intakes of chemicals, are more evident. Elevated concentrations of DDE and TCDD have been associated with the development of cancers such as breast cancer, leukaemia and thyroid cancer. Dioxin exposure may also be associated with immunotoxicity, reproductive diseases and neurotoxicity. Extreme exposure to chlorinated compounds has resulted in death [101].

In addition to recognition of the toxic properties of the Stockholm POPs, PAHs are also a significant concern; therefore, 16 PAHs are listed by the US EPA as having a possible carcinogenic nature. However, PAHs with a "bay" or "fjord" region in their molecular structure are the most likely carcinogens. For instance, when entering an organism, benzo-*a*-pyrene (BaP) is activated by a series of metabolic reactions that lead to the final carcinogenic metabolite, a reactive diol epoxide, which may bind covalently to DNA, leading to mutations and tumours [102]. Only BaP is classified by the International Agency for Research Cancer (IARC) as carcinogenic to humans due to extensive evidence of the induction of different tumours by BaP in in vitro and in vivo studies in mice and mammalian cell cultures [103, 104].

3.1 Emerging Endocrine-Disrupting Compounds

Despite the evidence concerning chlorinated compounds and PAHs, a general correlation between human diseases and exposure to other organic contaminants has yet to be proven. This is especially true for new emerging contaminants at low levels of exposure. The reasons for the lack of knowledge are insufficient field or semi-field studies, a lack of data concerning residual levels in the body, ecological background, dose–response relationships and contradictory scientific results. Frequently, the main effect associated with emerging contaminants is the alteration of functions in the endocrine system, that is, the contaminants act as endocrine disrupters. The most common endocrine disrupters found in groundwater, bodies of water, wastewater effluents and sewage sludge are triclosan; tributyltin; 17β-estradiol; bisphenol A; nonylphenol; the synthetic musks galaxolide and tonalide;

the pharmaceuticals paracetamol, ibuprofen, naproxen, diclofenac and fluoxetine; polybrominated diphenyl ethers (PBDEs); and perfluorinated compounds (PFCs) [88, 105–108]. In the following section, we have focused on three types of endocrine-disrupting agents with particular interest due to the extent of the research conducted on them: bisphenol A, PBDEs and PFCs. For the other potential endocrine-disrupting chemicals listed above, see [108–111] and the references therein.

3.1.1 Bisphenol A

Scientific results for bisphenol A toxicity are contradictory. In experiments with rodents, maternal exposure during pregnancy or early post-natal exposure of pups to low doses of bisphenol A (up to 2.5 mg/kg/day) showed developmental or reproductive effects in the offspring [112]. On the contrary, in a long-term study with minnows, the NOEC (no observed effect concentration, which is defined by the US EPA as "the highest tested concentration of an effluent or a toxicant at which no adverse effects are observed on the aquatic test organisms at a specific time of observation") related to survival, growth and development parameters ranged from 16 to 1280 μg/L. This concentration range is markedly above the measured concentrations in North American and European fresh waters (ranging from 0.081 to 0.47 μg/L and 0.01 to 0.05 μg/L, respectively) [113]. The authors concluded that the likelihood that bisphenol A in surface waters would cause an effect in fish, even if exposed for multiple generations, is low [114]. In humans, bisphenol A has been detected in serum, breast milk and urine due to environmental or direct exposure during production, use and disposal of bisphenol A-containing products [112, 115]. Following the scientific results on the toxicity of bisphenol A in animals, the European Food Safety Authority (EFSA), the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) declared that the actual effects of bisphenol A on human health need further research. The lack of scientific evidence of its toxicity at the current levels of exposure and major methodological flaws were the main reasons for their declaration [116]. Nevertheless, a global concern has been raised regarding the potential effect of bisphenol A on reproduction and neural and behavioural development in foetuses, infants and children. One of the most important worldwide actions concerning bisphenol A was to ban the manufacturing of baby bottles containing bisphenol A in the EU [117], in some states of the United States [118] and in Canada [119].

3.1.2 Polybrominated Diphenyl Ethers

PBDEs are a class of brominated flame retardants (BFRs) used in textiles, plastics and electronic products. The effects of BFRs are associated with three commercial mixtures of PBDEs: decaBDE, octaBDE and pentaBDE. In laboratory animal experiments, the toxicity of PBDEs was linked to damage to liver function and,

in the case of octaBDE and decaBDE, to an alteration of thyroid hormones and developmental neurotoxicity. Importantly, bacterial tests indicated that these three groups of PBDEs are not mutagenic [120]. Epidemiological studies on humans have focused on three main health effects: endocrine disruption, neurotoxicity and reproductive toxicity. Despite the lack of correlation between thyroid hormone levels and exposure to PBDEs, serum concentrations of PBDEs may be associated with alterations of hormones, as PBDEs may interact as antagonists or agonists at androgen, progesterone and oestrogen receptors [121]. Moreover, PBDEs may be inversely associated with thyroid-stimulating hormone (TSH) or cause a reduction in thyroid hormone T4, which may result in developmental neurotoxicity [120]. In fact, neurotoxicity is the greatest concern regarding PBDEs because there is enough evidence from animal data that suggests that pre- and postnatal exposure may cause behavioural alterations, particularly in motor performance and cognition [121].

3.1.3 Perfluorinated Compounds

Due to their persistence in the environment, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are the two major classes of PFCs found in the environment. Their use in a wide range of products has been reduced due to accumulating evidence of their toxicological effects and because they are now included in the Stockholm Convention. Regardless of industrial efforts to moderate their use, PFCs still remain in the environment. Consequently, serum concentrations in humans at levels proven to cause toxicological effects to laboratory animals have raised concern about their effect on human health [122–124]. A recent study has detected PFCs in human newborn cord blood at levels ranging between 30% and 79% of the maternal plasma, which demonstrates that PFCs pass, at least partly, the placental barrier [125]. Laboratory animal studies have demonstrated that hepatotoxicity (e.g. liver enlargement and induction of peroxisome proliferation), immunotoxicity and reproductive and developmental alterations are the main toxicological effects of PFOA and PFOS [123, 124].

3.2 Potential Toxicity of the Application of Biosolids in Agricultural Land

Epidemiological studies have frequently focused on the exposure to a single compound, usually at a higher concentration than that measured in the environment. Only a few studies exposing animals to a mixture of chemicals at low concentrations have been conducted. Even research using real contaminated environmental samples (e.g., wastewater, soil, sewage sludge) is negligible. To the best of our knowledge, only the studies by Bellingham and colleagues have been conducted with grazing animals exposed to sewage sludge applied to pasture [126, 127]. In their first study

[126], sheep foetuses were exposed to sewage sludge via maternal ingestion of pasture grass. The foetuses presented lower expression of genes of the gonadotrophin-releasing hormone (GnRH) and galanin systems in the foetal hypothalamus and pituitary gland [126, 127], both having an important role in the regulation of reproductive function. In a similar study [127], foetal and postnatal exposure of rams to sewage sludge caused a reduction in germ cell numbers in a significant fraction of the sheep, indicating sperm production alterations. The results of these studies are difficult to extrapolate to humans because direct exposure to sewage sludge applied to soils is insignificant. Although there is insufficient animal epidemiological data, the effects of the application of biosolids on pasture raise concerns about farm animal health, welfare and productivity [100].

4 Legislative Framework for Soil Protection and Sewage Sludge Use

Whereas water and air have been traditionally more protected, awareness of the need for soil protection, despite the fundamental role of soil in providing important ecosystem services, has been scarce. European experts argue that monitoring all the compounds with potential to enter the soil system is not feasible, and they propose a list of priority substances to be regulated instead. The list is based on the substances that may enter the soil at such concentrations that they pose a risk to human health, crops, and soil biological function and diversity. Several heavy metals (cadmium, copper, lead, zinc, mercury, arsenic, nickel and chromium) and organic contaminants [PAHs, PCDD/Fs, PCBs, hexachlorocyclohexane (HCH), DDT and dichlorodiphenyltrichloroethylene (DDE), among others] are included in the list. A concise description of the most relevant organic contaminants in European soils and their origins is provided in the second section of this chapter.

4.1 European Environmental Policies on Soil Protection

One of the main obstacles for effective protection of soil in Europe is that such a policy is spread across many EU laws and regulations, which are mainly focused on the protection of other environmental media or cover other objectives (e.g., air and water legislation and agriculture legislation) [128]. Because these policies are not directly oriented towards soil protection, soil has not been the subject of specific legislation or regulation for many years. A major legislative breakthrough occurred in 2002, when the preliminary communication by the commission “towards a thematic strategy on soil protection” created a common framework for soil protection. The strategy considered many stakeholders and different threats to soil quality and functions, including soil contamination [129]. The first proposal of the soil thematic

strategy was reflected in the sixth Environment Action Programme (EAP), published by the commission in 2002. In the sixth EAP, the main objectives regarding soil protection were the prevention of soil degradation, the preservation of its functions and the restoration of degraded soil [130]. The next step was the creation of extensive consultation processes during a 2003–2004 division into five working groups. Each working group was composed of experts from the public and private sector, research institutes, the European Environment Agency (EEA) and agricultural, industrial, environmental and consumer organisations [21]. The results from the contamination and land management technical group were launched in June 2004 as a set of recommendations for policy, monitoring and research focused on the priority objectives and targets of the soil thematic strategy. The targets were (1) the sustainable use of soil, (2) the preservation of soil as a resource and (3) the remediation of degraded soils. Having examined the results of the contamination technical group, the commission recognised the need for a Soil Framework Directive to be transposed to the national legislation of each member state [6]. The commission recommends that each member state create an inventory of contaminated sites based on a common definition of contaminated sites, as well as a common list of potential contaminating activities. Additionally, each member state must adopt a national remediation strategy on how to manage contaminated sites and declare a timeframe for carrying out remediation plans. Additionally, a list of potentially dangerous substances is to be compiled by each member state to prevent diffuse (non-point) contamination. The directive sets the obligation to provide a soil status report by the owner of the contaminated soil to the administration.

One year after the proposal of the Soil Framework Directive, the European Parliament adopted its first reading. However, in 2010, a few countries impeded the continuation of the process on the basis of excessive cost, administrative burden and subsidiarity. To date, the proposal remains on the council's table [131].

Nevertheless, since adoption of the strategy, the awareness of soil protection has risen, and as a consequence, numerous European projects and networks have been created, such as the CLARINET network[1] (contaminated land rehabilitation network for environmental technology in Europe) and the NICOLE[2] (network for industrially contaminated land in Europe). Other networks related to soil protection are the SNOWMAN network,[3] working as a funding body for sustainable use of soil and water, and EURODEMO+,[4] which encourages the demonstration of sustainable and cost-effective technologies for the treatment of contaminated soil and groundwater. The web portal EUGRIS[5] disseminates information related to soil and water management in Europe.

[1] http://www.eugris.info/DisplayProject.asp?ProjectID=4420

[2] http://www.nicole.org/

[3] http://www.snowmannetwork.com/

[4] http://www.eurodemo.info/

[5] http://www.eugris.info/

At the member state level, some countries, such as the Netherlands, a pioneer in the protection of soil, have their own national policies and regulations for the management of contaminated soils that were adopted before the Soil Framework Directive [132]. Protection guidelines vary from country to country, and specific legislation for soil contamination only exists for some member states, including the Netherlands, Italy, Austria, France, Belgium, Germany, the UK, Denmark, Spain and Finland [128].

4.2 Regulations for Use of Treated Sewage Sludge on Agricultural Land

Sludge is the solid residue originating from the process of domestic or urban wastewater treatment. The EU encourages the use of sewage sludge in agriculture through the Sewage Sludge Directive 86/278/EEC because of its rich organic matter content and the presence of other essential elements such as nitrogen and phosphorous [133]. The directive requires the pretreatment of sludge before its discharge to agricultural soil to provide protection to potential receptors (soil, vegetation, animal and human) against pathogens. Without any other specifications, the directive requires biological, chemical or thermal pretreatment. The pretreatment must be efficient enough to prevent the application of sludge to soil from being hazardous. For this reason, the sewage directive requires concentration limits for certain heavy metals (cadmium, copper, mercury, nickel, lead and zinc). It also prohibits the application of sewage sludge to "soil in which fruit and vegetable crops are growing or grown" and limits the application to "less than 10 months before fruit and vegetable crops are to be harvested."

The sewage directive was adopted over 20 years ago, and it was based on the existing knowledge of that time. Currently, the EU recognises the need for revision of the directive in light of new scientific evidence about potential risks to human health, the environment and soil quality. The proposal for the revision of the directive in the "3rd draft of the working document on sludge" sets stricter limits on heavy metals depending on the pH of the soil and includes chromium in the analysis [134]. The draft suggests that each treatment plant carry out a minimum number of chemical and biological analyses regarding the quantity of sludge produced per year. The proposal also includes an analysis of some organic contaminants that were not included in the directive (Table 2) [134]. Moreover, the EU is currently reviewing the possibility of analysing halogenated organic compounds, nonylphenol, linear alkylbenzene sulphonates, di(2-ethylhexyl) phthalate (DEHP), PAHs, PCBs and PCDD/Fs in sludge applied to agricultural land. Regardless of the debate on the type of organic compounds and their limits, no current guidelines exist for these compounds. Similarly, no regulation takes into account an analysis of new emerging contaminants in sewage sludge. Current biological and physicochemical technologies are unable to completely eliminate

Table 2 Differences between the main elements of the Sewage Sludge Directive 86/278/EEC [133] and the 3rd draft of the working document on sludge of the European Commission (2000) [134]

Element	Directive 86/278/EEC (1986)	Working document on sludge 3rd draft (2000)
Pretreatment technology	No specification of type, temperature and duration of the treatment: biological, chemical or heat treatment, long-term storage or any other appropriate process.	*Advanced treatments*: – Thermal treatment at >80°C – Thermophilic aerobic stabilisation: ≥55°C for 20 h – Thermophilic anaerobic digestion: ≥53°C for 20 h – Thermal treatment of liquid sludge (70°C for 30 min) followed by mesophilic anaerobic digestion (35°C with mean retention time of 12 days) – Lime conditioning: pH ≥ 12 and ≥55°C for 2 h – Lime conditioning: pH ≥ 12 for 3 months *Conventional treatment*: – Thermophilic aerobic stabilisation: ≥55°C with mean retention time of 20 days – Thermophilic anaerobic digestion: ≥53°C with mean retention time of 20 days – Lime conditioning: pH ≥ 12 for 24 h – Mesophilic anaerobic digestion: 35°C with mean retention time of 15 days – Extended aeration at ambient temperature (time adjusted to climatic conditions) – Simultaneous aerobic stabilisation at ambient temperature (time adjusted to climate conditions)
Limit values of heavy metals in sludge for use in land (mg/kg dry matter)	Cd: 20–40 Cu: 1,000–1,750 Ni: 300–400 Pb: 750–1,200 Zn: 2,500–4,000 Hg: 16–25 Cr: not considered	Cd: 10 or 250 mg/kg P[a] Cu: 1,000 or 25,000 mg/kg P Ni: 300 or 7,500 mg/kg P Pb: 750 or 18,750 mg/kg P Zn: 2,500 or 62,500 mg/kg P Hg: 10 or 250 mg/kg P Cr: 1,000 or 25,000 mg/kg P

Pathogens	Not considered	No *Salmonella* spp. in 50 g of sludge (wet weight) At least 6 Log_{10} reduction (or at least 2 Log_{10} in the case of conventional treatments) in *Escherichia coli* to less than $5{\cdot}10^2$ CFU/g of sludge.
Limit values of organic contaminants in sludge for use on land (mg/kg dry matter)	Not considered	AOX (500)[b] LAS (2,600)[c] DEHP (100)[d] NPE (50)[e] PAHs (6)[f] PCBs (0.8)[g] PCDD/Fs (100 ng TE/kg dry matter)
Emerging contaminants	Not considered	Not considered

[a]Limit values are different if they are related to phosphorus content of the soil (mg/kg of P) [134]
[b]Sum of adsorbable organic halogens
[c]Linear alkylbenzene sulphonates
[d]Di(2-ethylhexyl)phthalate
[e]Nonylphenol and nonylphenolethoxylates with 1 or 2 ethoxy groups
[f]Sum of acenapthene, phenanthrene, fluorine, fluoranthene, pyrene, benzo-*bjk*-fluoranthene, benzo-*a*-pyrene, benzo-*ghi*-perylene, indeno-1,2,3*cd*-pyrene
[g]Sum of the congeners 28, 52, 101, 118, 153, 180

low-biodegradable emerging contaminants from wastewater streams. As these contaminants are often hydrophobic, they directly accumulate in the sludge [88].

Despite increasing concerns related to potential human toxicity, evidence of adverse effects to the environment and endocrine-disrupting properties, more knowledge is required about the occurrence and detection of emerging contaminants in sludge as well as their degradability and actual risk. In this context, the following chapters of this book provide an overview of the current analytical methods for determining emerging contaminants in sewage sludge samples [33] and the different biological treatment processes available for degrading emerging contaminants in sludge [34–39].

5 Concluding Remarks

As the world population increases and clean sites become scarce, new technologies are needed to provide a clean and healthy environment for humans. The chemical industry produces tons of new, potentially hazardous chemicals every year in addition to those substances that are already known to be toxic. These chemicals are designed by humans for specific purposes that have no analogue in the biological world. However, many anthropogenic chemicals have structural resemblances to biologically produced molecules. Microbes inhabiting our soils, sediments and waters are often starved of nutrients and will readily develop ways to use emerging compounds in their metabolism. Microbial evolution is rapid, and for this reason, researchers are finding new genetic pathways for breaking down many anthropogenic chemicals that have not existed for more than a few decades. In this sense, microbes may be a useful resource for cleaning up contaminated environments.

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Analysis of Emerging Contaminants in Sewage Sludge

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Abstract This chapter summarizes the advances in knowledge regarding the analysis of emerging contaminants in sewage sludge samples. Pharmaceutical compounds, estrogens, illicit drugs, UV filters (sunscreens), brominated flame retardants, and perfluorinated compounds are selected as emerging contaminants of particular concern, as many of them display endocrine-disrupting properties. These substances are released as a consequence of human activities and enter the wastewater network after use in households and industry. Due to their physico-chemical properties, they tend to accumulate in sewage sludge during wastewater treatment, so the common practice of spreading sewage sludge over agricultural land can constitute a source of many important xenobiotic compounds. In this chapter, we discuss the use of different sample-preparation techniques applied to sludge, as well as the different approaches for mass spectrometry (MS). Finally, we review the available data concerning the occurrence of selected analytes in sewage sludges.

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Keywords Brominated flame retardants, Estrogens, Gas chromatography, Illicit drugs, Liquid chromatography, Mass spectrometry, Perfluorinated compounds, Pharmaceutical compounds, Polybrominated diphenyl ether, Tandem mass spectrometry, UV filters

Contents

Abbreviations

4DHB	4,4′-Dihydroxybenzophenone
4HB	4-Hydroxybenzophenone
4-MBC	3-(4-Methylbenzylidene) camphor
AM	Amphetamine
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
ASE	Accelerated solvent extraction
BE	Benzoylecgonine
BFR	Brominated flame retardant
BM-DBM	Butyl methoxydibenzoylmethane
CO	Cocaine
DAD	Diode array detection
DBDPE	Decabromodiphenylethane
DBT	Diethylhexyl butamido triazone
Dw	Dry weight
E1	Estrone
E1-3S	Estrone-3-sulfate

E2	17β-Estradiol
E2-3S	Estradiol-3-sulfate
E3	Estriol
ED	Electrochemical detection
EE2	17α-Ethynyl estradiol
EHMC	Ethylhexyl-methoxycinnamate
EHS	Ethylhexyl salicylate
EHT	Ethylhexyl triazone
EI	Electron impact
EPI	Enhanced product-ion
ESI	Electrospray ionization
EU	European Union
FLD	Fluorescence detection
FR	Flame retardants
GC	Gas chromatography
GPC	Gel permeation chromatography
HBB	Hexabromobenzene
HBCD	Hexabromocyclododecane
HLB	Hydrophilic–Lipophilic Balance
HMS	Homosalate
HRMS	High resolution mass spectrometry
IAMC	Isoamyl methoxycinnamate
IT	Ion trap
K_{OW}	Octanol-water partition coefficient
LC	Liquid chromatography
LDTD	Laser diode thermal desorption
LOD	Limit of detection
LOQ	Limit of quantification
LRMS	Low resolution mass spectrometry
MA	Methamphetamine
MAE	Microwave-assisted extraction
MDL	Method detection limit
MDMA	Ecstasy
MS	Mass spectrometry
MS–MS	Tandem mass spectrometry
NCI	Negative chemical ionization
N-EtFOSAA	Perfluorooctanesulfonamidoacetate
OC	Octocrylene
OT	Octyl-triazone
PBB	Polybrominated biphenyl
PBDE	Polybrominated diphenyl ether
PBEB	Pentabromoethylbenzene
PFA	Perfluorinated acid
PFBA	Perfluorobutanoic acid

PFBS	Perfluorobutanesulfonate
PFC	Perfluorinated compound
PFEtS	Perfluoroethanesulfonate acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonate
PFOSA	Perfluorooctanesulfonamide
PFPrA	Perfluoropropionic acid
PFPrS	Perfluoropropanesulfonate
PhACs	Pharmaceutically active compounds
PLE	Pressurized liquid extraction
PMALE	Pressurized membrane-assisted liquid extraction
POP	Persistent organic pollutant
QqLIT	Quadrupole linear ion trap
QqQ	Triple quadrupole
RP	Reverse phase
SBSE	Stir bar sorptive extraction
SIM	Selected ion monitoring
SLE	Solid–liquid extraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected reaction monitoring
TBBPA	Tetrabromobisphenol A
TD	Thermal desorption
TFA	Trifluoro acetic acid
TOF	Time of flight
UPLC	Ultra-performance liquid chromatography
USE	Ultrasonic extraction
WWTP	Wastewater treatment plant

1 Introduction

New studies are revealing the presence in the environment of substances that we use on a daily basis in our homes, industries, and agricultural fields. These substances are called emerging contaminants. Among the large variety of compounds, we highlight the pharmaceutical compounds, estrogens, illicit drugs, UV filters (sunscreens), brominated flame retardants (BFRs), and perfluorinated compounds (PFCs) due to their endocrine-disrupting properties.

The main points of collection and subsequent release of these contaminants into the environment are wastewater treatment plants (WWTPs), where they enter via domestic and hospital sewages or industrial discharges [1]. Some of them are more likely accumulated in sewage sludge, due to their moderate to high hydrophobic

properties. Thus, their sorption onto sludge constitutes an important process for their removal from the water column.

The high volume of sewage sludge produced nowadays is partly a consequence of the increase in the number of new WWTPs built to achieve the water-quality standards set by the European Union (EU) (Directives 91/271/EEC and 98/15/EEC). The EU considers that reuse of sludge should be encouraged, since it represents a long-term solution. In Europe, the agricultural use of sewage sludge is, along with disposal to landfills, the most popular disposal route. Of the total sludge produced in European countries, the percentage of reused sludge reached levels as high as 50% in Germany, 54% in Spain, 65% in France, and 71% in the United Kingdom [2]. Despite the valuable properties of sewage sludge, such as relatively high levels of organic matter, the widespread application of sewage sludge in agriculture needs to be critically evaluated in view of the concomitant presence of a variety of contaminants that may adversely affect environment and health. Sludges are treated before application to reduce odor and pathogen content. Moreover, the application of sewage sludge to soils is limited by guideline concentrations of heavy metals in the soil through the EU Directive on the use of sewage sludge in agriculture (COM 86/278). No similar guidelines exist for organic contaminants in sludge or soils at present. The European Union is currently studying the issue and may enact legislation on the application of sewage sludge containing organic pollutants (such as polychlorinated biphenyls, benzo[*a*]pyrene, and dioxins and furans) to agricultural land [3]. However, the presence of new emerging contaminants in sewage sludge is not yet evaluated.

In this context, we need to increase our knowledge about the occurrence of all these emerging contaminants in sewage sludge, with the aim of evaluating and reducing potential sources of these compounds entering the environment (e.g., the common practice of using biosolids as fertilizers in agriculture). However, one of the main drawbacks is the availability of reliable analytical methods able to determine these families of compounds from a variety of physical and chemical properties, and in a matrix as complex as the sludge.

This chapter reviews the published analytical methods for the determination of selected emerging contaminants in sewage sludge samples, including sample preparation techniques as well as instrumental approaches. We survey the current state of the art and examine future perspectives. Finally, levels of selected compounds found in sewage sludge samples are reviewed.

2 Emerging Contaminants

In this chapter, among the large variety of compounds, we include some of the emerging contaminants most commonly found in sewage sludge: pharmaceutical compounds, estrogens, illicit drugs, UV filters (sunscreens), BFRs, and PFCs.

2.1 Pharmaceutical Compounds

In a vast array of contaminants of anthropogenic origin reaching our water supplies, pharmaceutically active compounds (PhACs) are among the ones with the most continuous input into the environment, which is raising concerns of long-term consequences on human health [4]. PhACs are a group of chemical substances that have medicinal properties, and they are produced worldwide on a 100,000 tonne scale. Most of the modern drugs are small organic compounds with a molecular weight below 500 Da, which are moderately water soluble as well as lipophilic, in order to be bioavailable and biologically active. After the oral, parenteral, and/or topical administration, PhACs are excreted via liver and/or kidneys as a mixture of parent compound and metabolites that are usually more polar and hydrophilic than the original drug.

PhACs are designed to have specific pharmacologic and physiologic effects at low doses and thus are inherently potent, often with unintended outcomes in wildlife [5]. Their consumption increased last years and will continue to increase due to the expanding population, inverting age structure in the general population, increase of per capita consumption, expanding potential markets, patent expirations, new target age groups, etc. After their usage, a large fraction of these substances are discharged into the wastewater unchanged or in the form of degradation products that are often hardly eliminable in conventional WWTPs. Depending on the efficiency of the treatment and chemical nature of a compound, PhACs can reach surface and ground waters. In the worst-case scenario, they are encountered in the drinking water, in spite of the expensive treatment steps.

Among pharmaceuticals, antibiotics have become of special concern in recent years. The reason is that these substances are continuously being introduced into the environment and may spread and maintain bacterial resistance in the different compartments. Sulfonamides are very commonly used antimicrobials in humans but mainly in veterinary medicine, due to their broad spectrum of activity and low cost, being the second most widely used veterinary antibiotic in the EU. Their occurrence has been reported in all kinds of water matrices; their high excretion rates (after their intake by humans of livestock) and high water solubility make them very ubiquitous and persistent pollutants in the environment.

2.2 Estrogens

Steroid sex hormones, such as natural and synthetic estrogens, constitute a group of environmental emerging contaminants of particular concern, as they present endocrine-disrupting properties. In the last decades, these compounds have been widely used for human medicine and animal farming and consequently released into the environment. Free unchanged estrogens and less toxic conjugated forms are daily discharged into the aquatic systems through treated and untreated sewage

waters, and as a result of fish farming activities, manure runoff, and sewage sludge application in agriculture [1]. The presence of the natural estrogens 17β-estradiol (E2) and estrone (E1), and the synthetic estrogen 17α-ethynyl estradiol (EE2), in aquatic environments, even at very low levels (0.1–10 ng/L), has been linked with different estrogenic effects (e.g., intersex, increase of plasma vitellogenin levels) observed in several aquatic species [1, 6]. Contrary to excreted conjugated forms (less toxic and more polar), free estrogens are more likely to accumulate in sediments and sewage sludge, due to their moderate to high hydrophobic properties [log octanol-water partition coefficient (K_{OW}) = 3–5] [1].

2.3 Illicit Drugs

In the last years, drugs of abuse, which present volumes of production and usage comparable to some prescription or over-the-counter drugs, have received increasing interest by environmental analytical chemists as a new confirmed group of environmental emerging contaminants. The main source of these compounds in the aquatic environment is STP effluents, as direct disposal in natural streams is not likely. Some classes of illicit drugs, such as cannabinoids, are highly hydrophobic, with log K_{OW} between 5 and 7.6, and can therefore be expected to bind to sewage sludge [7]. Conversely, other drugs of abuse, such as amphetamine and related compounds, cocainics, LSD and opioids, present medium to polar characteristics, being thus expected to partition to a higher extent in the aqueous phase.

2.4 UV Filters (Sunscreens)

Sunscreen agents, also known as UV filters, have become very popular chemicals since they were shown to have a protective role against photoaging [8], photocarcinogenesis [9], and photoimmunosuppression [10] promoted by UV sun radiation. These compounds are extensively used in personal care products but are also present in a wide variety of industrial goods such as textiles, paints, or plastics to prevent photodegradation of polymers and pigments [11]. According to an exhaustive report on personal care products, UV filters experienced the highest sales growth, 13% in Europe from 2002 to 2003 reaching around 1,000 US $ mill (ACNielsen Global Services, 2004). However, recent concern has risen due to their potential for endocrine disruption and developmental toxicity [12, 13]. More data about UV filters toxicity in the aquatic environment have been described by Fent et al. [14] and Brausch and Rand [15].

UV filters enter the aquatic environment directly as a result of recreational activities when they are washed off from the skin, or indirectly through wastewater resulting from the use of personal care products, washing clothes, and industrial discharges. Residues of more polar organic UV filters have been found in all kinds

of water matrices [16–18] including tap water [19]. Due to the high lipophilicity and poor biodegradability of many UV filters, they end up in sewage sludge during wastewater treatment [20–23], and accumulate in river sediments [24, 25] and in biota [16–18].

2.5 Brominated Flame Retardants

BFRs are one of the last classes of halogenated compounds that are still being produced worldwide and used in high quantities in many applications. In order to meet fire safety regulations, flame retardants (FRs) are applied to combustible materials such as polymers, plastics, wood, paper, and textiles. Approximately 25% of all FRs contain bromine as the active ingredient. More than 80 different aliphatic, cyclo-aliphatic, aromatic, and polymeric compounds are used as BFRs. BFRs, such as polybrominated biphenyls (PBBs), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBPA), have been used in different consumer products in large quantities, and consequently they were detected in the environment, biota, and even in human samples [26, 27].

Due to the toxicological effect of PBDEs, the production and use of penta-, octa-, and deca-BDE mixtures have been banned in Europe. Moreover, and in response to increasing international regulations on BFR formulations, alternative FRs for achieving commercial product fire safety standards are being developed and used. Some of these non-BDE BFRs are pentabromoethylbenzene (PBEB), hexabromobenzene (HBB), and decabromodiphenylethane (DBDPE) [28].

HBCD is a brominated aliphatic cyclic hydrocarbon used as a flame retardant in thermal insulation building materials, upholstery textiles, and electronics. In 2001, the world market demand for HBCD was 16,700 tons, from which 9,500 tons was sold in the EU. These figures make HBCD the second highest volume BFR used in Europe [29]. HBCD may be used as an alternative for PBDEs in some applications. To date, there are no restrictions on the production or use of HBCD. As a result of their widespread use and their physical and chemical properties, HBCD are now ubiquitous contaminants in the environment and humans [30, 31].

2.6 Perfluorinated Compounds

PFCs have been manufactured since 1940s. Because of their properties, these compounds are employed in a wide variety of industrial and consumer products. Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) have been two of the most used and studied PFCs, but due to their resistance to degradation, widespread in the environment, bioaccumulation and toxicological properties, these two compounds are limited in use and production practically worldwide. However,

due to their high persistence and because they are the degradation products of other PFCs currently in use, PFOS and PFOA are two of the more frequently found compounds in the environment. Recently, PFOS has been included as a persistent organic pollutant (POP) under the Stockholm Convention for global regulation of production and use [32]. PFCs are also prime candidates for chemicals that will need authorization within the REACH regulation [33].

PFCs have been detected in environmental and biological samples being widespread around the world including water, soils and sediments, human samples, and even in remote areas such as the Arctic (atmosphere [34], Arctic Ocean [35], biological samples [36, 37] and few reviews have been published [38, 39]) or Antarctic (biological samples as penguins or seals [40, 41]).

3 Analytical Methodologies

Several methodologies have been published for the determination of selected emerging contaminants in sewage sludge. The most recently reported methods are summarized in Table 1, comprising extraction of the sludge sample, subsequent purification of the extract, and final analysis by either gas or liquid chromatography (GC or LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS–MS).

3.1 Sample Preparation

Sampling of sewage sludge must be done according to ISO-5667 [42]. Storage and transport must be carried out in the dark (in order to avoid photodegradation) at approximately 4°C. Pre-treatment must be done immediately in the laboratory by thickening by centrifugation, freeze drying to stabilize the sample, homogenizing and sieving. A number of precautionary measures must be taken in order to avoid sample contamination. All glassware used must be previously washed and heated overnight at 380°C, and further sequentially rinsed with different organic solvents and HPLC grade water. In addition, a set of operational blanks must be processed together with each batch of samples. Since some of the compounds analyzed undergo photodegradation, stock standard solutions and samples were always covered with aluminium foil and stored in the dark.

3.1.1 Extraction Techniques

There are a number of procedures described in the literature dealing with the extraction of emerging contaminants from solid matrices. For extractions of solid samples, Soxhlet is widely accepted as a robust liquid–solid extraction technique.

Table 1 Overview of methods applied to determination of emerging contaminants in sewage sludge

Compounds	Sample preparation	Instrumental analysis	LOD (ng/g)	Ref.
Pharmaceuticals				
43 compounds (analgesics and anti-inflammatory drugs, lipid regulator and cholesterol lowering statin drugs; psychiatric, psychoactive drugs; antibiotics; β-blockers; β-antagonists; diuretics; anticoagulants)	PLE: MeOH/H_2O, 1:2 (v:v), 100°C; SPE: Oasis HLB, pH 7	LC–QqLIT–MS	0.01–8	[93]
19 compounds (pharmaceuticals, steroid hormones, and EDCs)	USE: ACN:water (5:3)	UPLC–MS–MS	0.1–3[a]	[56]
15 compounds (antibiotics and others)	PLE: ACN:water (1:3), 100°C	LC–ESI–MS–MS	0.6–146	[138]
10 compounds (acidic pharmaceuticals, phenolic EDCs)	USE: MeOH/H_2O, derivatization	GC–MS	15–25	[109]
31 compounds (analgesics and anti-inflammatory drugs, antiulcer agent, psychiatric drugs, antiepileptic drugs, antibiotics, β-blockers, diuretics, hypoglycemic agents, lipid regulator and cholesterol lowering statin drugs, anti-histamines)	PLE: MeOH/H_2O, 1:2 (v:v), 100°C; SPE: Oasis HLB, pH 7	LC–ESI–MS–MS	0.35–160 (treated); 0.15–114 activated); 0.17–256 (MBRs)	[49]
27 compounds (analgesics and anti-inflammatory drugs, lipid regulator and cholesterol lowering statin drugs; psychiatric, psychoactive drugs; antibiotics; β-blockers; β-antagonists; diuretics; anticoagulants)	PLE: MeOH/H_2O, 1:1 (v:v), 60°C; SPE: Oasis HLB, pH 5.5	LC–ESI–MS–MS	2–580	[50]
20 compounds (antiepileptic drugs, antibiotics, anti-histamines, lipid regulators, analgesics and anti-inflammatory drugs, antihypertensives)	USE: MeOH/acetone; SPE: Oasis HLB (pH 2; pH 3; pH 6)	LC–ESI–MS–MS	0.26–250	[43]
8 compounds (antibiotics and antiepileptic)	USE: MeOH, 0.1 M acetic acid and 5% Na_2EDTA 2:1:1 (v:v:v); SPE: Strata-X	LC–ESI–MS–MS	−17	[44]

11 compounds (macrolides, sulfonamides, antihistamines, antiulcer agent and trimethoprim)	PLE: MeOH/H_2O (pH 3) 1:1 (v:v), 80°C; Filtration: 0.45 μm	LC–ESI–MS	2.0–11	[51]
6 compounds (lipid regulators, analgesics and anti-inflammatory drugs)	USE: MeOH/acetone; SPE: Oasis MCX	GC–EI–MS	4–40	[45]
12 antibiotics (fluoroquinolones, sulfonamides, cephalosporins, penicillins, nitroimidazoles, tetracyclines, macrolides)	USE: phosphate buffer pH 6	LC–ESI–MS–MS	−5.3	[46]
8 compounds (sulfonamides, macrolides, and trimethoprim)	USE: MeOH/acetone; PLE: MeOH/H_2O 1:1(v:v),100°C; SPE: Oasis HLB	LC–ESI–MS–MS	3–41	[52]
9 fluoroquinolones	USE: MeOH/H_2O 30:70 (v:v) 15 mM sodium tetraborate solution pH 4.2; SPE: Chromabond Tetracycline polypropylene columns	LC–ESI–MS–MS LC-FLD	MS(SIM): 0.3–7.5 MS(SRM): 0.6–6.8 FLD: 11–62	[47]
Triclosan and transformation products	MAE: MeOH/acetone 1:1 (v:v); SPE: Oasis HLB, pH 2.5	GC–MS–MS	0.8	[48]
Esteroid estrogens				
E3, E2-3S, E1-3S, 17α-E2, E2, EE2, E1, DES, E2-17A	Lyophilization, homogenization, sieving (<125 μm) PLE Extract filtration (0.45 μm)	LC–ESI–MS–MS	0.15–175	[51]

(continued)

Table 1 (continued)

Compounds	Sample preparation	Instrumental analysis	LOD (ng/g)	Ref.
E1, E2, E3, EE2	Centrifugation, freezing, lyophilization Tumbling Cleanup: SPE and LC-DAD Derivatization	GC–MS	5–10	[53]
E1, E2, EE2	USE Cleanup: GPC, silica gel, SPE and LC Derivatization	GC–(EI)–MS–MS	2–4	[54, 97]
E1, E2, E3, EE2	USE Cleanup: centrifugation, solid phase ion exchange, LLE and SPE	LC-ESI-TOF	nr	[55]
E1, E2, E1-3G, E1-3S, E2-3S, E2-3,17S, E2-3G, E2-3G-17S, E2-17G, EE2	Centrifugation, lyophilization, homogenization Soxhlet extraction Cleanup: SPE	LC–ESI–MS–MS	nr	[61]
E1, E2, E3, EE2	Lyophilization and sieving (2 mm) PLE Cleaning: SPE and LC	GC–EI–MS and LC–ESI–MS–MS	1–3 2–16	[62]
E1, E2	SBSE	GC–EI–MS	0.02[b]	[63]
E1, E2, 17α-E2, EE2, E3	Centrifugation, lyophilization, grinding, sieving USE Cleanup: SPE, derivatization with dansyl chloride, LLE with hexane, silica gel column chromatography	UPLC–ESI–MS–MS	0.1–0.3[b]	[56]

E1, E2, EE2, DES	Centrifugation, lyophilization, crushing, homogenization USE Cleanup: SPE	RR–LC–ESI–MS–MS	0.10–0.98	[57]
E1, E2, E3, EE2	USE Cleanup: SPE	LDTD-APCI–MS–MS	9.2–13.9	[58]
E1, E2, E3, EE2, E1-3S	Lyophilization Mechanical shaking Cleanup: SPE, GPC, SPE	LC–ESI–MS–MS	2.1–5.3	[59]
E1, E2, E3, EE2	USE Cleanup: SPE	LC–DAD–FLD	2.30–30.4[b]	[60]
Illicit drugs				
AM	Centrifugation USE Filtration and pH adjustment (pH 10) Cleanup: SPE and filtration	LC–ESI-IT–MS	2	[98]
MA	Homogenization PLE Cleanup: hexane	LC–ESI–IT–MS	nr	[99]
CO, BE, AM, MA	Lyophilization PLE Addition of water + centrifugation	UPLC–ESI–MS–MS	2–20	[100]
UV filters (*sunscreens*)				
4-MBC, EHMC, OC	Solid–liquid extraction with pentane/diethyl ether (1/1, v/v) and diethyl ether/dichloromethane (4/1, v/v). Cleanup: silica gel in hexane	GC–EI–MS	3–6	[20]

(continued)

Table 1 (continued)

Compounds	Sample preparation	Instrumental analysis	LOD (ng/g)	Ref.
OT	Solid–liquid extraction with pentane/diethyl ether (1/1, v/v) and diethyl ether/dichloromethane (4/1, v/v). Cleanup: silica gel in hexane	LC-DAD	57	[20]
BP3, OC, OD-PABA, 2DHB, DHMB, PMDSA	PLE with in-cell purification with aluminum oxide	UPLC–ESI–MS–MS	1.5–3.5	[21]
BP3, OC, EHMC, EHS, HMS, OD-PABA, 4-MBC, BM-DBM, IAMC, EHT, DBT	PMALE with low density polyethylene membranes	LC–APPI–MS	0.3–25	[22]
BP3, 4-MBC, EHMC, OC, OD-PABA, 4HB, 4DHB, BP1	PLE with in-cell purification with aluminum oxide	UPLC–ESI–MS–MS	0.2–19 (60 for BP1)	[23]
Brominated flame retardants				
PBDEs	Soxhlet: hexane:DCM (1:1); clean up: H_2SO_4, alumina SPE	GC–ECNI–MS	0.03–1.2	[139]
PBDEs	Soxhlet: toluene Clean up: 1.Multi layer SiO_2-$AgNO_3$, H_2SO_4, NaOH. 2. Macro Al_2O_3. 3. GPC Bio-Beads S-X3, 4. Mini Al_2O_3.	GC–EI–MS	nr	[140]
PBDEs	Soxhlet: DCM; clean up on silica, Florisil, alumina	GC-HRMS	nr	[141]
PBDEs	PLE: DCM, 100°C, 1,000 psi, cleanup: SEC (Enviosep-ABC)	GC-ECNI and GC-EI	nr	[73]
BDE-209 DBDPE	PLE: heptane, 100°C, 2 cycles; cleanup: aminopropyl gel	GC–ECNI–MS	1.9 ng 1.4 ng	[74, 75]

BDE-209 DBDPE	PLE: hexane:DCM (1:1), 100°C, 1,500 psi; cleanup: H_2SO_4, acidic silica gel and basic alumina	GC–NCI–MS	nr	[76]
PBDEs HBCD	Soxhlet: hexane/acetone (3:1, v/v), cleanup: GPC, Bio-Beads SX3	MDGC-ECD	1.3 6.4	[142]
TBBPA	Soxhlet: toluene; cleanup: 1. Multi layer SiO_2-$AgNO_3$, 2. GPC Bio-Beads S-X3, 3. HPLC on a nitro column, 4. micro alumina	GC–EI–MS	nr	[143]
HBCD	Soxhlet: acetone; cleanup: silica gel	GC-ECD	1.0	[144]
TBBPA	Centrifugation: DCM and MeOH 1:9; cleanup: Superclean ENVI-18 SPE, centrifugation with HCl 1% and DCM	LC–ESI–MS	60–180 pg	[145]
TBBPA	nr	LC–ESI–MS–MS	6–18 ng/mL	[146]
HBCD TBBPA	Soxhlet: hexane:acetone; cleanup: GPC, silica gel, H_2SO_4	LC–ESI–MS	nr	[114]
HBCD TBBPA	Soxhlet: hexane:acetone; cleanup: H_2SO_4, GPC, silica gel	LC–ESI–MS–MS	1.4–12	[146]
HBCD TBBPA	PLE in-cell extraction and cleanup: DCM	UPLC-LC-ESI–MS–MS	0.08	[147]

(continued)

Table 1 (continued)

Compounds	Sample preparation	Instrumental analysis	LOD (ng/g)	Ref.
Perfluorinated compounds				
PFOA, PFNA, PFDA, PFUnA, PFOS, 4:2, 6:2, 8:2, 10:2 diPAP	Ion pair extraction – 0.25 M $NaCO_3$ + 0.5 M TBAS, pH 10 – Extraction with MTBE	LC–ESI–MS–MS(QqQ)	0.0625 (PFCs)[a] 0.375 (diPAPs)[a]	[81]
PFBS, PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTeA, PFHxDA, PFODA	Solid acid liquid extraction – 7.5 mL (1% AcH) – Sonication 20 min at 60°C – Centrifugation – Supernatant separation and pellet with MeOH, sonication and centrifugation – 7.5 mL 1% AcH addition – SPE by Oasis HLB	LC–ESI–MS–MS(QqQ)	< 25[a]	[148]
PFHxS, PFOS, FOSA, PFOA, PFNA, PFDA, PFUnA, PFDoA	Solid acid liquid extraction – 7.5 mL (1% AcH) – Sonication 20 min at 60°C – Centrifugation – Supernatant separation and pellet with MeOH, sonication and centrifugation; – 7.5 mL 1% AcH addition; – SPE by Oasis HLB	LC–ESI–MS–MS(QqQ)	nr	[149]
		LC–ESI–MS–MS(QqQ)	1–5[a]	[137]

PFBS, PFHxS, PFHpS, PFOS, PFDS, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA	Solid acid liquid extraction – Sonication and centrifugation – Supernatant separation and solid with MeOH:1% AcH, sonication and centrifugation – SPE by Oasis HLB;			
PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTrA, PFTeA, PFHxS, PFOS	Solid–alkaline liquid extraction – Sonication/heat and overnight incubation – Neutralization (HCl); – ACN:MeOH (1/1), shaked – 2 mL of combined extracts (20 mL) + 98 mL water at pH 4 – Sonication – SPE by HLB cartridges	UPLC–ESI–MS–MS (QqQ)	1.8–6.8[a]	[83]
PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTeA	Solid–liquid extraction – MeOH, wrist-action shaker – Settled or centrifugation – 1 mL supernatant + 25 mg Envi-Carb graphitized carbon adsorbent + 50 μL AcH, vortexed and centrifugated	LC–ESI–MS–MS(QqQ)	1[a]	[107]
PFOA, PFNA, PFDA, PFUnA, PFOS, FOSA	Solid–liquid extraction – MeOH, ultrasonic bath – Extracts pooled + 1 L water – SPE by C18	LC–ESI–MS–MS(QqQ)	0.4–1.7	[86]

(continued)

Table 1 (continued)

Compounds	Sample preparation	Instrumental analysis	LOD (ng/g)	Ref.
N-EtFOSE, *N*-EtFOSAA, FOSAA, FOSA, *N*-EtFOSA, PFOSI (perfluorooctane sulfinate), PFOA, PFOS	Solid–liquid extraction – Sonication and centrifugation – Extract poured into a new tube + MeOH – Purification by dispersed solvent (ENVI-Carb) – (in parallel) SPE from headspace sampling	LC–ESI–MS–MS(QqQ)	0.1 μg/L (vial)[a] 46–49 pmol (microcosm)[a]	[85]
PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFHxDA, PFODA, PFBS, PFHxS, PFOS, PFDS, FOSA	PLE (MeOH solvent) – Evaporation to 1 + 30 mL water – SPE by Oasis WAX	LC–ESI–MS–MS (QqLIT)	50–2,772 pg/g[a]	[91]

For abbreviations of compounds, see text

Abbreviations: *ACN* acetonitrile, *APCI* atmospheric pressure chemical ionization, *APPI* atmospheric pressure photoionization, *DAD* diode array detection, *DCM* dichloromethane, *ECNI* electron capture negative ionization, *EI* electron impact, *ESI* electrospray, *FLD* fluorescence detection, *GC* gas chromatography, *GPC* gel permeation chromatography, *HRMS* high resolution mass spectrometry, *IT* ion trap, *LC* liquid chromatography, *LDTD* laser diode thermal desorption, *LLE* liquid–liquid extraction, *MAE* microwave-assisted extraction, *MDGC* multidimensional gas chromatography, *MeOH* methanol, *MS* mass spectrometry, *MS–MS* tandem mass spectrometry, *NCI* negative chemical ionization, *nr* not reported, *PLE* pressurized liquid extraction, *PMALE* pressurized membrane-assisted liquid extraction, *QqLIT* quadrupole linear ion trap, *QqQ* triple quadrupole, *RR* rapid resolution, *SBSE* stir bar sorptive extraction, *SPE* solid phase extraction, *TOF* time of flight, *UPLC* ultra-high-performance liquid chromatography, *USE* ultrasonic extraction

[a]Method quantification limits

[b]Instrumental detection limits (without matrix)

The main drawback of this technique is solvent and time-consuming. In the last decade, there have been efforts to develop extraction techniques that allow efficient extraction and reduced solvent volumes in shorter times, incorporating high levels of automation, such as pressurized liquid extraction (PLE) [commonly known as accelerated solvent extraction (ASE)].

In recent years, extraction methods for PhACs have usually been based on liquid partitioning with ultrasonic extraction (USE) [43–47], microwave-assisted extraction (MAE) [48], or the more advanced PLE [49–52]. When compared to the other extraction techniques, PLE provides good recoveries, saves time and organic solvent, which makes it become currently a preferred technique for PhAC analyses.

Extraction methodologies for estrogens have been performed with organic solvents and/or mixtures of them using a variety of techniques, including tumbling [53], shaking and USE [54–60], Soxhlet [61], and faster procedures such as PLE [51, 62]. In 2008, Tan et al. also described a method for determination of estrogens (among other EDCs), based on stir bar sorptive extraction (SBSE) with Milli-Q water prior to thermal desorption (TD) and GC–MS analysis [63], and according to the authors, the stir bars can be used more than 50 times with appropriate reconditioning.

As it is summarized in Table 1, drugs have been extracted from sewage sludge by USE or PLE using in all cases a mixture of methanol and acid as extraction solvent.

In the extraction of UV filters, PLE was the technique preferably used, although the more classical solid–liquid extraction technique was still used. Three consecutive extractions using pentane/acetone (1/1, v/v), pentane/diethyl ether (1/1, v/v), and diethyl ether/dichloromethane (4/1, v/v) were applied by Plagellat et al. [20] for the extraction of four UV filters namely 3-(4-methylbenzylidene) camphor (4-MBC), hethylhexyl-methoxycinnamate (EHMC), octocrylene (OC), and octyltriazone (OT). In the methods developed by Nieto et al. [21] and by Gago-Ferrero et al. [23], sewage sludge samples were extracted by PLE. Samples were mixed in the extraction cells with aluminium oxide in order to perform the purification step within the cell. The extracting solvents were methanol and a mixture of methanol/water (1/1, v/v), operating at 100°C and 10,000 kPa. PLE was also applied by Rodil et al. [24] for the extraction of UV filters, but in combination with nonporous polymeric membranes for improved purification. In the pressurized membrane-assisted liquid extraction (PMALE) method, sludge samples were confined in sealed low density polyethylene bags and extracted using a mixture of ethyl acetate/n-hexane (1/3, v/v) at 70°C and 10,000 kPa, resulting in a clear extract. The membrane encloses the sludge sample as barrier to remain particularly high molecular weight matrix molecules and also provides selectivity and specificity in terms of permeation and transport through the membrane. However, a quite important drawback of this proposed methodology is the lack of reproducibility in the manual preparation of the membrane bags.

Table 1 summarizes the recent analytical techniques applied to BFR analyses, including also new applications for emerging BFRs. Liquid–solid extraction, using Soxhlet apparatus, is a widely used standard technique for the determination of BFRs in sewage sludge. Typical solvents used for the extraction of BFRs are toluene, CH_2Cl_2, acetone, and hexane:acetone or hexane:DCM mixtures. The extraction time varied from 12 to 24 h [64–69]. Another traditional extraction method used by some authors is that based on a centrifugation methodology. Extraction was carried out using acetone and acetone:hexane mixtures as solvent extractors [70–72]. However, this technique is also time-consuming. PLE have also been evaluated and reported in some BFR studies [73–76] with heptane and hexane: CH_2Cl_2 mixture applied as solvent extractors. The temperature and pressure used varied from 100 to 150°C and from 1,000 to 1,500 psi, respectively. PBDE recoveries obtained using PLE method are similar to those obtained using the conventional Soxhlet extraction. However, it should be pointed that lower standard deviations were found with PLE, probably due to the automation of the system. The use of solid phase microextraction (SPME) has increased dramatically over the past several years, as it allows efficient extraction, reduced solvent consumption and analysis time, and it is easily automated. SPME was applied to the determination of some PBDE and PBB congeners in sludge [77]. Unlike conventional methods which involve solvent extraction and cleanup steps before instrumental analysis, the proposed method uses headspace extraction, and hard contamination of the chromatographic system is prevented. In this study, PBB-49, BDE-47, BDE-99, BDE-100, and BDE-154 were the selected analytes.

In the analysis of PFCs, we need to avoid undesirable matrix effects, and also the possible disturbance of some target compounds. For example, the hydrolysis of fluorotelomer compounds to fluorotelomer alcohol during the solvent extraction of soils, which was observed by Dasu et al. [78]. Main extraction procedures have been based on ion pair extraction, solid–liquid extraction, alkaline digestion, and PLE. Table 1 presents detailed information about different works published using these methodologies. Hansen et al. [79] obtained good sensitivity of the ion pair extraction approach using methyl tertiary butyl ether. Ion pair extraction procedure has been the basis of several procedures for sludge, and in general good recovery rates have been obtained [80]. However, Eon et al. [81] obtained low recovery rates for longer chain PFCs. Better recoveries, repeatability, and limits of detection (LODs) are in general obtained with solid–acid liquid extraction. This method consists in the ultrasonic assisted acid digestion of sludge [82]. Sample preparation by alkaline digestion has been also widely applied for the analysis of PFCs in complex matrices. This procedure is based on digestion with sodium or potassium hydroxide in methanol followed by solid phase extraction (SPE) [83, 84]. Solid–liquid extraction procedures are the most commonly used methodologies for the extraction of nonvolatile PFCs due to the facility and simplicity of the extraction [85–89]. It has been applied using mixtures of hexane and acetone or using methanol. Less reported has been the use of PLE [90–92].

3.1.2 Purification

Cleanup of the extracts is a key process when dealing with sludge matrices, since it not only removes matrix interferences, but may also cause losses of the target analytes in intense and long procedures, resulting in poor recoveries. The most effective cleanup of the extracts of sludge samples containing pharmaceutical residues has proved to be SPE. So far, the most comprehensive method is developed by Jelic et al. [93], who optimized and validated a protocol for simultaneous analysis of 43 pharmaceutical compounds in sewage sludge using PLE followed by SPE cleanup. Several other methods were developed using similar approach. Radjenovic et al. [49] used the PLE–SPE combination for the isolation of 31 pharmaceuticals from sludge samples proceeding from the conventional activated sludge treatment and pilot-scale membrane bioreactors. Barron et al. [50] used the same approach for sample preparation for analysis of 27 pharmaceuticals in soil and treated sludge. Also, Nieto et al. [51] and Göbel et al. [52] extracted the target compounds from sludge samples using the PLE–SPE. In all the mentioned methods, a hydrophilic–lipophilic balance (HLB) reversed-phase sorbent was used as SPE packaging. This sorbent has been found suitable for multi-residue methods in neutral pH condition, with a proper selection of the eluent (solvent) [94–96].

Purification methodologies for estrogens have been carried out by SPE with C18 [53, 54], polymeric [56, 60–62], silica [56–59] and anion exchange materials [59], preparative LC [53, 62], gel permeation chromatography (GPC) [54, 59, 97], liquid–liquid extraction [56], combination of them, or simple filtration [51]. However, the latter corresponds to the method with the highest reported method detection limits (MDL) [up to 175 ng/g dry weight (dw)].

Drugs have been purified by SPE in the analysis of amphetamine (AM) by Kaleta et al. [98], by various consecutive washing steps with hexane in the analysis of methamphetamine (MA) by Jones-Lepp and Stevens [99], and by simple centrifugation after addition of water, to separate the aqueous extract from a bottom sediment layer and a top fat layer, in the analysis of AM, MA, cocaine (CO), and benzoylecgonine (BE) by Langford et al. [100], who found little improvement in reducing matrix effects when applying SPE cleanup.

In the UV filter analyses, clear extracts were obtained which did not need any further time- and labor-consuming cleanup when using PLE with in-cell purification. In contrast, a thorough purification procedure was required for the cleanup of the extracts obtained by solid–liquid extraction. Columns packed with silica gel (previously activated) in hexane having Na_2SO_4 at the top of the column were employed for the purification of the extracts. The two first fractions obtained with 20 mL of hexane and 20 mL of hexane/diethyl ether (9/1, v/v) were discarded and 4-MBC, EHMC, and OC were collected with 50 mL of hexane/diethyl ether (9/1, v/v). OT was recovered with 70 mL of hexane/diethyl ether (3/2, v/v).

For PBDE analyses, the complexity of sludge matrix implies the use of multistep purification methods. Sludge extracts contain relatively large amounts of elemental sulfur, which would disturb the GC analysis, and must be removed.

Typical methods for sulfur removal are treatment with copper powder, silica modified with $AgNO_3$ in a multilayer silica column, or desulfuration with mercury. Sewage sludge samples contain also high levels of organic matter that must disturb the purification via column chromatography. For this reason, the purification step started with treatment with concentrated sulfuric acid to remove organic matter. A cleanup stage was then performed with the aim of purification as well as fractionation of the extract, using a great variety of adsorbents (e.g., silica, Florisil, and alumina). However, these conventional multi-step purification methods are time-consuming. PBBs, HBB, DBDPE, and HBCD were analyzed using the same protocols reported for PBDE determinations. However, TBBPA is a phenolic in its native state and a derivatization step prior to injection on the GC system is required. Different derivatization methods were found in the literature. Cariou et al. [101] used n-nonane/MSTFA (50:50, v/v) as derivatization reagent, leading to the diTMS derivative. Acetic acid anhydride could also be used to give the diacetylated derivatives [102]. Acetic acid anhydride and pyridine (1:1) at 60°C for 30 min was applied by Sellström and Jansson [103]. Some authors preferred to use diazomethane to obtain the TBBPA dimethyl ether derivative, reducing the polarity of the two hydroxyl groups [104, 105]. However, methylated TBBPA derivatives were analyzed, making it impossible to distinguish between dimethyl-TBBPA and TBBPA present in the sample.

The analysis of PFCs requires cleanup to remove co-extracted lipids and other matrix constituents. Without further cleanup, this may lead to enhancement or suppression of the electrospray ionization, resulting in inaccuracies [106]. Cleanup of extract can be performed by a washing step after sample enrichment on the SPE cartridge using a variety of commercial cartridges C18 [82], Oasis WAX cleaned-up by addition of Envi-carb (graphitized carbon) and 50 μL glacial acetic acid [107]. As a final cleanup step, extracts may be filtrated over, e.g., nylon filters to remove solids from the final extract, but care should be taken to avoid losses or contamination of the sample extract. Yamashita et al. tested several nylon filter types used for removal of solids from the final extract and found that some filters contained trace amounts of PFOS and PFOA [108]. A simple methanol-washing step reduced the filter-originating PFOS and PFOA to below limits of quantification (LOQ).

3.2 Instrumental Determination

There are a variety of analytical methodologies developed for the analysis of emerging contaminants selected for this chapter. In almost all cases, the instrumental analysis is based on the use of GC or LC coupled to MS or MS–MS. The selection of one or another technique depends primarily on the physicochemical properties of the compounds. We summarize the more recently developed methodologies for each of the families (Table 1).

3.2.1 GC–MS

GC–MS is applied in some methods for the analysis of pharmaceuticals in sludge [45, 109]. However, this technique can only be successfully applied for a limited number of nonpolar and volatile pharmaceutical compounds, while analysis of polar pharmaceuticals requires a time-consuming and often irreproducible derivatization. Consequently, LC–MS is the preferred technique in many laboratories. Some other detection techniques are also employed, such as diode array (DAD) and electrochemical (ED) and fluorescence detection (FLD). In the case of fluoroquinolones, FLD is still the favored technique.

A similar situation exists for the case of estrogens, which have also been analyzed by GC or LC coupled to MS or MS–MS. However, LC–MS offers the advantages, as compared to GC–MS, of not requiring prior compound derivatization and allowing the analysis of both free and conjugated estrogens in a single run.

GC–MS operated in electron impact (EI) mode was only sporadically used for the determination of some UV filters such as 4-MBC, EHMC, and OC. Separation was achieved on a 60 m $\times$ 0.25 mm i.d. DB-5 column, with 0.25-μm film thickness. For quantification of the compounds, data acquisition was performed in selected ion monitoring (SIM) mode recording three characteristic ions per compound. GC–MS allowed the differentiation between the two isomers (*cis*/*trans*) for 4-MBC and EHMC.

GC coupled to either negative chemical ionization (NCI)-low resolution mass spectrometry (LRMS) or EI-LRMS is an alternative more frequently used for PBDE and PBB analyses. NCI-LRMS is by far the most popular technique because it provides high levels of sensitivity. However, if high specificity and accuracy in quantification are needed (e.g., by using the isotopic dilution method), EI-LRMS shows better performance, but at the expense of lower sensitivity. GC-high resolution mass spectrometry (HRMS)-based analytical methods have also been developed for determination of congener-specific PBDE compounds, providing the most selective and sensitive method. However, HRMS needs sophisticated, expensive instruments that require a trained personnel and frequent maintenance. Moreover, an MS strategy on BFR analysis in sewage sludge was also developed based on the use of ion trap (IT)-MS.

HBCD can be determined by GC–MS, using methods similar to those developed for PBDE determinations. As the response factors of the three diastereomers do not appear to differ very much, HBCD can be quantified as total HBCD. However, the different isomers have not so far been separated by this technique. Moreover, because isomers of HBCD are thermally labile (it is known that HBCD decomposition takes place between 240°C and 270°C), elution from a GC column usually results in a broad, diffuse peak. In addition, a number of chromatographic peaks corresponding to different breakdown products were detected. These peaks could interfere with some BFR congeners (e.g., BDE-99) [102, 110]. TBBPA can be also determined by GC–MS; however, a derivatization step must be carried out prior to injection on the GC system.

3.2.2 LC–MS

As mentioned before, LC is the preferred technique for the analysis of pharmaceuticals. Among LC modes, reversed-phase with octadecyl C18-bonded or octyl C8-bonded silica packing is the most commonly used stationary phase. Although single MS has been used for quantification of pharmaceutical residues in the environment, MS–MS detection is preferred for increased analytical sensitivity and selectivity in complex matrices. LC–MS–MS is often applied with triple quadrupole (QqQ) or IT–MS. QqQ instruments can help to avoid false positive results if the ions of at least two ion–ion transitions are used in combination with at least one ion intensity ratio. IT–MS allows a reliable confirmation of analytes due to the possibility of multiple ion–ion transitions. Recently, a hybrid quadrupole linear ion trap (QqLIT)-MS has been applied for the analysis of pharmaceuticals in sewage sludge [93] providing very good sensitivity for the simultaneous analysis of 43 PhACs. Regarding the ionization techniques, electrospray ionization (ESI) is widely used in published works.

The analysis of estrogens in sludge has been reviewed by Kuster et al. [6] in 2004. Most of the LC–MS methodologies are based on reverse phase (RP) chromatography and ESI in the negative ion mode [51, 55, 57, 59, 61, 62], though ESI in the positive ion mode after derivatization of the compounds with dansyl chloride [56], which according to the authors, improves the analytical sensitivity about 20 times, and atmospheric pressure chemical ionization (APCI) combining both positive (for analysis of E2 and EE2) and negative ionization (for E1 and estriol (E3)) has also been used [58]. The latter conditions were employed in an ultrafast analytical method (15 s per sample), based on laser diode thermal desorption (LDTD)–APCI–MS–MS, in which the target analytes are not subjected to any previous chromatographic separation [58]. Finally, in most instances, detection has been performed in the selected reaction monitoring (SRM) mode recording two SRM transitions per compound, whereas quantification has often rely on the use of isotopically labeled surrogate/internal standards.

To date, several methodologies, all of them based on LC coupled to MS–MS, have already been reported to determine illicit drugs and their metabolites in aqueous matrices and to further estimate drug use at the community level [111]. However, only three analytical methods have been published so far in the peer-reviewed literature for the determination of illicit drugs, namely, AM, MA, CO, and its metabolite BE, in sewage sludge samples. In all methodologies, analysis has relied on the use of RP-LC or RP-UPLC (ultra-performance liquid chromatography) coupled to MS or MS–MS with ESI ionization working in the positive mode. With these methodologies, LODs in the range 2–10 μg/kg dw have been achieved. Matrix ionization effects have been observed to either induce signal suppression (up to 30% in the analysis of AM by Kaleta et al. [98] and 39% in the analysis of BE by Langford et al. [100]) or enhancement (110%, 62%, and 12% for AM, MA, and CO, respectively, as reported by Langford et al. [100]).

LC–MS–MS was also the method of choice for the analysis of UV filters in solid matrices. Both LC and UPLC have been applied in three out of the four methods available for the determination of UV filters in sludge. Separation was performed on C8 and C18 LC-chromatographic columns (Zorbax, Eclipse, Vydac, and Purosphere) using binary gradient elution of mobile phases consisting of water/methanol or water/acetonitrile. MS–MS detection was performed in SRM with ESI and atmospheric pressure photoionization (APPI) in both positive and negative modes. For each compound, two characteristics transitions were monitored. In addition to MS and MS–MS, diode array detection (DAD) was occasionally applied to the determination of OT. Spectra were recorded between 240 and 360 nm and discrete channels at 310 nm.

For the analysis of sewage sludge by PLE combining extraction and cleanup in a single step, and followed by LC–MS–MS analysis, the determination was fast and sensitive, affording LODs lower than 19 ng/g dw. Good recovery rates, especially given the high complexity of sludge matrix (between 70% and 108%) were afforded. The GC–MS-based method was somewhat more accurate, despite the additional purification step required, showing recovery rates in the range 88–101%, and more sensitive allowing LODs between 3 and 6 ng/g dw. As expected, DAD detection for OT was less sensitive, with a limit of detection of 57 ng/g dw.

Matrix effects were evaluated by Gago-Ferrero et al. [23]. Both signal suppression and signal enhancement were observed. The extent of these effects was found to be fairly dependent on the UV filter. Thus, quantification should be performed by standard addition or internal standard calibration. Since standard addition is a high time-consuming procedure, internal standard calibration with appropriate isotopically labeled compounds is the best option.

Traditionally, the analysis of BFRs has been developed using GC as the principal separation technique, due to the volatility of these compounds. However, GC analysis of some BFR compounds, such as HBCD or TBBPA, presented some drawbacks. That because, in recent years, methods employing LC–MS and LC–MS–MS have been developed offering good results. Guerra et al. [112] presented an overview of current analytical methods for selected BFRs, focusing on instrumental determination using LC–MS. Table 1 summarizes different LC–MS methods found in the literature for the analysis of different BFRs.

The most suitable LC–MS interface for TBBPA analysis is ESI operating in the negative mode. Tollbäck et al. [113] reported LODs 30–40 times lower than with APCI. In addition, it permitted the monitoring of the intact TBBPA molecule, as the soft ionization of ESI improved selectivity and accuracy. Similar results were found by Morris et al. [114]. In terms of sensitivity, LC–ESI–MS can be competitive with published GC–EI–MS techniques with LODs in the ppt range. Another proposed method is based on the use of LC–APPI–MS [115] to analyze TBBPA and its related degradation products working with a 100-C8 column. LC–QqLIT–MS was proposed to analyze TBBPA and related compounds [bisphenol A (BPA), monobromobisphenol A (MonoBBPA), dibromobisphenol A (DiBBPA), and tribromobisphenol A (TriBBPA)] in sewage sludge and sediment samples [116].

LC–MS or LC–MS–MS using ESI or APCI are versatile tools for the isomer-specific determination of trace levels of HBCDs, monitoring the specific transitions *m*/*z* 640.6 to *m*/*z* 78.9 and 80.9. Budakowsky and Tomy [117] showed that APCI has lower intensities than in a similar experiment with ESI. Consequently, the ESI mode was preferred for determining diastereoisomers in several studies. Different methods for the analysis of diastereoisomeric HBCD using LC–ESI–MS–MS and SRM were developed, obtaining LODs of 0.5–6 pg on-column [117, 118].

Some other LC–ESI–MS–MS methods have been developed for the simultaneous analysis of diastereoisomeric HBCDs and TBBPA [119, 120]. Two different LC–QqLIT–MS methods were developed by Guerra et al. [119] comparing an SRM experiment with an enhanced product-ion (EPI) experiment to analyze α-HBCD, β-HBCD, and γ-HBCD together with TBBPA and related compounds (BPA, MonoBBPA, DiBBPA and TriBBPA). The methods developed displayed excellent LODs in SRM mode (0.1–1.8 pg), but even better results were obtained in EPI mode (0.01–0.5 pg). In order to analyze sewage sludge, the previously optimized experiment involving SRM was used [119]. Sample extraction was based on the use of sonication-SPE, obtaining mLODs of 1.4–12 ng/g. Recent studies involving the use of UPLC–ESI–MS–MS for the analysis of HBCD and TBBPA through a C18-BEH column found iLODs of 0.03–0.06 pg on-column and mLODs of 0.080–0.082 ng/g for sewage sludge samples [121].

It is also important to note that matrix-related effects, either signal enhancement or more commonly signal suppression, can have a pronounced effect on quantitative measurements. Based on these observations, the use of isotope-labeled standards is helpful to achieve accurate analytical measurement data on the diastereoisomers. Several methods found in the open literature include use of both ^{13}C-labeled and d_{18}-labeled surrogates as recovery and/or instrument standards [118].

LC separation of PFCs has been mainly carried out with C18 and C8 columns. In spite of the widely used of RP-C18 columns for PFCs analysis, the interference producing the enhancement of spectral signal has been reported. RP columns with shorter alkyl chain bonded phases (e.g., C8, C6, phenyl, phenylhexyl) also separated the branch isomers, but to a lesser extent. To minimize the separation of branched isomers, the authors increased the LC column temperature to 35°C or 40°C [122–124]. Taniyasu et al. [125] explored the chromatographic properties and separation of short-chain PFCs on RP-C18 and ion-exchange columns. The results showed that the peaks of perfluoropropionic acid (PFPrA) and perfluoroethanesulfonate acid (PFEtS) were broad and not adequately resolved whereas that of trifluoro acetic acid (TFA) was not retained in the analytical column eluting with the solvent front. This suggested that RP columns are not suitable for the analysis of short-chain perfluorinated acids (PFAs), especially TFA. As a proper alternative, ion-exchange columns have superior retention properties for more hydrophilic substances enabling the analysis of short-chain perfluoroacids, TFA, PFPrA, perfluorobutanoic acid (PFBA), PFEtS, perfluoropropanesulfonate (PFPrS), and perfluorobutanesulfonate (PFBS) together with several long-chain perfluoroacids.

ESI operating in the negative ion mode has been the interface most widely used for the analysis of anionic perfluorinated surfactants. In addition, ESI has also been optimized for the determination of neutral compounds such as the sulfonamides perfluorooctanesulfonamide (PFOSA), perfluorooctanesulfonamidoacetate (*N*-EtFOSAA), and *t*-Bu-PFOS. The use of APPI has been explored in few works [126–128]. Takino et al. [126] found as the main advantage of this technology, the absence of matrix effects, but the LODs were considerably higher than those obtained by LC–ESI–MS–MS.

LC–MS–MS performed using QqQ mass spectrometer combined with SRM is one of the more widely applied detector, as well as, to be one of the better suited for quantification of PFCs. Nowadays, the performance of IT and time of flight (TOF) have been also considered for trace quantification of PFCs. PFCs contain carboxylic, sulfonic, hydroxy or sulfonamide group. They have acidic properties and can therefore dissociate. Therefore, ESI in the negative mode is best suited, allowing LODs in the pg–ng/g range. Pseudo molecular ions are formed such as $[M–K]^-$ for PFOS (*m*/*z* 499), $[M–H]^-$ for PFOA (*m*/*z* 413) and PFOSA (*m*/*z* 498), which are generally selected as precursor ions for MS2 experiments using IT and a triple quadrupole instruments. Berger et al. [106] have presented a comparison between IT, QqQ, and TOF instruments. MS–MS showed excellent specificity, but the matrix background is eliminated by the instrument, thus it cannot be visualized. Applying TOF–MS gives an estimation of the amount of matrix left in the extract, which could impair the ionization performance, and the high mass resolution of the TOF–MS instrument offers excellent specificity for PFCs identification after a crude sample injection. Recently, the analytical suitability of three different LC–MS–MS systems, QqQ, conventional 3D IT, and QqLIT, to determine trace levels of PFCs in fish and shellfish was compared [129]. In this study, the accuracy was similar in the three systems, with recoveries always over 70%. Precision was better for the QqLIT and QqQ systems (7–15%) than for the IT system (10–17%). The QqLIT (working in SRM mode) and QqQ systems offered a linear dynamic range of at least three orders of magnitude, whereas that of the IT system was two orders of magnitude. The main advantage of QqLIT system is the high sensitivity, at least 20-fold higher than the QqQ system. Another advantage of QqLIT systems is the possibility to use EPI mode and MS^3 modes in combination of SRM mode for confirmatory purposes of target analytes in complex matrices.

4 Occurrence in Sewage Sludge

During the last decade, a large amount of environmental data have been generated for different emerging contaminants, showing the wide distribution of these contaminants in the environment. However, although some compounds have been well studied and their levels have been reported for different matrices and different countries, for some others, data in the literature are very scarce. The content of emerging pollutants in sewage sludge from wastewater treatment plants gives an

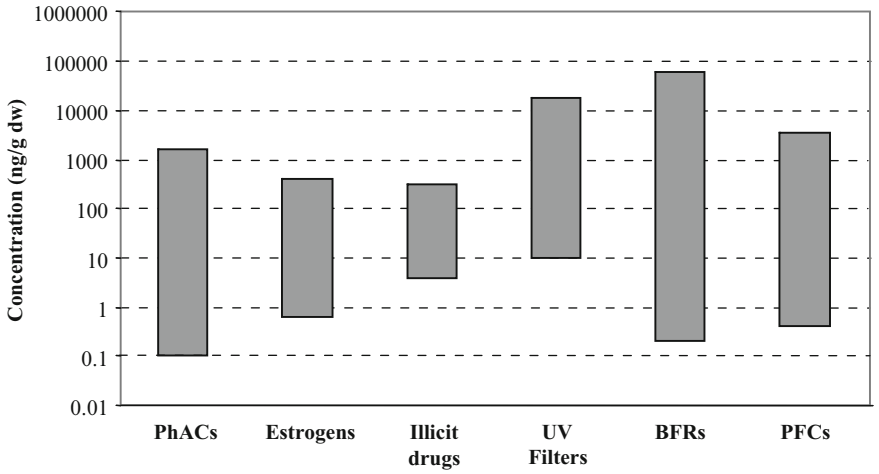

Fig. 1 Range of concentrations obtained for the different emerging contaminants in sewage sludge samples

indication of the general exposure and use of these compounds. The range of contaminants found in sewage sludge samples is a reflection of what is being used in society. Table 2 shows some of the studies carried out in sewage sludge samples from different countries, and Fig. 1 represented the range of contamination levels found for each of the selected emerging contaminants.

4.1 Pharmaceutical Compounds

While variety of pharmaceutical residues have been detected in effluent and surface water samples, only few works have been reported regarding their occurrence in sewage sludge samples.

Carbamazepine (anticonvulsant) is one of the most frequently detected pharmaceuticals in environmental and sewage treatment plant samples, known for its persistence. Its presence has been detected in treatment plants in concentration range from 1.7 ng/g in South China [56] to 120 ± 10 ng/g in Ireland [50]. In Spain reported levels of carbamezapine ranged from 11 to 42 ng/g [130] in one study, while Radjenovic et al. [49] reported levels between 74 and 90 ng/g.

Anti-inflammatory drugs such as Ibuprofen, acetaminophen, diclofenac, atorvastatin, and hydrochlorthiazide were detected in the highest concentrations in samples from three WWTPs in Spain [93]. These compounds were present in average concentrations from 43 to 117 ng/g, 42 to 103 ng/g, 28 to 75 ng/g, 21 to 65 ng/g, and 29 to 126 ng/g, respectively.

Antimicrobial agents as well as macrolide antibiotics and fluoroquinolones used in human and veterinary medicine are of particular interest due to their potential for

antibiotic resistance. Therefore, they were subject of analysis in all the recent publications. In Table 2 are presented the concentrations of macrolide antibiotic clarithormycin, though erythromycin, azythromycin, and roxithromycin were detected in sludge samples as well. Spongberg et al. [43] found clarithromycin in concentrations from 1.6 to 30.2 ng/g in a WWTP in Ohio (USA), and Göbel et al. [52] reported higher concentrations, up to 63 ng/g in WWTPs in Germany and Switzerland. Nieto et al. [51] reported very high levels for tylosin and roxithromycin ranging from 1,074 to 1,958 ng/g and from 337 ng to 1,453 ng/g, respectively. The most prescribed fluoroquinolones worldwide are ciprofloxacin and ofloxacin. These two compounds were detected in sludge samples in wide concentration range, reaching values of 778 ng/g for ciprofloxacin in Ohio (USA) [44], and 510 ng/g for ofloxacine in Austria [52].

Triclosan is an antimicrobial compound widely used in household products, including cosmetics, oral sanitary products, detergents, etc. Thence, it is not surprising that it was detected in concentrations of approximately 1,000 ng/g in treated sludge and 5,000 ng/g in biological sludge from an urban WWTP in Spain [48].

4.2 Estrogens

Of the various estrogens detected in sewage sludge, E3 has been the compound measured at the highest concentration (up to 406 ng/g dw), followed by EE (up to 313 ng/g), E2 (up to 203 ng/g), DES (up to 184 ng/g), and E1 (up to 137 ng/g), all of them showing the highest concentrations in sludge samples from Spain (see Table 2). Of the various conjugated estrogens, which have seldom been investigated [51, 61, 130], only estrone-3-sulfate (E1-3S) and estradiol-3-sulfate (E2-3S) have been detected, at concentrations up to 59 and 189 ng/g, respectively, in Spain [51, 130]. In terms of detection frequency, E1 seems to be the most ubiquitous compound [55] (detected in eight out of nine studies), followed by E2 (detected in seven out of nine studies), EE (detected in five out of seven studies), and E3 (detected in four out of seven studies). DES, a currently not-in-use synthetic estrogen, was investigated in sludge samples from Canada [58] and Spain [51, 130], and its finding in the latter suggests illicit use of this compound, perhaps for illegal growth promoting purposes.

4.3 Illicit Drugs

The investigations done by Kaleta et al. [98] and Jones-Lepp and Stevens [99] are to date the only ones that report occurrence of illicit drugs in sewage sludge, since Langford et al. [100] did not detect any of the four illicit drugs analyzed in sewage sludge samples from three Scottish STPs. Kaleta et al. [98] detected AM in all the

Table 2 Concentration levels (expressed in ng/g dw) of selected emerging contaminants in sewage sludge samples

Analyte	Location, country (year)	Concentration	Reference
Pharmaceuticals			
Carbamazepine	Spain	74–90	[49]
	Spain	10–13	[93]
	Ireland	120 ± 10	[50]
	China	nq-22.3	[138]
	USA	34.5	[44]
	USA	4.7–12.8	[50]
Diclofenac	Spain	nq-425	[49, 93]
	Japan	35 ± 7	[45]
	USA	10.4–23.1	[44]
Trimethoprim	Spain	nq-13.8	[49]
	Germany	87–133	[52]
	Switzerland	13–30	[52]
Clarithromycin	USA	5.2	[44]
	USA	1.6–30.2	[50]
	Germany	16–41	[52]
	Switzerland	25–63	[52]
Ciprofloxacin	USA	778	[44]
	USA	22.6–46.4	[50]
	Sweden	0.9–4.8	[52]
	Austria	230	[47]
Ofloxacin	Spain	44.6–86.6	[49]
	Sweden	0.1–2	[7]
	Austria	510	[47]
Triclosan	Spain	418–1,508	[48]
	Ireland	10–24.6	[43]
	Ohio, USA	320	[44]
Esteroid estrogens			
E1	Spain (2007–2008)	43–137	[51, 130]
	France (2008)	2–8	[62]
	Australia (2004–2005)	4.00–4.50	[63]
	Australia (2005)	17.5 ± 30.3;	[150]
	China (2008)	33.8 ± 51.3[a]	[61]
	China (2010)	9.7–22.9	[57]
	Canada	1.6–5.4	[58]
	UK	32–54	[59]
	Spain	up to 994	[60]
		nd	
E2	Spain (2008)	<250	[51]
	France (2008)	1–10	[62]
	Australia (2004–2005)	0.83–3.60	[63]
	Australia (2005)	2.2 ± 3.7;	[150]
	China (2008)	17.3 ± 30.0[a]	[61]
	China (2010)	nd	[57]
	Canada	3.9–10.2	[58]
	UK	41–57	[59]
	Spain	up to ~60	[60]
		11.9–203	

(continued)

Table 2 (continued)

Analyte	Location, country (year)	Concentration	Reference
α-E2	Spain (2008)	<250	[51]
	Australia (2005)	nd	[150]
	China (2008)	nd	[56]
E3	Spain (2007–2008)	104–406	[51, 130]
	France (2008)	nd	[62]
	Australia (2005)	nd	[150]
	China (2008)	nd	[56]
	Canada	45–69	[58]
	UK	up to 27	[59]
	Spain	4.85–35.6	[60]
EE2	Spain (2007–2008)	213–313	[51, 130]
	France (2008)	1–18	[62]
	China (2008)	nd	[56]
	China (2010)	nd	[57]
	Canada	47–55	[58]
	UK	up to ~200	[59]
	Spain	13.6–94.9	[60]
DES	Spain (2007–2008)	25–184	[51, 130]
	China (2010)	nd	[57]
E1-3S	Spain (2007–2008)	0.64–37	[51, 130]
	UK	up to 59	[59]
E2-3S	Spain (2007–2008)	0.64–189	[51, 130]
Illicit drugs			
AM	Austria (2006)	5–300	[98]
	Scotland (2011)	nd	[100]
MA	Milwaukee, WI, US (2007)	nd	[99]
	Los Angeles, CA, US (2007)	4	[99]
	Scotland (2011)	nd	[100]
CO	Scotland (2011)	nd	[100]
BE	Scotland (2011)	nd	[100]
UV filters (sunscreens)			
4-MBC	Switzerland (2001, 2003)	150–4,980	[20]
	Leipzig, Germany (2009)	73 ± 3[a]	[22]
	Catalonia, Spain (2010)	730–3,830	[23]
EHMC	Switzerland (2001, 2003)	10–390	[20]
	Leipzig, Germany (2009)	35 ± 5[a]	[22]
	Catalonia, Spain (2010)	nd-3,350	[23]
OC	Tarragona, Spain (2007)	700–1,842	[21]
	Switzerland (2001, 2003)	320–18,740	[20]
	Leipzig, Germany (2009)	585 ± 90[a]	[22]
	Catalonia, Spain (2010)	1,060–9,170	[23]
OT	Switzerland (2001, 2003)	700–27,700	[20]
BP3	Tarragona, Spain (2007)	10–20	[21]
	Leipzig, Germany (2009)	29 ± 3[a]	[22]
	Catalonia, Spain (2010)	nd-790	[23]
OD-PABA	Tarragona, Spain (2007)	132–170	[21]
	Leipzig, Germany (2009)	1.9 ± 0.3[a]	[22]
	Catalonia, Spain (2010)	nd	[23]

(continued)

Table 2 (continued)

Analyte	Location, country (year)	Concentration	Reference
EHS	Leipzig, Germany (2009)	280 ± 37[a]	[22]
HMS	Leipzig, Germany (2009)	331 ± 47[a]	[22]
BM-DBM	Leipzig, Germany (2009)	517 ± 78[a]	[22]
DBT	Leipzig, Germany (2009)	54 ± 12[a]	[22]
IAMC	Leipzig, Germany (2009)	20 ± 3[a]	[22]
EHT	Leipzig, Germany (2009)	928 ± 143[a]	[22]
2DHB	Tarragona, Spain (2007)	nd	[21]
4DHB	Catalonia, Spain (2010)	nd-620	[23]
BP1	Catalonia, Spain (2010)	nd	[23]
4HB	Catalonia, Spain (2010)	nd-150	[23]
Brominated flame retardants			
PBDEs	Spain (2005)	197–1,185	[139]
	Germany (2002–2003)	142–2,491	[140]
	Palo Alto, USA	nd-1,183	[141]
	North Carolina, USA (2002,2005)	nd-58,800	[73]
	Switzerland (2003 and 2005)	138–617	[142]
	Baden-Württemberg, Germany	45–461	[147]
Deca-BDE-209, DBDPE	12 different countries (1998–2006)	3.4–19,000, nd-220	[74]
	Stockholm, Sweden (2006–2007)	650–1,100, 66–95	[74]
	Spain (2002)	32–541, 0.2–15	[76]
HBCD	Sweden (2000)	nd-33	[144]
	The Netherlands, UK, Ireland (2002)	nd-9,120 39–597	[114] [143]
	Baden-Württemberg, Germany		
TBBPA	Montreal, Canada (2003)	300	[145]
	Switzerland (2003 and 2005)	nd-600	[142]
Perfluorinated compounds			
PFHxS, PFOS, PFOA, PFDA, PFUdA	New York State (2005)	PFOA = 18–241 PFDA = nd-91 PFUdA = nd-115 PFHxS = nd-18 PFOS = nd-65	[148]
PFOS, FOSA, PFOA, PFDA, PFUnA, PFDoA	Georgia (2005)	PFOS = 8.2–990 PFOA = 8.3–219 PFDA = 12–201 FOSA = nd-117 PFUnDA = 5.9–37 PFDoDA = 7.2–48	[137]
PFOA, PFNA, PFDA, PFUnA, PFHxS, PFOS, FOSA	Denmark	PFOA = 0.7–19.7 PFNA = 0.4–8.0 PFDA = 1.2–32 PFUnA = 0.5–4.4 PFHxS = 0.4–10.7 PFOS = 4.8–74.1 FOSA = 0.5–3.6	[86]
PFOA, PFOS	(2006)	PFOS = 236–5,383 PFOA = 454–4,780	[51]
	Ontario, Canada (2002)		[81]

(continued)

Table 2 (continued)

Analyte	Location, country (year)	Concentration	Reference
PFOS, (6:2, 8:2, 10:2) diPAP		PFOS = 100 PFCAs = 1.6–0.17 6:2 diPAP = 55–590 8:2 diPAP = 12–860 10:2 diPAP = 28–220	
PFOA, PFOS	Singapore (2006–2007)	PFOA = 6.5–54.4 PFOS = 13.1–617.7	[136]
PFOA, PFDA	Korea (2008)	PFOA = nq-5.3 PFDA = 2.2–11.8	[137]
PFBS, PFHxS, PFHpS, PFOS, PFDS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, FOSA	Hong Kong (2008)	PFBA = 3.1–111.4 PFPeA = 0.5–10.1 PFHxA = 0.3–27.8 PFHpA = 0.4–4 PFOA = 1.3–15.7 PFNA = 0.5–23 PFDA = 0.3–15.2 PFUnA = 0.4–7.8 PFDoA = 0.6–8.6 PFTrA = 0.2–19	[84]
PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFHxDA, PFODA, PFBS, PFHxS, PFOS, PFDS, FOSA	Catalonia, Spain (2010)	PFBA = nd-22.6 PFPeA = nq-17.2 PFHxA = nd-4.8 PFHpA = nq-4.5 PFOA = 7.0–30.3 PFNA = 1.0–2.4 PFDA = 6.1–23.5 PFUnA = nq-12.2 PFDoA = 2.7–11.3 PFTeA = nq-5.0 PFHxDA = nq-4.9 PFODA = nd-0.9 PFBS = nd-7.6 PFOS = 53–121.1 PFDS = nd-7.5 FOSA = nd-10.7	[91]

For abbreviations of compounds, see text
nd below limit of detection, *nq* below limit of quantification
[a]Average ± standard deviation

samples analyzed (sewage sludge from aeration tanks and processed sludge from 12 different STPs from Austria) at concentrations up to around 300 ng/g dw and presumed also the presence of ecstasy (MDMA). Meanwhile, Jones-Lepp and Stevens [99] found MA (4 ng/g dw) in Class A biosolids (biosolids that can be used as soil amendment without restriction) from an STP in Los Angeles, CA, but did not detect it in commercial biosolids Milorganite® from Milwaukee, WI.

4.4 UV Filters (Sunscreens)

To date literature on UV filters levels in sewage sludge is very scarce, and only results relative to some compounds are available. To the author's knowledge, there are only four studies [20–23] on the occurrence of UV filters in sewage sludge.

From the various compounds investigated, the most lipophilic 4MBC, OC, OT, and EHMC were the UV filters most frequently found (in most cases with 100% of detection). The highest concentrations observed by far corresponded to OT, with a maximum value of 27,700 ng/g dw [20]. This sunscreen agent is not susceptible to bio- and photo-degradation, and due to its high lipophilicity (log $K_{OW} = 8.1$) adsorbs onto sludge. As expected, significantly higher concentrations were observed in samples collected in summer and at WWTPs treating urban wastes from big cities. For instance in summer, OC showed twice the concentration that had in winter [21].

Degradation products and metabolites considered in the studies were also found in several samples. Gago-Ferrero et al. [23] reported for the first time the occurrence in sewage sludge of two major degradation products of BP-3 known to have estrogenic activity: 4,4′-dihydroxybenzophenone (4DHB) and 4-hydroxybenzophenone (4HB). In this study, results revealed the presence of UV filters in 15 WWTPs in Catalonia (Spain) at concentrations ranging from 40 to 9,170 μg/g dw. In a similar survey carried out by Plagellat et al. [20] in 14 WWTPs from Switzerland, 4-MBC, EHMC, OC and OT were detected in all the sludge samples analyzed showing mean concentrations in the range 110–5,510 ng/g dw. In comparison, lower concentrations were reported by Rodil et al. [22] for 4-MBC, EHMC, BP3 and OD-PABA (1.9–585 ng/g dw) in solely one sludge sample from a WWTP in Leipzig. Also in this sample residue of isoamyl methoxycinnamate (IAMC), butyl methoxydibenzoylmethane (BM-DBM), ethylhexyl salicylate (EHS), homosalate (HMS), diethylhexyl butamido triazone (DBT), and ethylhexyl triazone (EHT) were detected for the first time at concentrations from 20 to 928 ng/g dw.

The concentration levels observed in sewage sludge show that UV filters originate mainly from private households, but besides this, surface runoff and industrial discharges may be considered as additional sources. This indicates that in addition to sunscreens, cosmetics and other personal products, UV filters from plastics, textiles, and other materials can be released to the environment by either volatilization or leaching.

4.5 Brominated Flame Retardants

Although some compounds, such as PBDEs, have been well studied and their levels have been reported for different matrices (including sewage sludge), there are not enough data information about HBCDs and TBBPA, and in the case of the emerging compounds (HBB, PBEB and DBDPE) only a few studies have been published.

The first European results from 1988 reveal levels of about 20–30 ng/g dw of PBDEs in sewage sludges collected in Sweden [65], being deca-BDE-209 the

compound found at the highest concentrations in most sludges. A survey conducted in three WWTPs in Sweden showed levels of PBDEs of about 200 ng/g dw, along with minor contribution of other BFRs (e.g., TBBPA and HBCD) [71]. Öberg et al. [72] studied the distribution and levels of BFRs in 116 sewage sludge samples from 22 municipal WWTPs in Sweden between October 1999 and September 2000. Their results showed that there were considerable variations in the concentrations of BFRs in sewage sludge, and that there was a statistically significant variation among municipal WWTPs, implying influence from local sources. Two examples of industries that can give specific contributions are the electronics (TBBPA) and textile industries (BDE-209), and the highest concentrations of each pollutant were indeed found in samples with such connections. Other studies have also been carried out in different European countries. Christensen et al. [131] reported on the PBDE concentration in sewage sludge samples from Bjergmarken, Roskilde, Denmark, with values around 500 ng/g dw. Another work, with samples from The Netherlands [132], showed total PBDE levels up to 230 ng/g dw. HBCD and TBBPA were also found in sewage sludges from Ireland, The Netherlands, and UK [66]. HBCD levels ranged from not detected to 9,120 ng/g dw, whereas TBBPA concentrations ranged from not detected to 600 ng/g dw. Few studies have been conducted in USA [133, 134]. Hale et al. [133] reported levels from four regions of the USA, with values in the range 1,000–2,290 ng/g dw for tri- to hexa-BDEs, and 85–4,890 ng/g dw for deca-BDE-209, with mean values of about 1,600 ng/g dw and 1,000 ng/g dw, respectively. Concentrations of BDE-47, -99, -100, and -209 exceeded those of the major PCB congeners and other halogenated contaminants present. In general, substantially higher levels have been reported in sludge in USA than in European sludge.

Works including a wide range of different BFRs were recently published. In the first, sewage sludge from 32 WWTPs in south Germany was analyzed [45]. HBB was detected in all sludge with concentrations in the range 4.5–2,468 ng/g dw. Moreover, all 32 samples contained PBDEs in the range 45–462 ng/g dw. Finally, TBBPA was detected in all samples with an average concentration of 16 ng/g dw and a maximum concentration of 62 ng/g dw.

In some countries, such as The Netherlands, most of the sewage sludge is burned in specific sludge incinerators. In other countries, sewage sludge may be deposited in landfills. That procedure may delay input of BFRs into the environment through contaminated leachate water. The higher BFR concentrations found in the USA samples are of environmental concern, because disposal of sewage sludge by application to agricultural and other land is widely practised in the USA.

4.6 Perfluorinated Compounds

During recent years, several works have been published reporting the concentrations of PFCs found in sludge. In most of these works, PFOS continues to be the more frequently found compound, partly because is a very stable

compound and partially because is the end product of the degradation of other PFCs currently in use. In general, the concentrations of PFCs found in these studies were in the range of ng/g dw, sometimes reaching to concentrations in the range of μg/g dw [135]. For example, Yu et al. studied the presence and mass flows of PFOS and PFOA in two different WWTPs from Singapore in 2006–2007 receiving different amounts of industrial and urban wastewater [136]. In all the samples, the presence of PFOS and PFOA was confirmed and the concentrations ranging from 13.1 to 702 ng/g dw for PFOS. The sample presenting the highest values of PFCs pollution was from the anaerobic digester. In this case, the presence of PFCs was directly related to industrial use. Another example was reported by Ma et al. [84]. The authors studied the presence of PFCs in three WWTPs from Hong Kong in 2008. Sludge was presented as the major sink of PFCs (0.2–7,305 ng/g dw). In another study, Guo et al. evaluated the fate of PFCs in WWTPs (municipal, livestock, and industrial) from Korea in 2008 [137]. The results concluded that PFCs in sludge from a livestock WWTP were below the LOD, whereas industrial wastes presented high concentrations of PFCs, as it was expected. Navarro et al. [89] studied the presence of PFCs in 20 domestic WWTPs in Spain. In this case, the influence of inhabitants was studied but no correlation was found between the concentration of PFCs and the number of inhabitants attributed to the WWTPs. This result supports the previous reported works.

5 Conclusions and Perspectives

Over recent years, different methodologies for the analysis of emerging compounds have been developed. However, for some of these pollutants, very few studies are referred to the analysis of sewage sludge.

Sample preparation procedure plays a fundamental role in developing analytical methodology for complex matrixes, such as sludge. Techniques, such as PLE, that provide fast, safe and easy preparation procedures, and use smaller amounts of samples and solvents receive significant attention. In general, and regarding the instrumental determination, developed methodologies are based on the use of MS, coupled to either GC or LC. LC–MS offers an improvement over GC–MS, since the derivatization step is avoided for the more polar compounds. The required sensitivity needs the use of MS–MS. Different configurations have been applied, being the most common the use of QqQ. However, in recent years other MS configurations have also appeared in diverse works, such as QqLIT or Q-TOF. In general, LODs reached with LC–MS–MS methods have been slightly higher than those obtained with GC–MS, but LC–MS has shown advantages in terms of versatility and less complicated sample preparation.

The application of developed methods to the analysis of emerging contaminants in sludge samples showed the presence of all the contaminants in concentration levels in the range of ng/g dw. These findings indicate that thorough control and monitoring of organic contaminants, including the emerging contaminants, is

needed in order to ensure safe management of sewage sludge, of which it is estimated 45% is employed in agriculture, 18% is accumulated in open dumps, and 17% is incinerated.

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Fate of Emerging Contaminants During Aerobic and Anaerobic Sludge Treatment

Guillermina Hernandez-Raquet

Abstract The detection of a wide range of biologically active contaminants in sewage has raised the question as to whether these pollutants are disseminated in the environment via wastewater effluents. Numerous studies have shown that in wastewater treatment plants (WWTP) pollutants are removed from liquid effluent by biological degradation or by sorption into biosolids. Thus, in a general assessment of the environmental fate of emerging pollutants, adsorption must be taken into account in their elimination from the water. However, despite abundant information existing on the removal rates and residual pollutant concentration in liquid effluent, little information exists on the occurrence and fate of emerging pollutants concentrated in the solid fraction of wastewater (sewage sludge or biosolids).

The aim of this review is to present a comprehensive evaluation of the presence of three classes of emerging contaminants in sludge: alkylphenols, hormones, and pharmaceuticals. In particular, the fate of these compounds during sewage and sludge treatment by aerobic and anaerobic processes is addressed.

Keywords Activated sludge, Anaerobic treatment, Hormones, Nonylphenol, Pharmaceuticals

Contents

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Abbreviations

APE	Alkylphenolic polyethoxylated
BOD	Biological oxygen demand
CAPEC	Alkylphenoxy-ethoxycarboxylate
COD	Chemical oxygen demand
E1	Estrone
E2	Estradiol
E3	Estriol
EE2	Ethinylestradiol
ER	Estrogen receptor
HRT	Hydraulic retention time
K_d	Partition coefficient
LAS	Linear alkylbenzene sulfonates
LOD	Limit of detection
MeEE2	Mestranol
NP	Nonylphenol
NP1EC	Nonylphenoxy-acetic acid
NP1EO	Nonylphenol monoethoxylate
NP2EC	Nonylphenoxyethoxy-acetic acid
NP2EO	Nonylphenol diethoxylate
NP*n*EO	Nonylphenol polyethoxylates
OP	Octylphenol
PEC	Predicted environmental concentration
Pg	Progesterone
SRT	Solid retention time
T	Testosterone
TKN	Total Kjeldahl nitrogen
WWTP	Wastewater treatment plant

1 Introduction

There exists a growing concern about the release into the environment of a wide range of organic compounds known as emerging contaminants. Emerging contaminants include chemical compounds such as detergents, pharmaceuticals as well as natural and synthetic hormones. These compounds are characterized in terms of their biological activity. Contaminants such as natural and synthetic hormones, octylphenol and nonylphenol derivating from alkylphenolic detergents, as well as plasticizers are known as endocrine disruptors. They are able to interact with the endocrine system and alter functions such as reproduction, growth, and development in wildlife populations and humans and so have been associated with the reduction of fertility, changes in sex ratio and imposex incidence observed in wild fish populations [1]. In addition, endocrine-disrupting contaminants are suspected in

the increase in the incidence of breast cancer and the decrease in the quality and quantity of sperm in humans. Emerging contaminants such as pharmaceuticals have been designed to stimulate a specific response in animals and humans. However, after their use and excretion, they may affect nontargeted organisms; in this way, pharmaceuticals may induce negative responses in wildlife. For instance, antibiotics are known to induce resistance to antibiotics in bacteria, while other compounds seem to increase the toxicity of complex mixtures of pollutants [2]. Though, the impact of pharmaceuticals such as anti-inflammatory drugs, antibiotics, and mutagenic compounds used in the treatment of cancer is not completely understood. They are suspected of inducing abnormal physiological processes in living organisms [3]. Hence, the continuous presence of emerging contaminants in the environment is an actual risk for wildlife and humans.

Depending on their origin and use, emerging contaminants enter the environment via very diverse routes. Chemical compounds of industrial use, such as detergents, enter the environment via regulated or unregulated discharge to surface water or from WWTP. Pharmaceuticals and natural and synthetic hormones reach the environment mainly via industrial or urban WWTP. Veterinary medicines and animal hormones are released through direct excretion in the field or by manure spreading on land, and these contaminants can reach aquifers and groundwater via runoff and leaching from manure-dressed soils. Regarding the diversity of emerging contaminants and the wide range of routes by which they enter the natural environment, over the last 15 years great effort has been made in developing analytical methods to assess their occurrence in diverse environmental matrices. Thus, several studies have reported trace levels of emerging pollutants present in sewage, wastewater effluents, surface, ground and drinking water, soils, sediments, and sludge [4–6].

Currently, abundant literature stated on the level of emerging compound contamination in divers aqueous matrices such as industrial and agricultural effluents, raw sewage and WWTP effluents from urban origin, surface, ground and drinking water [7–9]. In contrast, little information exists on the occurrence and fate of emerging contaminants in solid matrices, even though these pollutants may display hydrophobic properties. Thus, it is expected that some fraction of them will be adsorbed to sewage sludge, manure, or sediments. In most of the WWTP around the world, sewage is treated biologically by activated sludge combining aerobic and anoxic processes. During such treatment, emerging contaminants are partially eliminated from the liquid effluent via biological degradation or by sorption into sewage sludge. Indeed, as much as 80% of hormones (ethynylestradiol, estrone), 50% of nonylphenol and 30% of pharmaceuticals (e.g. triclosan) may be adsorbed into sludge [10–12]. Thus, several studies have suggested that sorption of emerging pollutants by solid organic matter is the main mechanism involved in eliminating pollutants from the water phase [10, 11].

Sewage sludge is an abundant by-product of wastewater treatment processes. For 2006, a production of more than 16 million tons was estimated for the USA and the European Union [13, 14]. Due to its high content in organic matter and nutrients, sewage sludge is used as a fertilizer or conditioner for soils. Indeed, sludge spreading on land is considered as one of the more economic and environmentally

sustainable approaches for sludge management and nutrient recycling. Sludge spreading is widely practiced: about 30% and 50%, respectively, of the sludge produced annually in Canada and USA are applied to land [15]. Fifty percent to seventy percent of the sludge produced is spread on land [5, 16]. In addition, agricultural sludge resulting from manure treatment is also applied to land.

Prior spreading, sludge is generally stabilized using anaerobic digestion (about 70% of sewage sludge is anaerobically digested), mesophilic or thermophilic aerobic treatment or composting [17]. Despite the importance of sludge spreading practices, however, little information exists on the presence of emerging contaminants in sludge substrates or on their fate during sludge treatment. Similarly, little information exists on the microorganisms involved in the biological attenuation of such contaminants.

This chapter has three main objectives: firstly, to summarize the behavior of emerging contaminants during sewage treatment because it determines their transfer to biosolids; secondly, to provide a comprehensive overview of the current knowledge regarding the occurrence of these pollutants in sludge. Finally, the potential routes for the elimination of emerging contaminants in both aerobic and anaerobic conditions are discussed.

2 Alkylphenolic Detergents

Due to their cleaning and surfactant properties, alkylphenolic polyethoxylated (APE) detergents are used in the formulation of industrial and household products. They are used in the paper industry, metal production, and in oil extraction. APE are also used in making paints, detergents, pesticides, plastics, and resins [18]. Indeed, with the anionic detergents LAS (linear alkylbenzene sulfonates), nonionic alkylphenol ethoxylated detergents represent most of the worldwide production of detergents. LAS do not present estrogenic activity and are almost fully eliminated (93–97%) by WWTP [19]. In contrast, APE and their derivates nonylphenol (NP) and octylphenol (OP) are estrogenic and persist during the biological treatment of sewage. With an annual consumption worldwide of nonylphenol polyethoxylate (NP*n*EO, where *n* indicates the number of ethoxy units) in excess of 600,000 tones, it is by far the most commercially important APE, representing 90% of total production [20]. This means that the main detergent derivate will be NP [21].

Polyethoxylated NPEO are weakly estrogenic and easily degraded by microbial activity [22]. In contrast, its short-chain metabolites nonylphenol-monoethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), and NP are estrogenic and more recalcitrant to biodegradation. The estrogenic activity of NP was discovered by chance by the Soto's team in the early 1990s on account of its ability to induce the proliferation of breast cancer cells MCF7 [23]. Thereafter, the ability of NP to compete with natural hormones for binding to the nuclear estrogen receptor was elucidated [24]. NP is tenfold more estrogenic that its parent polyethoxylated NPEO and is the most recalcitrant derivate of APE surfactants. In addition, NP is

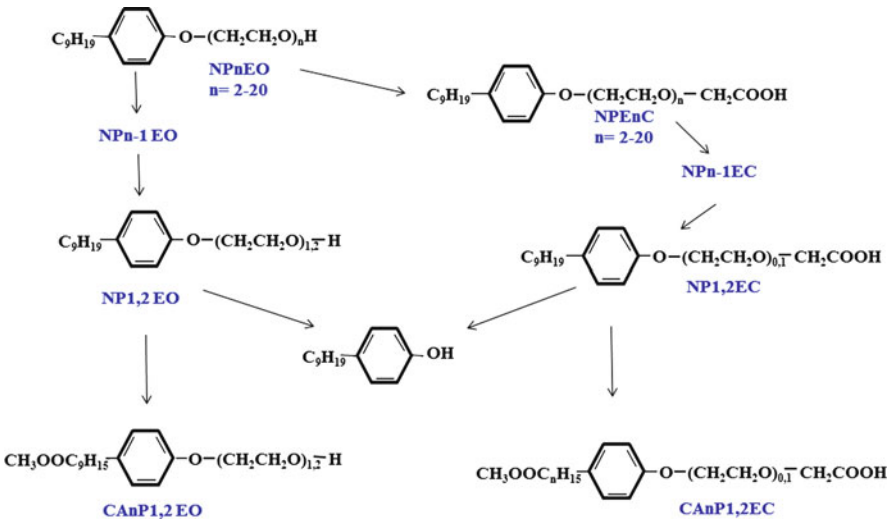


Fig. 1 Degradation pathway of polyethoxylated nonylphenols

reported as inhibiting testicular growth and inducing vitellogenin production in male fish (a protein associated with egg production), even at low microgram-per-liter levels [25, 26]. The estrogenic effect of NP and its accumulation in several organisms both vertebrate and invertebrate has also been demonstrated [27–30]. Due to its estrogenic properties, NP has been included in the list of priority pollutants in the European Union Water Framework Directive [31]. Moreover, in 2003, the European Parliament endorsed a directive for restricting the use of nonylphenol and nonylphenol ethoxylates in the formulation of cosmetics, personal care products, and some industrial applications, authorizing their use only in processes where these substances are destroyed or fully recycled [32].

The estrogenic properties of NP have aroused a lot of interest in determining the degradation pathway of NPEO in different environmental conditions. Comprehensive studies in NPEO degradation have been conducted in full-scale wastewater treatment plants which are the main receptacle of such pollutants [33]. A general scheme of NPEO degradation pathways is presented in Fig. 1. During sewage treatment, the cleavage of the terminal ether bond of long-chain NPEO leads to the formation of shorter ethoxylated compounds, mainly NP2EO and NP1EO, which are further transformed into NP [22, 34, 35]. These metabolites are more resistant and estrogenic than the parent compound. In aerobic conditions, NPEO are also degraded by ω-carboxylation of the terminal alcohol forming ethoxycarboxylated nonylphenols such as nonylphenoxy-acetic acid (NP1EC) and nonylphenoxyethoxy-acetic acid (NP2EC). Such carboxylates can also be transformed into NP. Carboxy-alkylphenoxy-ethoxycarboxylates (CAPEC) are also produced via simultaneous oxidation of the alkyl and ethoxyl groups [36].

The degradation of NPEO and its metabolites has been confirmed in laboratory experiments using simplified conditions. Aerobic respirometric tests have demonstrated the easy mineralization (transformation to CO_2) of long-chain NPEO, after 35 incubation days [37]. Similar results have also been reported for NP degradation in mesophilic aerobic reactors, using activated sludge as the inoculum [38]. However, in such aerobic respirometric tests, more than 50% of the initial amount of NP was still not degraded after 35 days period, suggesting that NP was adsorbed into the biomass, limiting its further degradation [37]. The degradation of NP in anoxic environments has also been reported in experiments performed under sulfate-reducing [39, 40] and nitrate-reducing [41] conditions but not under methanogenic conditions. Under nitrate-reducing conditions, NP degradation by adapted cultures seems to start at the linear nonyl chain but no degradation was observed when NP presented a branched alkyl chain [41]. Nevertheless, under anoxic conditions, the degradation rates are slower than those measured in aerobic conditions.

The sorption of NP into the solids has important consequences for its fate during wastewater treatment. During sewage treatment, NPEO degradation is accompanied by the production of metabolites that differ in their physicochemical properties, particularly in respect to its hydrophobicity. Therefore, the distribution of NPEO and its metabolites within the sewage matrix evolves through the wastewater treatment process. Long-chain NPEO detergents are hydrophilic compounds that remain in the liquid phase. However, as the NPEO ethoxylated chain is shortened, losing its hydrophilic moieties, they became less hydrophilic. Consequently, the compounds NP1EO, NP2EO, and NP, displaying a high octanol/water partition coefficient (log K_{ow} between 4.17 and 4.48 [42]), will be sorbed into the lipophilic sludge fraction. Thus, based on mass balance calculations carried out in diverse sewage treatment plants, it has been estimated that up to 60% of the total amount of NPEO entering WWTP can be sorbed into the solids [10, 43]. Similarly, the levels of these compounds increased in sediments exposed to WWTP effluent [44, 45].

Worldwide surveys on the presence of alkylphenolic detergents in sewage sludge have shown that most of sludge samples displayed high levels of NP, NP1EO, and NP2EO (Table 1). NP1,2EO, and NP concentrations ranged from the limit of detection (LOD) to 3,150 mg kg_{dw}^{-1}, a level much higher than that proposed by the European Union regulations (50 and 400 mg kg_{dw}^{-1}; EU 2000, 2003). Sludge stabilized by aerobic or anaerobic processes showed NP concentrations ranging from, respectively, 0.1 to 800 mg kg_{dw}^{-1} and 0.6 to 2,540 mg kg_{dw}^{-1} (see references in Table 1). NP concentrations in sludge vary regionally, depending on the use of NPE surfactants: low NPEO concentrations were recorded in Denmark (0.3–67 mg kg_{dw}^{-1}) and Australia (< 0.4 mg kg_{dw}^{-1}), whereas high NPEO levels, ranging from 14.3 to 3,150 mg kg_{dw}^{-1} were reported in Spain [46]. High NP concentrations were also reported in California and New York, with mean values of 754 and 1,500 mg kg_{dw}^{-1}, respectively [47, 48]. NPEO concentrations in sludge from different WWTP vary as a function of the sewage treatment process used. In general, lower levels of NP1,2EO, and NP are detected in WWTP using aerobic treatment based on activated sludge; in such systems, raw, dewatered, and thermally

Table 1 Concentrations of nonylphenol and nonylphenol mono- and di-ethoxylates in sewage sludge

Type of sludge	NP + NP1,2EO (mg kg_{dw}^{-1}) min–max	Mean value (mg kg_{dw}^{-1})	Number of samples	Type of wastewater treatment	Country	Reference
Aerobic stabilization	80–500	250	8	nm	Switzerland	[35]
Aerobic stabilization	200–1,270	798	5	nm	Switzerland	[10]
Aerobic digested sludge	0.02–0.4	0.11	1	Activated sludge + nitrification/ denitrification	Australia	[9]
Aerobic/extended aerobic treatment	4.9–10	7.4	2	Activated sludge	Australia	[158]
Anaerobic digestion	450–2,530	1,010	30	nm	Switzerland	[35]
Anaerobic digestion	900–2,540	1,410	24	nm	Switzerland	[10]
Anaerobic digestion	707–1,030	830	5	Activated sludge	USA	[47]
Anaerobic digestion	348	348	1	Activated sludge	Italy	[52]
Anaerobic digestion	942–1,901	1,496	5	Activated sludge	USA	[48]
Anaerobic digestion	103–856	327	18	Activated sludge/chlorination	Canada	[53]
Anaerobic digestion	1,600	1,600	1	Activated sludge	USA	[148]
Anaerobic digestion	1.7–513	111	5	Activated sludge	Australia	[158]
Anaerobic digestion	109–122	114	1	Thermal hydrolysis	Australia	[158]
Anaerobic digestion	0.6–91.1	44	3	nm	Australia	[158]
Raw sludge	50–2,668	620	69	nm	Spain	[46]
Dewatered sludge	LOD-212	22	18	Activated sludge or aerated lagoon	Canada	[53]
Dewatered sludge	22–650	136	36	Primary + chemical and activated sludge	Norway	[159]
Dewatered sludge	2.5–3.7	nm	3	Activated sludge	Germany	[50]
Dewatered sludge	342	342	1	Activated sludge	Italy	[52]
Dewatered sludge	72–808	242	16	Activated sludge/chlorination	Canada	[53]
Dewatered sludge	193	193	1	Activated sludge	USA	[61]
Dewatered sludge	510	510	1	Activated sludge	USA	[148]

(continued)

Table 1 (continued)

Type of sludge	NP + NP1,2EO (mg kg_{dw}^{-1}) min–max	Mean value (mg kg_{dw}^{-1})	Number of samples	Type of wastewater treatment	Country	Reference
Dewatered sludge	8.9	8.9	1	Activated sludge	Greece	[160]
Thermal treated	130–492	303	2	Activated sludge/chlorination	Canada	[53]
Thermal treated	511–587	544	1	Activated sludge	USA	[47]
Thermal dried	14–3,150	458	41	nm	Spain	[46]
Dried sludge	297–394	346	2	Activated sludge	USA	[148]
Composted sludge	LOD-193	65	3	Activated sludge	USA	[47]
Composted sludge	18–363	140	30	nm	Spain	[46]
Composted sludge	9–505	201	4	Activated sludge	USA	[148]
Chemically stabilized (lime)	498–953	730	2	Activated sludge	USA	[47]
Chemically stabilized (lime)	7.6–43	23	2	nm	Australia	[158]
nm	LOD-1,193	128	149	nm	Germany	[161] (1987–1989)
nm	0.3–67	15	20	nm	Denmark	[162]
nm	LOD-360	27	100	nm	Germany	[49] (1994–1995)

LOD below the limit of detection, *nm* not mentioned

treated sludge displayed NP1,2EO and NP concentrations of about 265 mg kg_{dw}^{-1}. When sludge is composted, the corresponding average level is about 135 mg kg_{dw}^{-1}; this value reflects both the sludge dilution with composting material as well as NPEO degradation during the composting process. In anaerobically digested sludge, high levels of short-chain NP1,2EO and NP are generally observed; it results from the high NP sorption into sludge, the reduction of solid mass due to digestion, with the consequent increasing concentration of these compounds in the residual solid, and NP production during anaerobic digestion (from long-chain NPEO) accompanied by a limited degradation of this compound under anaerobic conditions. Nevertheless, in the recent years, the concentrations of NP1,2EO and NP in sludge tended to decrease as a consequence of government pressure exerted on industries to restrict the use of alkylphenolic detergent in their processes [49, 50].

Due to the high levels of short-chain NPEO and NP found in anaerobically digested sludge and the interest of this process for sludge stabilization (up to 70% of sludge is anaerobically digested) and energy production (methane), the study of the fate of NPEO during sludge digestion has received a lot of attention. Several studies concluded that NP1EO, NP2EO, and NP are recalcitrant to biodegradation under methanogenic conditions; thus, these compounds may accumulate during the anaerobic digestion of sludge [35, 51–53]. This was clearly demonstrated in controlled batch experiments carried out with ^{14}C-labeled NP, NP1EO and NP2EO and using inocula from different sources: an anaerobic digester, sludge landfill, and municipal waste landfill. These experiments showed that, whatever the origin of the inoculum, NP1,2EO transformation led to the accumulation of NP which was no further degraded [51]. Moreover, high NP concentrations (20 mg g_{dw}^{-1}) may inhibit anaerobic gas production [51, 54]. In contrast, mass balance calculations done for untreated and digested sludge in batch experiments showed that, even if the final NP concentration increased after anaerobic treatment, a fraction of NPEO was not transformed into NP or other metabolites [52]. This suggests that NPEO was partially eliminated by anaerobic digestion. Similarly, in continuous reactors feed with mixed sludge (50:50, primary:secondary sludge) naturally contaminated with NPEO, a net removal of 25% for such compounds was measured [55]. However, in other experiments using continuous reactors fed with secondary sludge spiked with NP, and taking into account the concentrations of NP and solids measured in the feed and treated sludge, the mass balance analysis showed that NP was completely recalcitrant to biological degradation under methanogenic conditions [56]. In these last experiments, the levels of NP and estrogenic activity measured by the MELN in vitro test were compared, showing that neither NP nor estrogenic activity was eliminated during sludge digestion. The contrasting results obtained in such experiments led to more recent research to compare NP removal from different types of sludge: primary, secondary, mixed sludge or thermally treated sludge. In these experiments, carried out in continuous anaerobic reactors under mesophilic conditions, between 20% and 60% removal of NP was observed [57]. Nevertheless, in these experiments, as well as in those preceding, the resulting digested sludge contained higher levels of NP per gram of dw than the sludge prior to anaerobic treatment. Overall, the results obtained in

continuous reactors suggest that NP degradation is conditioned by several parameters such as the length of treatment, the physicochemical properties of the sludge and the composition and metabolic activity of the microbial community.

The treatment of sludge contaminated with NP1,2EO and NP by mesophilic and thermophilic aerobic processes has also been studied. Experiments carried out in batch cultures (60°C) and in continuous reactors (55°C) showed that, respectively, only 66% and 40% of the initial NP and NP1,2EO amounts were removed [58, 59]. In contrast, experiments done in continuous reactors under mesophilic temperatures (35°C) showed that these compounds were completely removed [56]. These results are in accordance with previous observations showing that NPEO are more easily transformed in aerobic environments [38].

The fate of NPEO has also been studied during sludge composting under mesophilic (35°C) or thermophilic conditions (50°C, 55°C, and 65°C). In laboratory experiments using a mixture of sludge with straw and plant residues or municipal solid waste, a similar decrease of NP, NP1,2EO levels was observed at 35°C, 50°C, and 55°C. However, at 65°C and above, a big accumulation of NP was observed [60]. In another study, NP degradation was monitored during composting (55°C) of both naturally contaminated sludge and NP-spiked sludge. Under such conditions, about 95% of the initial concentrations were eliminated after 15 and 45 days of incubation for, respectively, unspiked and spiked experiments [61]. This suggests that aerobic processes such as composting and extended sludge aeration ensure efficient removal of NP1,2EO and NP. Otherwise, these data suggest that at temperatures higher than 55°C, the microorganism involved in NP metabolism was less or not active [60].

Recently, others methods for sludge treatment have been developed, combining aerobic and anaerobic biological sludge treatment or physicochemical treatment (ozonation, thermal, mechanical, or chemical treatment) with biological aerobic or anaerobic processes. In experiments using anaerobic digestion followed by aerobic treatment to eliminate NP1,2EO and NP present in sludge, it was shown that NP1EO and NP2EO were completely eliminated whereas a residual, but low, NP level was still apparent after aerobic treatment [56]. In contrast, in the control experiment treating sludge by aerobic mesophilic processes only, the NP and total NPE were completely eliminated. Other authors have applied thermophilic aerobic treatment prior mesophilic anaerobic sludge digestion. In such experiments, a removal of about 45% of NP and NP1,2EO was observed; values that were equivalent to the efficiency of the aerobic thermophilic process alone [59]. The main objectives of the anaerobic treatment of sludge are: firstly, to reduce sludge volume and the level of its fermentable organic matter; secondly, to produce energy (methane). Thus, combining anaerobic digestion with the aerobic treatment of sludge seems to be advantageous because it permits both a high methane production level with efficient NP removal. This configuration may also reduce the energy required for aeration of anaerobically digested sludge that contains a lower amount of solids than raw sludge. Experiments combining ozonation with the anaerobic digestion of sludge showed a significant increase (about 50%) of NP removal [59]. Such treatment also reduces to the same extent the solid content of sludge.

Consequently, the treated sludge presented similar levels of NP and NP1,2EO per gram of dry weight as sludge before treatment. Nevertheless, before validating the use of such advanced processes, the assessment of their economic and environmental cost is still needed [62].

Little information exists on the microflora involved in NP degradation during sludge treatment. Indeed, most of the information on NP-degrading microorganisms has been obtained in simplified laboratory experiments performed in liquid media and under aerobic conditions. In such conditions, different species belonging to *Candida* and *Sphingomonas* have been identified for their capacity to degrade linear or branched NP as sole carbon source [38, 63, 64]. Under sulfate-reducing conditions, using soil bacteria as inoculum for a simplified aqueous medium, five isolates were obtained; the most efficient, related to *Bacillus niacin,* was able to degrade 98% NP (50 mg kg_{dw}^{-1}) after 77 days of incubation [40]. Recently, *Pseudomonas* and *Alcaligenes* species have also been found in denitrifying enrichments [41]. Nevertheless, further research is still needed to identify the microorganisms involved in NP degradation in complex sludge systems, particularly in anaerobic environments.

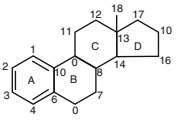
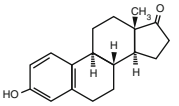
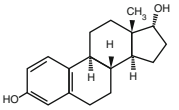
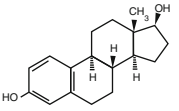
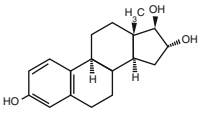
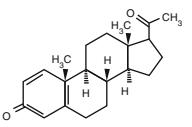
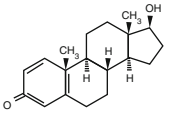
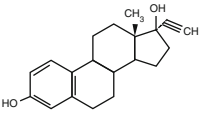
3 Hormones

Hormones (estrone E1, estradiol E2, estriol E3, testosterone T, progesterone Pg) are chemical messengers naturally produced by the endocrine system of humans and animals. They are involved in cell communication throughout the organism. Hormones participate in the control of important physiological functions such as growth, development, and reproduction. Hormones are C18 steroids, made up of by 18 carbon atoms distributed in three hexagonal (A–C) and one pentagonal (D) rings (Table 2). The different substitutions in the cyclopentane–phenanthrene nucleus differentiate these molecules. In synthetic estrogens, the addition of ethinyl and methyl groups to estradiol forms ethinylestradiol (EE2) and mestranol (MeEE2), compounds used to control reproduction (contraceptive pills) and to regulate hormonal disorders in humans. Estrogens dissolve poorly in water, with solubility of about 13 mg L^{-1} for the natural compounds, while EE2 and MeEE2 display solubility of, respectively, 4.8 and 0.3 mg L^{-1} (Table 2). Steroid hormones show moderate affinity with solids (Log K_{ow} 2.4–4 [65]) which means that a fraction of these chemicals will be sorbed to sludge, sediments and the particulate matter in sewage.

After exerting their action in the organism, natural and synthetic hormones are catabolized in the liver by conjugation to glucuronide and/or sulfate moieties, forming more polar conjugated forms which are excreted via urine. This is the main route of hormone excretion in humans and pigs. A fraction of hormones is also excreted in a free form via feces; in animals such as sheep and cattle this is the main route for hormone excretion (Table 3) [66, 67].

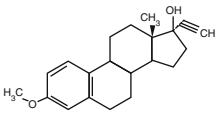
The natural estrogens E1–E3, as well as the synthetic estrogen EE2, have received considerable attention in the last decade as they are able to induce strong

Table 2 Hormone properties

Compound	Chemical structure	Molecular weight	Solubility (mg L^{-1})	Log K_{ow}[a]	Log K_{ow}[a]
Cyclopentane phenanthrene					
Estrone		E1	270.4	13.0	3.43
17-α-Estradiol		αE2	272.4		
17-β-Estradiol		βE2	272.4	13[b]	3.94
Estriol		E3	288.4	13[b]	2.81
Progesterone		Pg	314.4	7[c]	3.05
Testosterone		T	288.4	18–25	3.32
Ethinylestradiol		EE2	296.4	4.8	4.15

(continued)

Table 2 (continued)

Compound	Chemical structure	Molecular weight	Solubility (mg L^{-1})	Log K_{ow}[a]	Log K_{ow}[a]
Mestranol		MeEE2	310.4	0.3	4.67

[a]Log K_{ow} octanol/water partition coefficient
[b][65]
[c][163]

Table 3 Daily excretion of hormones by human and animals

Species	Category	Fecal excretion contribution (%)	Urine excretion (μg day^{-1})	Excreted hormones
Humans	Cycling female	5[a]	16–35	βE2 < E1 = E3
	Pregnant female		5,270–7,030	βE2 < E1 ≪ E3, P[b]
	Noncycling female		7–13	E3 < E1 = βE2
	Male		7–9	T[c], βE2 < E1 = E3
Cattle	Calves	58–67[a]	15	αE2, E1, βE2
	Cycling cows		99	αE2, E1, βE2
	Pregnant		2,176–104,320	αE2, E1, βE2, P
	Bulls		1,616	T, A[d], αE2, E1, βE2
Pigs	Cycling sows	4–12[a]	100	αE2 < βE2, E1, E3
	Pregnant		10,876	P, αE2 < βE2, E1, E3
	Boars		4,000	T, E1, αE2 βE2
Sheep	Cycling ewes	87–90[a]	3	βE2 < E1 ≪ αE2[a]
	Pregnant		52	
	Rams		3	
Chickens	Female	69[a]	2.5	E1, βE2, E3
	Male		2	E1, βE2, E3, T
	Laying hens		6	

[a]Information does not concern either the sex or the stage of development
[b]Includes progesterone derivates pregnanediones, pregnenolones and pregnanediols
[c]α- and β-testosterone
[d]Androstenedione [67, 79–83]

estrogenic responses at ng per liter levels. Indeed, in comparison with synthetic chemical displaying estrogenic activity (called xenoestrogens), and with the exception of pharmaceutical hormones, natural hormones display the highest affinity for binding to nuclear estrogen receptors (ERs) and present the greatest estrogenic potency. The relative estrogenic activity for different xenoestrogens, with E2 estrogenicity arbitrarily fixed at 1, display values of 2.46 for EE2, 1.76 for E3, 0.025 for E1, 0.015 for genistein, 0.0066 for bisphenol A, and 0.0032 for nonylphenol, as measured by the MELN in vitro test [68, 69]. In contrast, conjugated

hormones are inactive compounds showing very little or no estrogenic activity. However, after their excretion, conjugated hormones undergo deconjugation by the intestinal and fecal flora result in active free hormones [70]. Thus, conjugated hormones represent a reservoir of estrogenic activity.

The best documented negative effects of hormones observed in natural environments are those induced by estrogens. For instance, estrogen hormones are responsible for the induction of vitellogenine production in male fish; estrogens also alter the sexual development (imposex) of wild fish populations and inducing feminization [71–73]. Nevertheless, studies performed in controlled conditions have demonstrated that other phyla are also impacted by such exposure. For instance, the mollusk *Potamopyrgus antipodarum,* when exposed to EE2 at a 1-ng L^{-1}level, displayed an increase in embryo production while the larval development in the crustaceans *Balanus amphitrite* and *Elminius modestus* was altered by exposure to E2. DNA damage has also been reported in such species [74, 75]. Some evidence also exists for the impact of estrogen in plants: for example, a higher phytoestrogen content was measured in alfalfa irrigated with wastewater containing between 10 and 300 ng L^{-1} of E1 and E2 [76]. The consequence of human exposure to estrogens is difficult to assess due to the presence in the environmental of a wide range of contaminants. Even so, the increase in reproductive alterations as well as the increase in breast and testicular cancer observed in the last decades is likely to have resulted, at least partially, from exposure to estrogens [77, 78].

The main source of hormones is thus the excretions of the human population and livestock. The make-up and rate of hormone excretion by humans and animals varies as a function of their sex, age, state of reproduction and development, and for animals, their species (Table 3 [67, 79–83]). Human excretions are generally collected in sewers, while animal hormones are concentrated in manure; thus, high levels of free and conjugated estrogens have been measured in raw sewage and manure [67, 79, 84–88]. Human hormones will eventually enter the environment mainly via wastewater treatment plants while hormones produced by animals reach the environment via direct excretion in fields or by manure spreading (treated or not) on the land.

Due to the high endocrine-disrupting potency of estrogen hormones, their occurrence and fate in sewage treatment plants and in manure disposal/treatment units have been abundantly documented. Similarly, their degradation pathways, as well as the microflora involved in their degradation, have been widely studied in controlled experiments [89]. On this account, this last aspect will be not treated here, with preference to discussing the occurrence and fate of hormones in wastewater, sludge, and manure.

For human hormones, several studies carried out in urban WWTP in different countries around the world have shown wide variability in hormone concentrations in raw sewage. Free hormones levels ranged from < 0.5 to 670 ng L^{-1} for E1, < 0.5 to 125 ng L^{-1} for E2, 2 to 660 ng L^{-1} for E3, < 0.2 to 70 ng L^{-1} for EE2, while conjugated hormones varied from non-detected levels (nd) to 29 ng L^{-1} for E1, 1.5 to 8.5 ng $L^{-1}$for E2, 33 to 253 ng L^{-1} for E3, and nd to 11 ng L^{-1} for EE2 [89]. In some cases, conjugates may constitute up to 60% on the total amount of hormones

entering WWTP [90]. Nevertheless, despite the variability observed in hormone levels, their concentration, make-up and ratio of free:conjugated hormones measured in sewage is a function of the characteristics of human population (sex composition, age, and contraception practices), the size of sewers, and the deconjugation and degradation rates occurring in the sewers. Thus, the hormone levels in raw sewage can be accurately estimated by mathematical models based on such parameters [81].

As regards the hormones produced by animals, their concentration in manure is less well documented that the presence of human hormones in sewage. Animal manure is a mixture of urine, feces, residual feed, bedding, and cleaning water. Thus, very high variations in hormone levels in manure can be expected as a function of farm practices. For instance, in raw liquid effluents or slurry from pig farms, levels of 5,300, 665, 1,250 and 2,600 ng L^{-1} of E1, αE2, βE2 and E3, respectively, have been reported [87]. For dairy raw wastewater, the corresponding levels for E1, αE2, βE2, were, respectively, 873, 2,282, and 643 ng L^{-1} while E3 was not detected [88]. The conjugated hormones E1, αE2, and βE2 have also been detected in manure, mainly in sulfate forms, whereas glucuronide forms were never detected [91]. Farm wastewater is frequently stored in a series of lagoons; in such cases, the hormone levels vary greatly depending on the dilution and the lagoon (primary or secondary lagoons), and also from on farm to another. For example, in two different studies very contrasting hormone levels were found in primary lagoons: in the first, levels of 10,000, 2,500 and 2,500 ng L^{-1} of, respectively, E1, αE2, βE2 were detected (E3 was not measured [92]). In the second study, much lower levels were detected, with E1, αE2, βE2, and E3 levels of, respectively, 3,172, 360, 275, and 3,000 ng L^{-1}. Such differences certainly resulted from the distinct characteristics and manure handling practices of each farm (e.g. number of animals, dilution) as well as waste age. Very high and variable levels of hormones have also been reported for solid manure, ranging from <100 to 1,416 ng $g_{dw}^{-1}$of αE2, LOD-1,500 ng $g_{dw}^{-1}$of βE2 and 98–4,800 ng $g_{dw}^{-1}$of E1 [92–94].

The fate of hormones, irrespective of their origin, will be a function of the physicochemical properties of the hormones, the conditions prevailing in the sewage or manure disposal or treatment unit and the microbial activity. As hormones tend to be sorbed by the organic matter, the concentration of solids prevailing in these systems will greatly affect the fate of hormones.

The fate of hormones in urban WWTP has been abundantly documented. Several studies reported high removal rates for hormones from the liquid phase of sewage, particularly in those WWTP using activated sludge processes. For instance, removal rates of 40–96%, 40–100%, 40–96%, and 40–98% for, respectively, E1, E2, E3, and EE2 were reported in WWTP all around the world. Nevertheless, residual hormone concentrations of < 0.3–100 ng L^{-1} of E1, < 0.2–20 ng L^{-1} of E2, < 1–275 ng L^{-1} of E3, and < 0.3–7.5 ng L^{-1} of EE2 have been detected in treated effluents, which shows that hormones were not completely removed by the treatment processes [82, 84, 86, 90, 95–97].

Due to their lipophilic properties, hormones tend to be divided up between the liquid and solid phases, making sorption an important parameter for hormone elimination from the liquid phase of sewage. In laboratory studies based on an

empiric partition coefficient (K_d) and assuming no hormone degradation, it was estimated that up to 70%, 79%, and 76% of E1, E2, and EE2, respectively, would be sorbed into sludge during sewage treatment [98]. Thus, in actual WWTP using only enhanced precipitation (e.g. addition of $FeCl_3$), low removal rates of estrogens were recorded and up to 43% E2, 24% E1, and 100% EE2 were associated with the solid fraction of the treated effluent [99]. In contrast, in WWTP using activated-sludge processes, various studies based on mass balance calculation and taking into account hormone concentrations in solid and liquid phases of sewage concluded that the main mechanism for hormone elimination was the microbial degradation. It seems that the low concentration of solids (from 300–500 mg L^{-1} in raw sewage to 4–5 g L^{-1} in the biological reactor) and by the high level of colloids that interact with hormones decrease their sorption into the solids [99, 100]. In such systems, hormone sorption into sludge represents less than 10% of the total amount of hormones entering the system [85, 86, 90]. Other parameters of the treatment process, including hydraulic and solid retention times (HRT, SRT), redox potential, carbon load, temperature, concentration of solids, floc size, aromatic composition and salinity, have also been cited as determining the efficiency of hormone removal. Nevertheless, their specific influence on the overall performance of the process remains difficult to assess [86, 101–104].

As to hormones produced by animals, their fate during manure disposal varies greatly because manure is generally held for variable periods (from a few months to many years) in a wide range of structures (pits, ponds, lagoons or, for solid manure, in piles) without any specific treatment before being spread [105]. In dairy, pig and poultry farms holding manure in lagoons, a decrease in hormones levels was observed through successive lagoons [91, 93, 106]. Nevertheless, in such systems, it is difficult to differentiate hormone biodegradation from dilution or elimination by sorption and precipitation. In structures such as pits or ponds, where anaerobic conditions prevail, very high levels of hormones are frequently reported, suggesting that hormones are not easily removed from manure [92, 93]. The elimination of hormones from animal slurries by aerobic biological treatment has been studied in laboratory experiments as well as in actual farms. In such aerobic conditions, 90% elimination of hormones and higher has been reported [107, 108]. Nevertheless, in these studies the fraction of hormones sorbed into the solids was not considered. In a recent study done in our laboratory, measuring hormones in both the liquid and solid phases, we observed that hormones contained in swine manure were not or only removed slightly when manure was put in anaerobic tanks. In contrast, when manure was aerobically treated, high hormone removal rates were obtained [109, 110].

The occurrence and fate of hormones in sewage sludge have received much less attention when compared to hormones present in liquid effluents. For information concerning the presence of hormones in liquid effluents see chapter by Eljarrat et al., this volume [172].

The fate of hormones during sludge treatment has been also poorly documented. Two surveys based on full-scale anaerobic reactors treating sludge concluded that hormones were poorly eliminated or not at all in methanogenic conditions [85, 111]. These results are in accordance with laboratory experiments performed in

simplified anaerobic conditions (liquid media inoculated with sludge [112]). Such experiments showed that E2 was transformed into E1 which was further partially removed (17%), whereas EE2 was not degraded. Similarly, in experiments using methanogenic reactors with sludge spiked with hormones, no EE2 elimination was observed while E1 was reduced to E2 which was not further degraded [113]. Only one study reported hormone degradation in anaerobic sludge reactors. Indeed, Carballa et al. [114] reported 85% of removal for E1, E2, and EE2 contained in sludge treated by mesophilic digestion (37°C). Similar values were also reported for thermophilic reactors (55°C), except for EE2 (75%). Such high degradation rates seem to have been a function of a long adaptation period.

The fate of hormones present in manure or sludge produced during manure treatment and displaying a high solid content (> 20 g L^{-1}) can be compared to the fate of hormones observed in sewage sludge. In a recent study carried out in our laboratory, we assessed the fate of hormones in controlled continuous bioreactors fed with manure (50 g_{dw} L^{-1}) naturally contaminated with hormones (about 29 μg L^{-1}). Our results showed that under mesophilic anaerobic conditions (35°C) no hormone removal was observed. Similarly, in anaerobic thermophilic reactors (55°C) inoculated with two different inocula (from digesters treating sewage sludge or solid horse manure) no hormone degradation was observed. Similar observations were recorded with an anaerobic tank where sludge from manure treatment, containing high levels of hormones, was held: hormone levels stayed constant for a period of 4 months [109, 110]. These observations confirm the recalcitrance of hormones during anaerobic treatment as reported in previous studies [85, 111–113]. Only Carballa's study reported hormone removal in such conditions. However, in this last study, hormones were spiked into sludge which can strongly affect the sludge's adsorption and degradation capabilities [57].

To our knowledge, no study directly concerns the aerobic treatment of hormones contained in sludge, but the high rates of hormone removal observed during sewage or manure treatment by aerobic processes suggest that estrogens can be removed in aerobic conditions [84, 87, 109]. Indeed, experiments conducted in aerobic conditions in a trickling filter treating manure showed removal of estrogens varying from 44% to 99%, depending on the compound [87, 109]. Similarly, in experiments done in our laboratory under aerobic/anoxic mesophilic conditions (35°C), or coupling anaerobic digestion with aerobic/anoxic treatment to eliminate hormones from swine manure, we observed hormone elimination higher than 40% and 80%, for the respective processes. In these last conditions, despite hormone removal occurring only during the aerobic/anoxic phase, a positive effect of anaerobic digestion on further hormone removal was observed. It is possible that anaerobic digestion, by modifying the manure structure, facilitates hormone desorption, increasing the availability of hormones for aerobic microbial activity [109, 110]. During composting of manure, a standard aerobic treatment process, it was observed that hormone levels decreased by more than 80% after 3 months of incubation [93]. Overall, these data suggest that hormone removal involves mainly aerobic processes.

Despite no specific study existing on the fate of hormones during sludge treatment by the addition of lime, some data exist on the presence of hormones in

limed sludge (see chapter by Eljarrat et al., this volume [172]). Lime-stabilized sludge displays low hormone levels, suggesting hormone desorption from sludge. Indeed, in experiments using bisphenol A, which has a pK_a similar to that of estrogens (10.3), it was desorbed from sludge, probably by deprotonation at the high pH levels (>12) that can be reached during such sludge treatment [115].

The environmental impact of hormones seems to be mainly related to liquid effluents. However, in the light of the high levels of hormones present in solid matrices such as manure or sludge from WWTP with poor hormone removal rates (e.g. enhanced precipitation [116]) there, it is still a need to implement treatment processes to efficiently remove hormones from sludge and manure.

4 Pharmaceuticals

The term pharmaceutical includes more than 4,000 chemicals used to control and treat different kinds of diseases in humans and animals. Pharmaceuticals include analgesics, anti-inflammatories, anti-epileptics, β-blockers, compounds used to prevent and treat parasites and microbial infections (parasiticides and antibiotics) and those for combating cancer. Hormones are also a class of pharmaceuticals; but due to their particular involvement in endocrine disruption, they have been discussed in a separate section (see above).

The estimated consumption of pharmaceuticals per 1,000 people, based on the average medical and veterinary prescriptions in Australia and USA, is about 180 g day^{-1}. This is equivalent to a pharmaceutical consumption, for the USA alone, of about 20,000 tons per year [117]. A similar level of consumption can also be expected in other developed countries. A large amount of these pharmaceuticals enter the environment primarily following therapeutic use as a result of their excretion in urine and feces in their original form, or as their conjugates or metabolites [118]. Pharmaceuticals also enter the environment when expired or unwanted drugs are flushed down the toilets. So, as with hormones produced by humans and animals, pharmaceuticals mainly enter the environment after passing through wastewater treatment plants, by direct excretion of animals into the land and by manure-spreading practices [118–120]. In addition, in the particular case of fish farming, pharmaceuticals may also be directly introduced into the environment [121].

The potential number of pharmaceuticals that may contaminate the environment is very large. Consequently, it can be expected that the effects of exposure to these substances on living organisms will be very variable. The possible impact of pharmaceuticals will be a function of the specific action for which each compound was designed, plus the eventual impact on nontargeted functions or organisms. For instance, a rapid decline in the vulture population in India and Pakistan, observed in the early 2000s, was related to the birds consumption of dead cattle treated with diclofenac, a widely used anti-inflammatory. This agent may produce, as a side effect, kidney disorders in mammals but in vultures it caused unexpected renal failure [122]. Information exists on the negative effects of pharmaceuticals on

Daphnia magna, a zooplankton organism commonly used in ecotoxicological studies. The exposure of this organism in laboratory tests to clofibric acid ($\geq$10 μg L^{-1}), a cholesterol-regulating pharmaceutical, induced a higher production of male offspring [123]. Similar changes in sex ratios were observed when *D. magna* was exposed to triclosan, an antimicrobial agent. The chronic exposure of this organism to fluoxetine (36 μg L^{1}), an antidepressant, increased fertility while exposure to a mixture of fluoxetine and clofibric acid resulted in increased mortality and the incidence of deformities [123]. Similarly, experiments performed with *Thamnocephalus platyurus* exposed to 28 pharmaceuticals, including anti-inflammatory agents, biocides, β-blockers, and drugs acting on the nervous system, showed that 17 of these compounds were toxic or very toxic for this crustacean [124]. The effect of pharmaceuticals in plants has also been documented; the main risk seems to be its transfer to and accumulation in the plants. For instance, greenhouse experiments shown that chlortetracycline antibiotics are absorbed by corn, green onion, and cabbage growing in soil amended with manure containing antibiotics [125]. The effects of exposure to pharmaceuticals at the community level have been assessed in multispecies experimental systems. In such conditions, the exposure to diclofenac induced an increase or decrease of bacterial and cyanobacterial biomass, depending on the season and the level of contamination; the composition of bacterial and protozoan communities was also modified [126]. Similar changes in community composition have also been observed in plankton and microbial communities exposed to antibiotics [127–129]. A well-documented effect of pharmaceuticals in the environment is the increased incidence of bacteria resistant to antibiotics in environments exposed to such compounds [129–131]. This exposure may greatly affect human populations because resistance to antibiotics may be transferred to pathogens and the commensal bacteria of human, livestock, and crops. Another important issue is the endocrine-disrupting effect of some pharmaceuticals. Indeed, using the estrogen reporter test YES and E-Screen assay, it has been demonstrated that compounds including fibrates, furosemide, and tamoxifen were able to bind and interact with the estrogen receptor [132]. Nevertheless, although some evidence exists on the negative impact of pharmaceuticals on the environment, most of these ecotoxicological evaluations have been based on the toxicity observed at very high concentrations that far exceed the expected concentrations in the natural environment. Indeed, the predicted environmental concentrations (PEC) estimated mathematically, based on production volumes and potential dilution, are much lower than the levels actually used for toxicology assays. At PEC levels, the ecological risk of most of these pharmaceuticals is unknown. Otherwise, in the environment, organisms are chronically exposed to mixtures of pharmaceuticals instead of single compounds. The consequences of such exposure have been little documented. The ecotoxicological impact of pharmaceuticals has thus not yet been completely assessed despite their widespread presence in the environment.

The occurrence of pharmaceuticals in different areas of the environment, including sediments, surface, drinking, and groundwater has been exhaustively summarized by Heberer [120], Khetan and Collins [133], Monpelat et al. [134], and Fatta-Kassinos et al. [135]. Thus, in this paper we will focus our attention on the behavior of pharmaceuticals during wastewater and sludge treatment processes.

As mentioned above, wastewater treatment plants represent one of the main sinks for pharmaceuticals before their dispersal in the environment. The pharmaceuticals most frequently detected in raw sewage include three main classes of compound: antibiotics, β-blockers, and analgesic-anti-inflammatories (Fig. 2 [136]). These compounds are present in raw sewage at concentrations varying from 100 ng L^{-1} up to levels of around 10 μg L^{-1}. Numerous studies indicate that the degradation of pharmaceuticals in WWTP varies from no removal to their complete mineralization. The elimination of pharmaceuticals is a function of a molecule's properties, the treatment processes applied and the conditions prevailing during the process [118, 137–139]. Consequently, the concentrations reported in treated effluents from WWTP vary widely: from trace levels for pharmaceuticals that are well eliminated to concentrations similar to those found in raw sewage, when a pharmaceuticals is poorly or not removed (Fig. 3 [136]). Widely used compounds such as the analgesics ibuprofen and naproxen are readily biodegradable (> 80% [139, 140]). Similarly, despite the great consumption of paracetamol, this compound is rarely found in treated effluents due to its degradability [141]. Nevertheless, the degradation of a wide range of pharmaceuticals in WWTP is rarely complete. Widely consumed compounds such as diclofenac, the β-blockers nadolol and atenolol and the diuretic furosemide display removal rates of, respectively, 58%, 60%, 59%, and 50% but these rates may vary up to 50% between different WWTP, depending on the biological treatment [140]. The removal of the antibiotics ofloxacin, norfloxacin, ciprofloxacin, sulfamethoxazole, tetracycline, and enrofloxacin also varied from 18% to 100%, in different WWTP [137, 142] but compounds such as lincomycin and spiromycin were, in some cases, not removed [143]. Carbamazepine, a commonly prescribed antiepileptic, has been detected in similar concentrations in raw sewage and treated effluents, showing its resistance to biological degradation [86, 144, 145]. Because the physicochemical properties of pharmaceuticals vary widely in molecular weight, structure, and functionality, it is not surprising that their degradation is also very variable. In an attempt to determine the main factors governing the removal of pharmaceuticals, Santos et al. [145] assessed, by correlation and principal component analysis, the relationship between pharmaceutical properties, influent load, and the characteristics of treatment processes. The authors concluded that a correlation exists between the total Kjeldahl nitrogen (TKN) load entering the raw sewage, the removal of biological (BOD) and chemical oxygen demand (COD), the elimination of TKN and oil and the hydraulic retention time; other parameters, such as the total suspended solids and pharmaceutical concentration, also influence the removal rate.

Recently, with the development of more advanced and sensitive analytical methods, studies on the fate of pharmaceuticals in WWTP have taken into account the analysis of pharmaceuticals sorbed into sludge (Table 4, and references therein). The antiseptics triclocarban and triclosan and the antibiotic ofloxacin have been reported in sludge at concentrations up to 441, 133, and 58 mg kg_{dw}^{-1}, respectively. Compounds such as the anti-inflammatory ibuprofen, the antiepileptic carbamazepine and the antidepressant fluoxetine have also frequently been reported though at lower levels (11, 6 and 3 mg kg_{dw}^{-1}, respectively), whereas the β-blockers were found at low

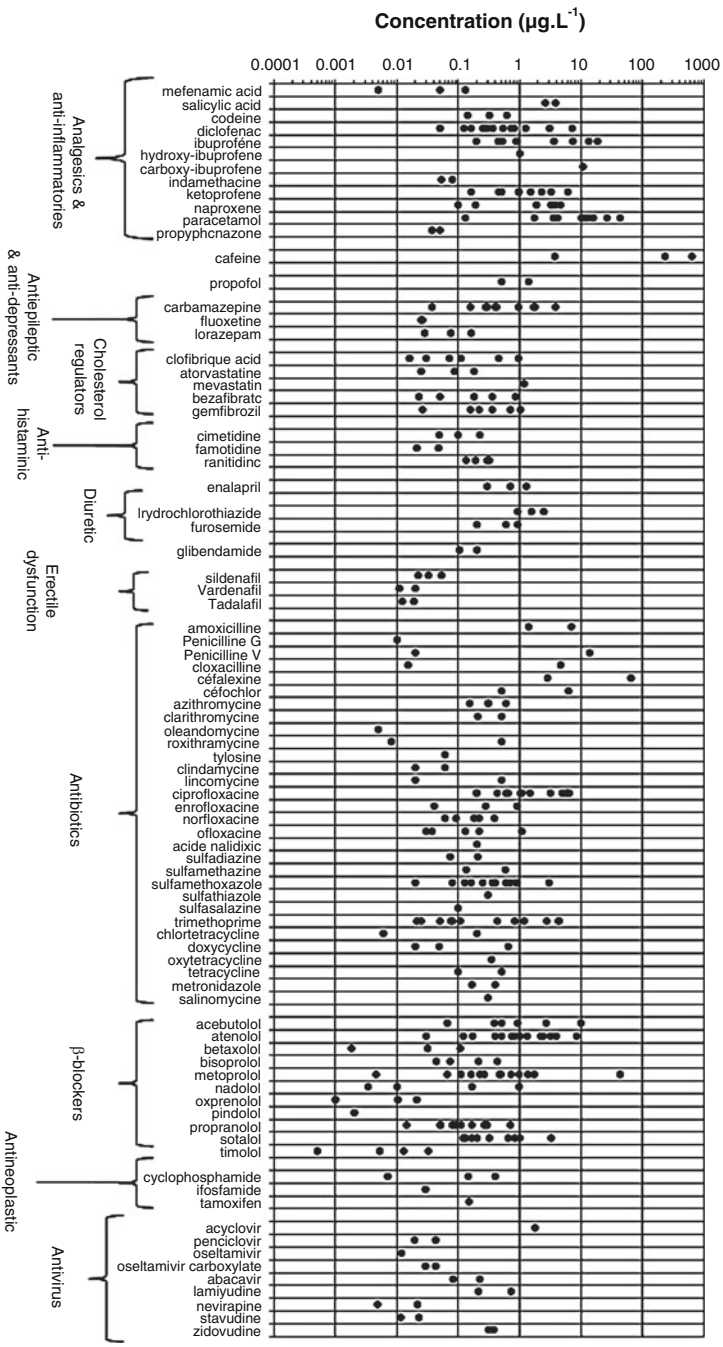


Fig. 2 Concentrations of main pharmaceuticals found in raw sewage [136]

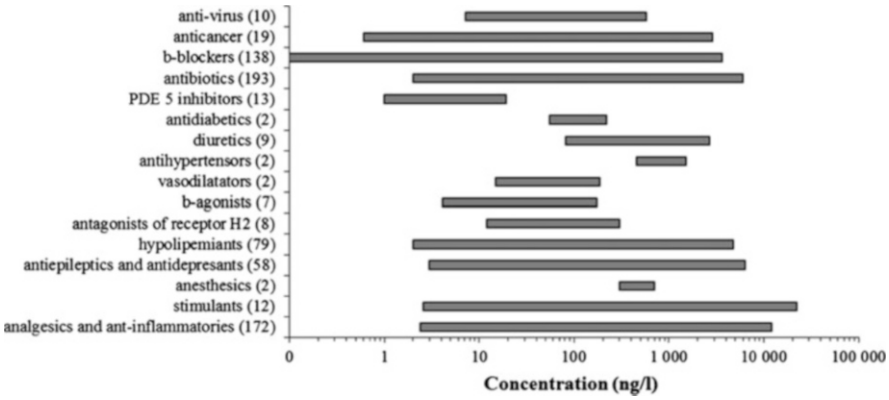


Fig. 3 Concentrations of main families of pharmaceuticals found in treated effluents from urban wastewater treatment plants [136]

levels in only a few samples (up to 85 μg kg_{dw}^{-1}). Pharmaceutical concentrations reported in studies carried out in different countries and with different types of sludge showed wide variations, which result from differences in the local consumption of pharmaceuticals and type of sewage treatment. Nevertheless, environmental monitoring done in WWTP in Spain and Chine showed no seasonal variation in the concentration of different pharmaceuticals detected in sludge, data which reflects the continuous consumption of such chemicals over the year [146, 147].

The presence of certain pharmaceuticals at high levels in sludge, such as triclosan and triclocarban, can be explained by their physicochemical properties. Indeed, these lipophilic compounds, displaying Log K_{ow} values of 3.5–4.2 and 4.5–4.8, respectively, are likely to be associated with sludge. However, some of the pharmaceuticals detected in sludge are not lipophilic (Table 4). For instance, high levels of hydrophilic compounds such as tylosin (up to 1–4 mg kg_{dw}^{-1}), tetracycline (0.038–5.2 mg kg_{dw}^{-1}), and ofloxacin (up to 58 mg kg_{dw}^{-1}) have been detected in different sludge samples. These data suggest that regardless of these pharmaceuticals properties, they are not completely removed by wastewater treatment and are sequestered in the sludge fraction. Pharmaceuticals need to be fairly soluble in water to be readily adsorbed orally by humans and animals and consequently are often ionic compounds with low Log K_{ow} coefficients (Table 4). Ionic compounds do not significantly bioconcentrate and would thus not be expected to significantly sorb to sewage sludge. However, pharmaceuticals containing polar functional groups (amine or carboxylic functional groups) may interact with the organic matter and/or minerals, leading to sorption regardless of their lipophilicity. The organic carbon content of sludge is frequently cited as an important parameter determining the sorption of pharmaceuticals. However, different studies have failed to find a correlation between levels of pharmaceuticals and the organic carbon content of sludge [148, 149]. It seems that the physicochemical properties of pharmaceuticals are not the only factor determining their sorption into sludge. Other parameters such as changes in the

Table 4 Concentrations of pharmaceuticals in sewage sludge

Class	Compound	Log K_{ow}[1]	Maximum concentration ($\mu g\ kg_{dw}^{-1}$)	Mean value (minimum value) ($\mu g\ kg_{dw}^{-1}$)	Reference
Analgesic/anti-inflammatory	Codeine		30	LOD	[164]
			328	(10)	[165]
	Diclofenac	4.51		LOD	[160]
				67	[139]
			87	43 (LOD)	[166]
			209 ± 149	144 (LOQ)	[150]
			74.9 ± 4	57	[167]
			23.09	16.7	[168]
	Ibuprofen	3.97		300	[139]
			359	246	[164]
			114	65 (24)	[166]
			117 ± 6	84	[167]
			520	160	[150]
			11,900	(99)	[165]
	Ketoprofen		21.1 ± 1.3	13.3 (LOD)	[167]
	Naproxen	3.18		54	[160]
			273	119	[164]
			5.9 ± 0.7	3.4	[167]
			1,020	(21)	[165]
Antihistaminic	Cetirizine			83	[139]
	Cimetidine		893	504	[164]
			9,780	(7)	[165]
			6.1 ± 0.5	3	[167]
	Diphenhydramine	3.27	1,740	1,166	[164]
			7,000	609	[148]
Anti-epileptic	Carbamazepine		28	11	[129]
			2.9	2.4	[147]
		3.27	238	163	[164]

(continued)

Table 4 (continued)

Class	Compound	Log K_{ow}[1]	Maximum concentration ($\mu g\ kg_{dw}^{-1}$)	Mean value (minimum value) ($\mu g\ kg_{dw}^{-1}$)	Reference
			42	29 (11)	[166]
			12.7 ± 1	11	[167]
			79 ± 6	56	[150]
			6,030	(9)	[165]
			34.5		[157]
			12.8	7.7	[168]
			390	46	[148]
β-Blocker	Atenolol	0.16–1.95		< 50	[139]
			85 ± 39	31 (LOQ)	[150]
			10.8 ± 1	8	[167]
β-Blocker	Metoprolol	1.88–2.28		310	[139]
			34	18	[147]
			21	16 (12)	[166]
	Propranolol	3.37–3.48	7.8	6.2	[147]
			26	15 (LOQ)	[166]
			44 ± 17	37	[150]
Antidepressant	Citalopram		960	620 (440)	[139]
	Fluoxetine	1.22	258	171	[164]
			3,130	(12)	[165]
			125 ± 51	97	[150]
			1,500	608	[148][e]
	Paroxetine	1.37	60 ± 31	50	[150]
Antidiabetic	Metformin		456	305	[164]
Antianginal	Diltiazem		109	45	[164]
			225	(1)	[165]
Antifungal	Miconazole		1,100	777	[164]
			9,210	(14)	[165]

	Thiabendazole		370	110	[164]
			239	(8)	[165]
Antiseptic	Triclocarban	3.5–4.2	5,088	3,467	[147]
			48,100	36,060	[164]
			441,000	(187)	[165]
			nm	51,000 ± 1,500	[154]
	Triclosan	4.53–4.8	16,790	5,580	[16]
			11,200	3,700	[158]
			1,187	883	[147]
			19,700	12,640	[164]
			133,000	(430)	[165]
			320		[157]
			55,000	30,000 (20,000)	[153]
			16,800	5,580 (90)	[156]
			10,500	2,709	[148]
Hypolipidemic	Gemfibrozil		159	152	[164]
			33.9 ± 5	27	[167]
			119 ± 71	57	[150]
			2,650	(12)	[165]
			3.7	2.9	[168]
			420	236	[148]
Stimulant	Caffeine		80	50	[169]
			643	248	[164]
			1,110	(65)	[165]
			5.2	4.8	[168]
			1,200	218	[148]
Antibiotic	Acetaminophen	0.46	389	160	[129]
			419	177 (13)	[166]
			103 ± 9	74	[167]
			145 ± 51	52	[150]
	Anhydrotetracycline	0.34	880	392	[164]

(continued)

Table 4 (continued)

Class	Compound	Log K_{ow}[1]	Maximum concentration ($\mu g\ kg_{dw}^{-1}$)	Mean value (minimum value) ($\mu g\ kg_{dw}^{-1}$)	Reference
			1,960	(94)	[165]
	Azithromycin	4.02	1,220	838	[164]
			128 ± 171	85	[150]
			6,530	(10)	[165]
			158	98	[170]
	Chlortetracycline		368	168	[129]
			43	23	[164]
			1,010	1,010	[165]
			12.7	13.7	[168]
	Ciprofloxacin	0.28	10,800	6,858	[164]
			47,500	(75)	[165]
			778		[157]
			46.3	34.5	[168]
	Clarithromycin	3.18	95	66	[164]
			47 ± 3	25 (LOQ)	[167]
			617	(9)	[165]
			5.2		[157]
			30.2	15.8	[168]
			63	32	[170]
Antibiotic	Doxycycline	−2.08	313	225	[129]
			1,780	966	[164]
			5,090	(51)	[165]
			296		[157]
	Erofloxacin	0.7	29	nd	[164]
			66	(12)	[165]
	4-Epianhydrotetracycline		399	261	[164]

	4-Epichlortetracycline		93	nd	[164]
			2,160	(126)	[165]
	4-Epitetracycline		3,040	2,376	[164]
			4,380	(47)	[165]
	Erythromycin	3.06	68	30	[129]
			183	81	[164]
			110 ± 53	51	[150]
			3.6		[157]
	Isochlortetracycline		36	nd	[164]
	Lomefloxacin		16	nd	[164]
	Minocycline		2,630	1,884	[164]
	Norfloxacin	−1.03	418	289	[164]
			1,290	(99)	[165]
	Ofloxacin	−0.39	8,140	5,446	[164]
			178 ± 275	57 (LOQ)	[150]
			58,100	(74)	[165]
	Oxytetracycline	−1.12	765	293	[129]
			114	87	[164]
	Ranitidine		30	21	[164]
	Roxithromycin		1,446	867 (337)	[166]
			1,800	1,550	[171]
			131	35	[170]
	Sulfamethoxazole		40	19	[129]
			3	nd	[164]
			178	70 (LOQ)	[166]
			28 ± 6	14	[150]
			651	(4)	[165]
			160	155	[148][e]
Antibiotic	Sulfanilamide		87	nd	[164]
			15,600	(191)	[165]

(continued)

Table 4 (continued)

Class	Compound	Log K_{ow}[1]	Maximum concentration ($\mu g\ kg_{dw}^{-1}$)	Mean value (minimum value) ($\mu g\ kg_{dw}^{-1}$)	Reference
	Sulfathiazole		63	36 (LOQ)	[166]
	Sulfapyridine		38	18 (LOQ)	[166]
			197	62	[170]
	Tetracycline	−1.3	282	282	[129]
			2,790	1,914	[164]
			5,270	(38)	[165]
			180		[157]
			15.7	(LOQ)	[168]
	Trimethoprim	0.91	60	26	[164]
			17	9 (LOQ)	[166]
			11.2 ± 1	7 (LOQ)	[167]
			43 ± 7	28	[150]
			204	(12)	[165]
			133	61	[170]
	Tylosin	1.63	1,958	1,516 (1,074)	[166]
			4,000	2,950	[171]

nm not mentioned, *nd* not detected, *LOD* below the limit of detection, *LOQ* below the limit of quantification

composition of the inlet or differences in the processes used in different WWTP, the conditions prevailing into the system (e.g. suspended solid concentration, water and sludge retention time, pharmaceutical load in the influent) and the structure and activity of biomass, can also influence the removal and repartition of pharmaceuticals between the liquid and solid phases of sewage [118, 150, 151].

Studies based on mass balance calculations have estimated that between 50% and 79% of triclosan is effectively degraded in WWTP, 2–6% is released in the treated effluent but 15–50% is linked to sewage sludge [152, 153]. Similarly, triclocarban is poorly removed from WWTP (21%), a small fraction is release via treated effluents (3%) but most of the amount entering to the WWTP is released in sludge (76% [154]). Nevertheless, in other studies concerning 13 pharmaceuticals (atenolol, cetirizine, citalopram, dextropropoxyphene, diclofenac, furosemide, ibuprofen, metoprolol, naproxen, oxazepam, sulfamethoxazole, tramadol, and trimethoprim) it was reported that for most of these compounds, sorption involved less than 1% of the amount entering in raw sewage; only cetirizine, citalopram and sulfamethoxazole were sorbed in higher proportions of 6.7%, 3.1%, and 3.1%, respectively [139]. Similar results were obtained for carbamazepine, for which sorption into sludge represented about 0.15% of the amount entering the WWT plant [144]. Thus, it seems that pharmaceuticals sorbed into sludge do not represent a big proportion of sink of pollution. However, due to the potential negative impact of pharmaceuticals on living organisms, it remains vital to determine the fate of these contaminants during sludge treatment.

Very few studies have dealt with the behavior of pharmaceuticals during sludge treatment. Studies based on the comparison of pharmaceutical concentrations in sludge coming from different full-scale sludge treatment processes suggest that most of these compounds are not easily removed from sludge; nevertheless this statement is compound- and condition dependent. A recent USA survey of sludge used for spreading on land showed that sludge frequently contained high levels of carbamazepine, diphenhydramine, and fluoxetine independently of the process used for sludge stabilization (drying, composting or anaerobic digestion) suggesting that they were not removed by such sludge treatment processes [148]. Nevertheless, compounds such as triclosan were detected at concentrations lower in aerobically treated sludge (220 $\mu g\ kg^{-1}$) than in digested sludge (mean of 5,580 $\mu g\ kg^{-1}$), suggesting that triclosan was more efficiently removed in aerobic conditions when compared to its poor removal observed in anaerobic conditions [155, 156].

In order to determine more precisely the fate of pharmaceuticals during sludge treatment, different experiments have been conducted in controlled conditions. In continuous anaerobic reactors treating sludge spiked with pharmaceuticals, Carballa et al. [114] observed removals higher than 80% for naproxen, sulfamethoxazole, and roxithromycin, while 40% and 23% of ibuprofen and iopromide, respectively, were eliminated at both mesophilic (37°C) and thermophilic (55°C) temperatures. For diclofenac and diazepam, elimination of about 60% was observed in mesophilic conditions while in thermophilic conditions, 38% and 73% of these two compounds, respectively, were eliminated. In these experiments, the sludge retention time (15 or 30 days) did not seem to influence pharmaceutical removal

[114]. In another study, it was observed that triclocarban was not transformed during anaerobic digestion of sludge in batch conditions after a period of 19 days of incubation [154]. Consequently, the digested sludge displayed a higher concentration of this compound than the sludge prior digestion (50 mg kg_{dw}^{-1} [154]).

During the aerobic thermophilic treatment of sludge, ibuprofen and galaxolide were removed, respectively, 95% and more than 75% of initial concentrations after 20 days of incubation. However, even after 45 days incubation no complete elimination was observed [61]. Observations in composted sludge, which is considered to be an aerobic treatment, showed that several pharmaceuticals, including carbamazepine, diphenhydramine, fluoxetine, and triclosan, were not significantly removed by composting at mesophilic or thermophilic temperatures [148].

After sludge treatment (aerobic or anaerobic), sludge is generally stored for a period before spreading on land. The fate of six antibiotics, one antibacterial and one antiepileptic during such a period, was assessed by Chenxi et al. [157] using batch conditions in an aerobic or anaerobic environment. In addition, the effect of sun irradiation was also tested. The authors reported no removal of carbamazepine, triclosan, and ciprofloxacin whatever the experimental conditions tested. Tetracycline and doxycycline were slightly removed (77 days) while the elimination of erythromycin and clarithromycin was relatively fast during the period studied. Clindamycin was eliminated rapidly in the first 2 days (>50%) but afterwards elimination was very slow. A significant positive effect of aeration was observed for tetracycline and erythromycin removal. In contrast, no effect of sun radiation was observed, indicating the low impact of photo-degradation on pharmaceuticals removal.

Overall, the elimination of pharmaceuticals during sludge treatment seems to be a function of the molecule as well as of the conditions prevailing into the treatment system. Data from full-scale and lab-scale monitoring suggest that aerobic conditions permit more efficient elimination of a wider spectrum of molecules. Nevertheless, further research is still needed to identify the main parameters governing the removal of pharmaceuticals.

5 Conclusions and Perspectives

There is a widespread concern that emerging contaminants are contaminating the environment. Diverse studies worldwide have demonstrated the presence of detergent derivates, natural and synthetic hormones and pharmaceuticals as well as their potential negative impact on living organisms. Abundant information exists on the effect of acute exposure to single molecules for few some living organisms. However, emerging contaminants are present in the environment as complex mixtures. To date, there has been little effort to determine the ecological risk of chronic exposure of living organisms to such mixtures.

A great effort has also been realized to identify the physicochemical parameters that favor the elimination of numerous molecules. Partitioning of pollutants between liquid and solid phases of wastes (urban or animal), biological aerobic

treatment, HRT and SRT seem to play a significant role on the removal of pollutants. Nevertheless, further research is still needed to identify the main parameters governing the removal of emerging contaminants, particularly for pharmaceutical molecules from solid matrices. Such research should also include the economic evaluation of using advanced technology for pollutant treatment.

Substantial progress has been made to identify the conditions that favor the biodegradation of various emerging pollutants; it is well known that wide range of molecules is removed by the aerobic metabolism of bacteria. Nevertheless, little knowledge exists about the elimination of hormones and most of pharmaceuticals in anoxic or anaerobic conditions. Similarly, the microbial communities or strains involved on the elimination of emerging pollutants, as well as the characterization of their metabolic pathways, have been poorly documented. Integrating such information in treatment technologies may help to define better strategies for the protection of the environment.

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Biodegradation of Emerging Organic Contaminants by Composting

Mathava Kumar

Abstract Sewage sludge is a rich source of organic matter and nutrients, which can be utilized for soil improvement and plant growth. The utilization of sewage sludge for plant growth and conversion of sludge into renewable resource is often restricted by the presence of hazardous organic matter (HOM) including di-(2-ethylhexyl) phthalate (DEHP) and antibiotics. HOM enters the sewerage system through various domestic and industrial sources; they are mostly hydrophobic in nature and recalcitrant to microorganisms. HOM has the tendency to accumulate in the sludge during biological wastewater treatment processes and, subsequently, raise the difficulty of further sludge treatment and/or disposal. The application of sewage sludge containing HOM can create risk to ecosystems. The stability and biodegradation of DEHP and antibiotics during composting are discussed in this chapter. Finally, the recent advances in the bioremoval of these HOM are briefly summarized.

Keywords Antibiotics, Biodegradation, Composting, Emerging contaminants, Sludge treatment

Contents

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1 Introduction

The drastic growth and development in both industrial and domestic sectors has increased the production and usage of artificial chemicals that subsequently increase the amount of these wastes drained into the domestic sewage system. Wastewater catchments receive organic pollutants from three main sources such as household disposal, fossil fuel spillages, and urban runoff. The properties and composition of wastewater are more complicated than past decades, consequently increasing the loading and difficulty of handling the sewage treatment systems. In recent years, hazardous and persistent organic pollutants have been identified very frequently in municipal wastewater treatment plants [1–4]. The lipophilic/hydrophobic substances are efficiently removed from wastewater and concentrated principally in sewage sludge during sedimentation of wastewater. The solids removed from wastewater, i.e., sludge, contain many organic and inorganic substances. Some of these organic pollutants accumulated in sewage sludge are harmful and recalcitrant to biodegradation and can cause risks to humans and animals when disposed without proper treatment. Several researchers have investigated the likely xeno-estrogenic effects of organic pollutants such as endocrine disrupting chemicals (EDCs) [5–7]. The exposure to EDC can produce irretrievable effect to reproductive systems of living beings. More than 60 organic and inorganic compounds are included in the priority EDC list suggested by the US and Japanese governments.

Sewage sludge is an unwanted and inevitable by-product of wastewater treatment process. The treatment and disposal of sludge may account for over 50% of both the capital investment and operational costs of a conventional activated sludge-based wastewater treatment system. One of the most important decisions in the design of a municipal wastewater treatment system is the selection of the final disposal of sewage sludge, as appropriate disposal or reuse of sewage sludge is beneficial to the management of wastewater treatment plants. Ocean disposal of sewage sludge has been banned by many European nations, and, on the other hand, incineration and sanitary landfill of sewage sludge are not preferred due to the production and transformation of secondary pollutants.

Di-(2-ethylhexyl) phthalate (DEHP) is the main HOMs, which is frequently detected in sewage and sludge samples. Owing to its high hydrophobicity and complex molecular structure, DEHP is difficult to break up using normal biological processes. Subsequently, the concentration of DEHP identified in the sludge cakes of municipal sewage treatment plants exceeds the restricted values by EU for land

application of sewage sludge (The European Directive 86/278 and 91/156 as Waste Basis Directive). Therefore, the concentration of DEHP in sewage sludge has to be reduced below the levels suggested by the USEPA and the EU before land application. To efficiently reduce the DEHP, the selection of a proper method for the degradation of DEHP and/or other organic pollutants was also an important task. Many conventional treatment techniques can be employed for the stabilization of sewage sludge. Among the techniques, composting is the cheapest and simplest method adopted for the recycling of organic wastes, i.e., sewage sludge. Considering the current status of environmental policies on sludge management as well as the main disposal routes of sewage sludge, composting is the most acceptable method because of its low environmental impact. Most of the organic wastes can be converted into reusable sources by composting. These organic wastes include municipal solid wastes (MSW), animal manure, household wastes, agricultural wastes, and sewage sludge. As a whole, the production and quantity of sewage sludge along with recalcitrant HOMs become serious issues in the management of sludge disposal in sewage treatment plants.

2 Hazardous Organic Matter in Municipal Sewage Sludge

Sewage sludge contains abundant nutrients and hydrocarbons that are highly valuable for recycling as soil fertilizers. On the other hand, sewage sludge also contains a portion of bio-resistant organic matter, which is harmful and may cause diseases to humans even at trace levels. The presence of HOMs reduces the appeal of converting sludge into renewable resources. The organic compounds generally observed in sewage sludge are polychlorinated terphenyls, naphthalenes, chloropesticides, halogenated hydrocarbon solvents, aromatic hydrocarbon solvents (BTEX), chlorobenzenes, polyaromatic hydrocarbons, phenols, chlorophenols, phthalates, petroleum hydrocarbons, surfactants (LAS and nonylphenol), organotin compounds, and 2,4-dichloroaniline [4]. However, there are no existing regulations for the land disposal of HOMs in sewage sludge in Taiwan. On the other hand, many developed countries have defined safety values for the disposal of sewage sludge in various environmental sources, i.e., inland, water, and sea disposal. In many developed countries, the land application of sewage sludge has been a popular disposal route in comparison with traditional disposal methods such as incineration or sanitary landfill. Therefore, stringent disposal standards have been enforced for the disposal of sewage sludge along with organic matter and heavy metal concentrations. The safety values suggested by the EU for the land application of organic compounds and dioxins in sewage sludge are shown in Table 1. Many researchers carried out different kinds of survey to report groups of hazardous organic pollutants in different types of sewage sludge samples. The types of hazardous organic pollutants identified in sewage sludge are represented in Table 2. It can be briefly concluded from Table 2

Table 1 Limit values of EU for the land application of organic compounds and dioxins[a]

Compounds	Limit values (mg/kg-dry matter)
AOX (sum of halogenated organic compounds)	500
LAS (linear alkylbenzene sulphonates)	2,600 (1,300)[b]
DEHP (di-(2-ethylhexyl) phthalate)	100 (50)[b]
NPE (nonylphenol and nonylphenol ethoxylates with one or two ethoxy groups)	50 (10)[b]
PAH (sum of various polycyclic aromatic hydrocarbons)	6 (3)[b]
PCB (sum of polychlorinated biphenyls)	0.8
	(ng TE/kg dry matter)
PCDD/F (polychlorinated dibenzodioxin/dibenzofuran)	100

[a]The Directive 86/278 (Waste Basis Directive) recommended by the EU
[b]Values in parentheses are the limit values valid from July 1st, 2000

that the composition of sewage sludge varies largely based on the nature of sewage treatment, i.e., combined industrial wastewater, municipal sewage treatment, or individual sewage treatment. The organic pollutants along with various HOMs determined in the sewage sludge include DEHP, one of the family of phthalate esters (PAEs), which is the major and the most detected organic pollutant in municipal sewage sludge [1]. Concentrations of DEHP in sewage sludge in previous studies were detected in the range of 105–153 mg/kg (dry weight basis). The concentrations of DEHP detected in the sewage sludge and soil of various countries are shown in Table 3.

3 Characteristics of PAEs

PAEs are widely used industrial chemicals [1, 16, 17]. They serve as important additives to impart flexibility in polyvinyl chloride (PVC) resins. PAEs are highly suitable as plasticizers due to their thermal stability, fluidity, and low volatility. Plasticizers are used in the preparation of building, home furnishing, transportation and clothing materials, and, to a limited extent, in food packaging and medical products. PAE is a mixture of branched isomers, i.e., isomeric di-*iso*-octyl phthalate (DIOP), or linear isomers, i.e., linear di-*n*-octyl phthalate (DnOP). The release of PAEs into the environment during manufacture, use, and disposal has been addressed by several researchers [10, 18–20]. Eighteen commercially important PAEs are used in the plastic manufacturing process. However, dimethyl phthalate (DMP), diethyl phthalate (DEP), dipropyl phthalate (DPP), di-*n*-butyl phthalate (DnBP), diisobutyl phthalate (DIBP), diallyl phthalate (DAP), dihexyl phthalate (DHP), DnOP, diisononyl phthalate (DINP), butylbenzyl phthalate (BBP), and DEHP are the most indispensable part of PAEs used in the PVC manufacturing process [16, 21–23]. PAE production in the world is around 2.7 million metric tons (t) per year, out of which, 200,000 t is produced in the USA and 350,000 t is produced in both Japan and Germany.

Table 2 Hazardous organic pollutants identified in sewage sludge in different studies

References	Organic pollutants	Concentrations	Sample types
Fürhacker and Haberl [8]	Benzene, toluene, xylene, and volatile chlorinated hydrocarbons, PCB and pesticides	Trace level close to the detected limits for each volatile compounds	Sewage sludge
Rogers [3]	Organochlorine pesticides and PCBs, chlorophenols and chlorophenoxy acids, organophosphorus compounds, nitrosamines and nitroaromatics, mineral oils, alkylphenols, lipids, acrylamide monomer, *phthalate esters*, surfactants and related residues, chlorobenzenes, PCDD and PCDF, PAH, and pharmaceutical chemicals	0.03–0.5 μg/g for PCBs and organochlorine pesticides, 0.1–2,400 μg/l for chlorinated phenols, 0.3–53 μg/l for nitrosamines, 620 mg/l for mineral oils, 0.45–2.5 g/kg for alkylphenols, 12–1,250 mg/kg for phthalate esters, 0.01–40.2 mg/kg for chlorobenzenes, 80–7,700 pg/g for PCDD and PCDF	Sewage sludge, agrochemical sewage
O'Connor [9]	*Phthalate esters* (*DEHP*), PAHs, PCBs, chlorinated pesticides and hydrocarbons, acid-extractable pentachlorophenol (PCP) and dinitrophenol (DNP), volatile aromatics (benzene, trichloroethylene), alkyl amines (nitrosamines)	>100 mg/kg for phthalate esters, 1–10 mg/kg for PAHs, 0.5–1 mg/kg for PCBs, <1 for chlorinated pesticides and hydrocarbons, 1–5 mg/kg for PCP, <1 mg/kg for DNP, 1–10 mg/kg for volatile aromatics, <1 mg/kg for nitrosamines	Sewage sludge and sludge-amended soils
Eljarrat et al. [10]	PCDD, PCDF, PCB congeners (28, 52, 101, 138, 153, and 180)	1.9E02–5.3E02 ng/g for PCBs, 1.1E02–7.4E02 pg/g for total PCDD and PCDF	Sewage sludge
Schnaak et al. [4]	Polychlorinated terphenyls, naphthalenes, chloropesticides, halogenated hydrocarbon solvents, aromatic hydrocarbon solvents (BTEX), chlorobenzenes, polyaromatic hydrocarbons (EPA 610), phenols, chlorophenols, *phthalates*, petroleum hydrocarbons, LAS and nonylphenol (NP), organotin compounds and 2,4-dichloroaniline	1 μg/kg to 10 mg/kg for chlorine-contained compounds; 10 μg/kg to 1 g/kg for solvent and phenols; 1 mg/kg to 10 g/kg for EPA610, DEHP, LAS, and mineral oils	Sewage sludge

(continued)

Table 2 (continued)

References	Organic pollutants	Concentrations	Sample types
Wilson et al. [11]	Volatile organic compounds (VOCs), PCBs, chlorophenols (CPs), PCDDs	13,000 μg/kg for VOCs, 270 μg/kg for PCBs, 410 μg/kg for CPs, and 8,000 ng/kg for PCDDs	Sewage sludge and sludge-amended soils
Mangas et al. [12]	Petroleum aliphatic hydrocarbons (AHCs), linear alkylbenzenes (LABs), PCB, and PAH	180–550 μg/g for AHCs, 10–25 μg/g for LABs, 5–20 ng/g for PCB, and 100–200 ng/g for PAH	Sewage sludge and sludge-amended soils
Litz [2]	PCDDs, PCDFs, AOX, PCBs, PAHs, LAS, nonylphenol, tributyltin oxides, *DEHP*	Risk assessment were conducted in this study, concentrations were not addressed	Sewage sludge
Fromme et al. [13]	Phthalates (BBP, DBP, *DEHP*), bisphenol A (BPA), bisphenol F (BPF)	0.004–1.363 mg/kg for BPA, 27.9–154 mg/kg for DEHP, others were detected in low amounts	Sewage sludge
Fauser et al. [5]	Phthalates (*DEHP*, DPP, DBP, BBP, DnOP, DnNP), nonylphenols (NP and NPDE), LAS	13.1–44.3 μg/l for total inlet DEHP, 2.75–9.65 μg/l for NP, 37.1–216.8 μg/l for NPDE, 2.47–4.53 mg/l for LAS	Wastewater and sludge
Petersen et al. [14]	*DEHP*, NP/NPE, LAS, PAH	27–55 mg/kg for DEHP, 12.5–60 mg/kg for NP, 110–2,870 mg/kg for LAS, 3.38–9.15 mg/kg for PAH	Sewage sludge (collected from anaerobic digested tank and aeration tank)
Aparicio et al. [15]	DEHP, NP, nonylphenolethoxylates with one or two ethyl groups (NPEs), PCBs	122.09–1,651.85 mg/kg for DEHP, 26.32–1,432.61 mg/kg for NP, 30.93–1,700 mg/kg for NPEs, 0.01–0.192 mg/kg for PCBs	Sewage sludge

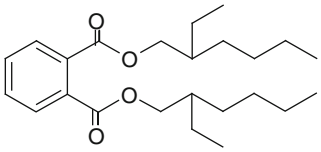
DEHP is the most widely employed industrial chemical among the 18 PAEs. DEHP makes up more than 90% of PAE-containing plastic refuses [18]. DEHP is used to improve the fluidity of the PVC matrix, but the weak physical bonding between the DEHP and the PVC matrix promotes the leachability of DEHP into the environment. This leaching process makes DEHP a ubiquitous pollutant [24]. Several phthalates have been detected in water systems, sediments, soil, air, and living organisms. In Western Europe, approximately 250 t of DEHP per year is leaching from landfills to groundwater.

Table 3 DEHP concentration detected in sewage sludge and soil of various countries

Authors	Sample types	DEHP concentration (mg/kg dry weight)	Countries
Staples et al. [16]	MSSP[a]	136–578	USA
Schnaak et al. [4]	MSSP[a]	170	Germany
Marttinen et al. [17]	MSSP[a]	58–80	Finland
Sablayrolles et al. [25]	MSSP[a]	159–173	France
Bagó et al. [26]	MSSP[a]	149–512	Spain
Jasen and Jepsen [27]	MSSP[a]	2–68	Denmark
Irvine et al. [28]	SoilP[b]	10–25,000	USA

[a]Sludge cake from municipal sewage treatment plant
[b]Contaminated soil collected near a PVC manufacturing plant

Table 4 The physicochemical properties of DEHP (*source*: Staples et al. [11])

Compound	Di-(2-ethylhexyl) phthalate (DEHP)
Formula	$CB_{24B}HB_{38B}OB_{4B}$
Structure	
Molecular weight	390.56
Specific gravity	0.986 (20/20°C)
Melting point	–47°C
Boiling point	384°C
Flash point	199°C
Vapor pressure	1.0E−7 mmHg
(log KB_{owB}[a])	7.54 (average value)
Aqueous solubility[b]	0.6–400 μg/l at 20°C
Henry's constant	1.71E−5 atm m^3/mol
Half-life	2,000 years (aqueous hydrolysis) 0.2–2.0 days (atmospheric photo-oxidation)
Toxicity	It is carcinogenic, hepatotoxic, nephrotoxic, and mutagenic to mammals Chronic toxicity may be predominant for DEHP; no obvious acute toxicity Bioconcentration factor (BCF) is high, and the persistence is medium

[a]log KB_{owB}: octanol/water partitioning coefficient (relative hydrophobicity index). log $KB_{owB} > 3$ reveals that compounds favor to adsorb onto the surface of particulate matter defined by the Danish Environmental Protection Agency
[b]Values were estimated and predicted under standard conditions derived from Staples et al. [11] and Brown et al. [29]

3.1 Di-(2-ethylhexyl) Phthalate

DEHP has a branch-like structure with two side alkyl chains, each chain comprised of eight carbons. This molecular structure reflects DEHP's poor water solubility compared to PAEs containing shorter alkyl chain lengths. DEHP can readily accumulate in lipids of aqueous biota when present in water due to its high

hydrophobic property [26]. The consumption of DEHP in drinking water can cause serious health concerns because of the testicularatrophy and hepatocellular carcinomas that have been proven in rats and mice [24, 30, 31]. In addition, there has also been an increasing focus on likely xeno-estrogenic effects of DEHP and its metabolites [16, 32, 33]. Due to these reasons, DEHP has been categorized as EDCs. The detailed physicochemical characteristics of DEHP are given in Table 4.

3.2 Environmental Fate and Transformations of DEHP

The fate and biodegradation/bioaccumulation of hydrophobic/lipophilic compounds such as DEHP in different environments are mainly based on water solubility. The loss of DEHP from wastewater treatment facilities, landfills, and sludge-amended soil is partially a function of aqueous solubility. Rogers [3] investigated the fate and the behavior of 15 environmental organic pollutants in sewage treatment plants, and reported that the major mechanisms controlling the fate of DEHP in the environment are sorption (onto solid surface/association with fats and oils), chemical degradation (abiotic processes, e.g., hydrolysis), biodegradation, and volatilization. Sorption and volatilization of an organic pollutant can be predicted by log $K\mathrm{B}_{\mathrm{owB}}$ and Henry's law constant (Hc), respectively, and their values mainly depend on the type and molecular structure of an organic pollutant. Generally, an organic compound with log $K\mathrm{B}_{\mathrm{owB}} < 2.5$ does not preferably sorb on a solid matrix in aqueous systems. On the other hand, organic compounds with log $K\mathrm{B}_{\mathrm{owB}}$ between 2.5 and 4.0, or >4.0, have medium and high potential for sorption, respectively. The volatilization loss of an organic pollutant in the environment can be estimated using Hc and Hc/Kow. An organic compound with $\mathrm{Hc} > 1 \times 10\mathrm{P}^{-4\mathrm{P}}$ and $\mathrm{Hc}/KB_{\mathrm{owB}} > 1 \times 10\mathrm{P}^{-9\mathrm{P}}$ has high volatilization potential whereas the compound with $\mathrm{Hc} < 1 \times 10\mathrm{P}^{-4\mathrm{P}}$ and $\mathrm{Hc}/KB_{\mathrm{owB}} < 1 \times 10\mathrm{P}^{-9\mathrm{P}}$ has low volatilization potential.

The interactions between Hc, log $K\mathrm{B}_{\mathrm{owB}}$, and vapor pressure are used to explain the tendency of DEHP accumulation and/or sorption in various environments. Therefore, the partitioning behavior of PAEs with colloidal and particulate organic carbon is an important factor in the measurement of DEHP in real world samples. Turner and Rawling [34] found that the partitioning behavior of DEHP between aqueous and solid phases was a function of particle concentration and particulate organic carbon. The presence of dissolved organic carbon (DOC) plays an important role as a carrier to enhance the interactions between hydrophobic organics and DOC, thus increasing the solubility of DEHP when a considerable amount of DOC is present in water [35]. It can be further emphasized that water solubility is a key factor affecting the extent of biodegradation of organic matter in the aqueous phase. Ejlertsson et al. [10] emphasized that water solubility and the degradability of side chains that esterify phthalic acid are some of the influencing factors in the degradation of PAEs.

4 Biodegradation of PAEs

The transformation of PAEs in the environment can be attributed to abiotic and biotic hydrolysis processes. Several intermediates or end products are produced during abiotic or biotic degradation of PAEs. Hydrolysis of PAEs produces acids and alcohols [36, 37]. However, biotic degradation is responsible for rapid depletion of PAEs in the environment [38, 39]. PAEs can be biologically degraded by a two-step hydroxylation process [37, 40]. The degradation pathway involves sequential cleavage of the ester bond to yield the phthalate monoester, and then phthalic acid is further metabolized before entering the tricarboxylic acid (TCA) cycle to produce carbon dioxide and water. The end product after the TCA cycle is further mineralized into COB_{2B} and $HB_{2B}O$ by aerobes or CHB_{4B} and $HB_{2B}O$ by anaerobes, respectively. Moreover, the hydrolysis process can be enhanced by the presence of metal ions, anions, or organic material that serves as catalysts [16].

Biodegradation is the major process which affects the environmental fate of PAEs. Gram-positive and Gram-negative bacteria and actinomycetes are responsible for the degradation of PAEs in aerobic and some anaerobic environments [41]. Although some individual microbes are capable of completely mineralizing PAEs, more efficient metabolism appears to result from mixed microbial populations [16]. Researchers indicated that hydrolysis of PAEs into monoester and alcohol is the common microbial metabolism under both aerobic and anaerobic conditions. However, the detailed metabolic pathways of PAEs under anaerobic conditions are poorly documented. On the other hand, many researchers hypothesize that the monoester is degraded into phthalic acid and subsequently is degraded into COB_{2B} and water following the similar degradation pathway reported for benzoate [36]. Based on a recent comprehensive review, Ejlertsson and Svensson [42] proposed a more detailed biodegradation pathway of PAEs under aerobic and anaerobic conditions (Fig. 1).

Wang et al. [37] revealed that the metabolic breakdown of PAEs by microorganisms is considered to be one of the major routes of environmental degradation for these widespread pollutants. In addition, to discuss the types of microorganisms used to degrade DBP, Wang et al. [40] further investigated the biodegradation of phthalic acid ester in soil by introducing indigenous microorganisms. The results demonstrated that the adsorption of DBP by soil conformed to the Freundlich equation. The indigenous bacteria were capable of degrading phthalate in soil. The inoculation of the soil with DBP-degrading bacteria (DBP degrader) enhanced the DBP degradation rate. The enumeration of microorganisms indicated a good correlation between phthalate degradation and the microbial count. The data also indicated that inoculation of the soil microorganisms with DBP degrader enhanced the DBP biodegradation when compared with microorganisms capable of biodegrading xenobiotics. Thus, using the DBP degrader seems to be a promising strategy for removing hazardous compounds from the environment. Equally, the microbial degradation by anaerobic sludge of three phthalate, listed as priority pollutants by both China National Environmental

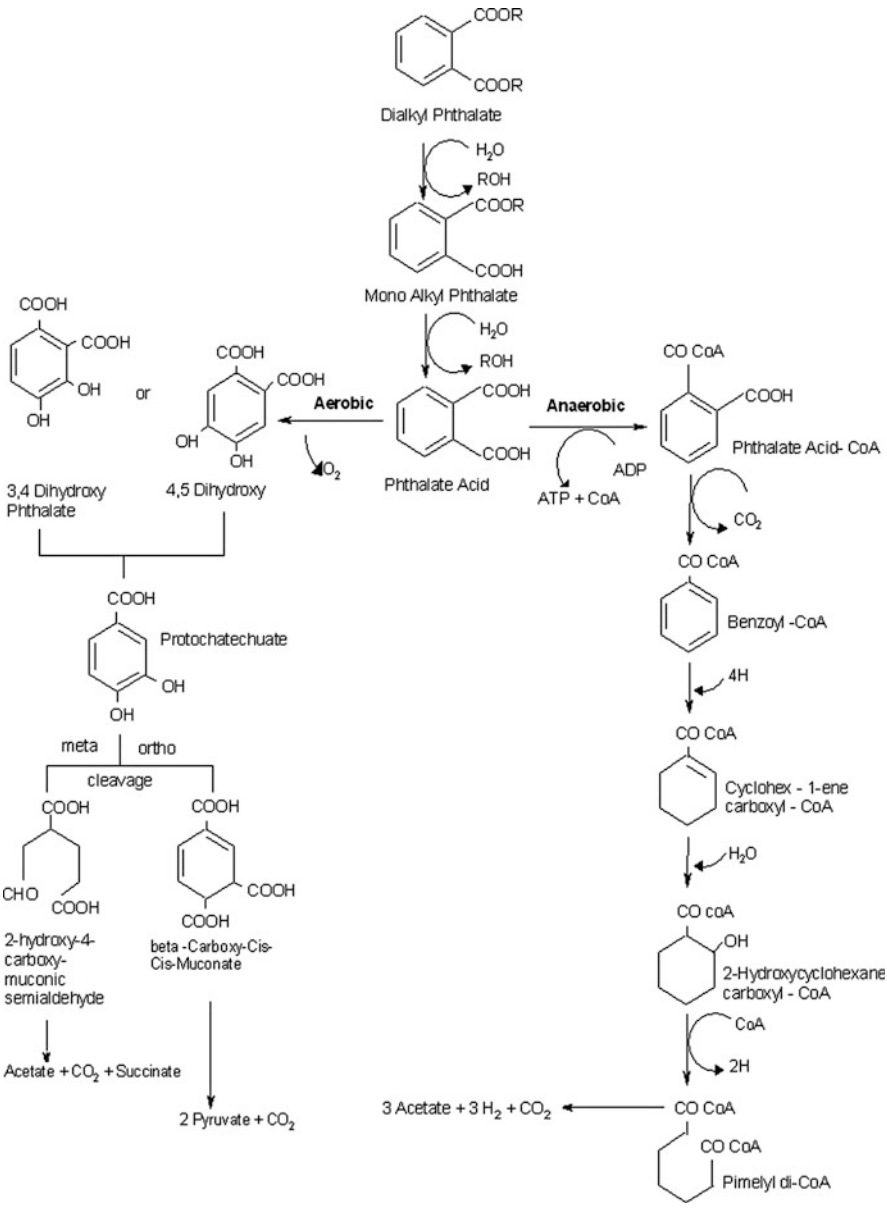


Fig. 1 Biodegradation pathway of phthalate esters in the environment

Monitoring Center and the USEPA, namely, DMP, DBP, and DOP was investigated by Wang et al. [43]. The experimental results indicated that the biodegradation rate and the biodegradability of the three phthalates under anaerobic conditions appeared to be related to the length of the alkyl-side chains. More than 90%

DMP and DBP with the short alkyl-side chain phthalates can be degraded, whereas the DOP degradation under the same experimental conditions appeared to be relatively slow. It is noteworthy that both the ester groups and the phthalate ring were mineralized at a significant rate. The kinetics study demonstrated that biodegradation of the three phthalates conformed to a first-order model with respect to their concentrations.

On the contrary, the simple first-order model cannot fully describe all of the degradation of PAEs. Madsen et al. [38] investigated the kinetics of DEHP mineralization in sludge-amended soil. A biphasic model with two independent kinetic expressions was proposed in this study to describe the whole degradation behavior of DEHP in sludge-amended soil. The initial mineralization activity fits well by first-order kinetics ($r^2 > 0.97$), whereas mineralization in long-term incubations (>40 days) was described better by fractional power kinetics ($rP^{2P} > 0.95$). The mineralization activity was much lower in the late phase presumably due to a decline in the bioavailability of DEHP caused by diffusion-limited desorption. Also, the results further emphasized that aerobic DEHP mineralization was four to five times faster than anaerobic mineralization. DEHP mineralization in sludge-amended soil was much more temperature-sensitive than was DEHP mineralization in soil without sludge. It is obvious that indigenous microorganisms in sewage sludge appear to govern the biodegradation of DEHP in sludge-amended soil. The concentration of DEHP remaining was more than 68% in the absence of oxygen after 1 year. A significant fraction of DEHP in sludge-amended soils may escape mineralization under in situ conditions. Consequently, the authors suggest that optimizing aerobic DEHP degradation in sewage sludge prior to field application appears to be a more efficient strategy for reducing the long-term DEHP levels than optimizing degradation in sludge-amended soils. Similar results were obtained by Cheng et al. [44]. Some laboratory-scale land-simulated experiments were performed to investigate the biodegradation behavior of DEHP in sewage sludge under various conditions (moisture, temperature, sunlight, and ventilation). The results indicated that DEHP could be degraded in sludge under the conditions of adequate ventilation, sufficient sunlight, and proper moisture content. However, about 70% of DEHP remained in sludge after 189 days of reaction time and it proved that DEHP is persistent in the environment. No degradation or reduction of DEHP was found in sterilized-sludge samples. The phenomenon obtained here is well supported by Madsen et al. [38] that indigenous microorganisms in sewage sludge play an important role in dominating the degradation of DEHP in sludge and sludge-amended soils.

Roslev et al. [39] revealed that microbial degradation is believed to be the principal sink for DEHP in aquatic and terrestrial systems, such as sewage, soils, sediments, and surface waters. Degradations of DEHP by indigenous and inoculated microorganisms in sludge-amended soil were performed in this study. The results exhibited that depletion of DEHP due to microbial mineralization was appreciably slower in assays performed with sludge-amended soil than in sludge assays performed without sludge. The degradation kinetics is complex, and DEHP appears to become increasingly less bioavailable during incubation for more than

4 weeks. The limited contributions of the soil microorganisms to the degradation of DEHP in sludge-amended soil were probably due to the immobilization of DEHP and its metabolites in the sludge matrix (which is so called the "aging effect"). The results summarized that indigenous microorganisms in the sewage sludge were responsible for the majority of the DEHP degradation in sludge-amended soil. The findings described above reflect a significant amount of DEHP (and its metabolites) with poor biodegradability and extractability (e.g., bound residues) that may remain in sludge-amended soil for extended periods of time despite the presence of microorganisms that are capable of degrading the compounds. However, it is uncertain whether this old DEHP poses a potential in situ risk (e.g., when the pollution exceeds 100 days).

In addition, Roslev et al. [39] pointed out that biodegradation of DEHP in aged environmental samples may be overestimated when conventional chemical extraction processes were used to isolate DEHP from the sample matrix. Calculating only the concentration difference leads to inaccurate results because it owes the reduction of DEHP to poor extractability rather than to actual microbial mineralization. This phenomenon is more pronounced when DEHP is diffused into soil micropores or is immobilized in the soil matrix while the pollution increasingly ages. Klinge et al. [45] also indicated that hydrophobic contaminants sorbed to sludge in wastewater treatment plants and enter the soil environment when the sludge is applied to agricultural fields. These compounds adsorb strongly to organic matter, and thus, their bioavailability is limited in organic-rich systems like sludge-amended soils. Due to the strong interferences of matrix effects, extracting hydrophobic compounds from organic-rich systems in order to obtain real concentrations is heavy and complicated.

5 Composting

Composting can be defined as the biological decomposition and stabilization of organic substrates, under conditions that allow development of thermophilic temperatures ranging from 35°C to 75°C as a result of biologically produced heat [46]. The end product of composting, i.e., compost, is a stable product rich in nutrients and free of pathogens that can be beneficially applied to land [47, 48]. Composting is a combined process which links physical, chemical, and biological factors with the ultimate goal of producing a humus-like, safe, and stable final product that can be used as soil improvement or fertilizers for plant growth. Compost can improve soil conditions and plant growth, and can reduce the potential for erosion, runoff, and nonpoint source pollution [46, 49]. Hassouneh et al. [47] revealed that composting is not a simple process, but the sum of a series of complex metabolic processes and transformations, brought on by the activity of a large mixed microbial population. These processes, however, vary according to the composition of composting materials. Moreover, high-temperature aerobic composting under thermophilic phase can kill most pathogens and parasites' eggs [50].

Several researchers emphasized that compost is also an excellent material to use to prevent the acidification and the deterioration of soil productivity [8, 51–54]. Several factors including moisture content, temperature, aeration rate, and carbon to nitrogen ratio have direct influence on composting process, which has been reviewed in detail in the past.

Sludge stabilization by composting is considered the least expensive method used worldwide for the purpose of sludge utilization [47, 50, 55]. The sewage sludge can be applied to land directly after digestion and dewatering or after co-composting with other materials. However, the temperature during composting has to be maintained over 55°C for at least 3 days. Composting, in general, requires huge spaces for operation, which is a major problem. The expansive land requirement for operation of composting increases the overall cost of treatment. Therefore, to lower the cost, the treatment facility can be constructed near the sewage treatment unit. In addition, the marketing of compost as a soil conditioner or fertilizer can further minimize the overall cost of composting. Most organic matter can be biodegraded more rapidly in the thermophilic stage caused by the high activity of microorganisms during the composting process. This stage can last for several weeks or months depending on the size of the system and the composition of the ingredients [51, 56]. DEHP is resistant to being fully biodegraded during the conventional sludge digestion process [57]; however, the biodegradation of DEHP can be stimulated by thermophilic aerobic sludge digestion (TAD) at the temperatures of approximately 60–70°C [58]. Most microorganisms cannot survive under thermophilic condition and therefore are killed during thermophilic stage.

6 Biodegradation of DEHP in Composting Processes

Metabolic breakdown of PAEs by microorganisms is considered as one of the major routes of environmental degradation for these widespread pollutants [37]. Owing to the low vapor pressure and thermal stability of DEHP, volatilization may not occur in environmental systems. In other words, loss of DEHP caused by a physicochemical pathway was insignificantly compared to biodegradation [16, 40]. Cheng et al. observed that the loss of DEHP from the sterilized sludge cakes was negligible after the completion of 100 days of aeration experiments [44]. PAEs with shorter side alkyl chains are relatively water soluble, i.e., DBP and DEB are readily biodegradable under aerobic and anaerobic conditions. Amir et al. [59] reported that DEHP underwent either a hydrolysis or de-esterification step to form monoesters (MEHP). Phthalic acid was produced after the biotransformation of MEHP. The rates of MEHP degradation were of the same order as those of DEHP, meaning that de-alkylation and de-esterification were the major biodegradation mechanisms [59]. A list of DEHP biodegradation studies by composting are shown in Table 5. The concentrations of DEHP remained almost unchanged when composting time was extended in many investigations (more than 30 days). Other phenomena such as adsorption, assimilation, and the poly-condensation can also explain the reduction

Table 5 Biodegradation of DEHP in composting processes

Reference	Composting method	Feeding materials (bulking agent)	Organic pollutants	Degradation
Hartlieb et al. [60]	In-vessel composting (1.8 m^3)	Municipal biowaste (shredded shrubbery)	DEHP, pyrene and simazine	86% after 370 days
Moeller and Reech [61]	In-vessel composting (10 l, insulated)	Municipal solid waste (garden park waste)	DEHP (38–160 mg/kg dry weight in MSW compost)	96–99% after 25 days
		Sewage sludge (straw and garden park green waste)	DEHP (3–6 mg/kg dry weight in sewage sludge compost)	91% after 25 days
Marttinen et al. [17]	Compost bins (220 l)	Raw municipal sewage sludge	DEHP (60 μg/g dry weight)	58% after 85 days
	Rotary drum (5 m^3)	Anaerobically digested municipal sewage sludge (tree bark and peat)		34% after 85 days
Amir et al. [59]	Platform (semi-industrial and industrial trials)	Lagooning sludge (straw)	Phthalic acid esters (DEHP concentration 6.26 mg/kg)	91% after 180 days
		Activated sludge (grass)	Phthalic acid esters (DEHP concentration 28.67 mg/kg)	94% after 135 days
Cheng et al. [62]	In-vessel composting (110 l)	Sewage sludge (sawdust)	DEHP (333 mg/kg dry weight in sludge cake)	85–88% after 18 days

of DEHP detection. DEHP is adsorbed onto organic matter such as ligno-cellulosic fractions. The binding between DEHP and these organic matters can be very tight especially in the stabilization (maturated) phase.

6.1 Kinetics and the Influence of Experimental Parameters on DEHP Degradation

The first-order kinetic model was used in most cases to describe the behavior of DEHP degradation by various biological treatments under aerobic or anaerobic conditions [16, 41, 43, 63]. However, a simple first-order model is insignificant to describe the degradation of PAEs especially in long-term treatment trials, such as sludge-amended soil and composting. Poor regression values ($r^2 < 0.6$) were obtained when a simple first-order kinetic model was used to describe whole

degradation behavior with two distinct degradation phases. Roslev et al. [39] and Madsen et al. [38] proposed an empirical biphasic model with two independent kinetic expressions to describe the two distinct phases of DEHP degradation in sludge-amended soil. The initial DEHP mineralization activity was well described by first-order kinetics, and the mineralization of the long-term trials (>40 days) was described by fractional power kinetics.

The first-order and fractional power kinetics were also used to describe the behavior of DEHP biodegradation in the thermophilic phase, including the initial mesophilic phases (phase I) and the phase thereafter (phase II), respectively [62]. The fractional power kinetic model parameters, i.e., K and N, were calculated by (1)–(3) and derived from a plot of log (C/C_0) versus log(t). The half time ($t_{0.5}$) of DEHP degradation in phases I and II was calculated using first-order and fractional power kinetic equations (3), respectively.

$$\frac{C}{C_0} = Kt^{-N} \tag{1}$$

$$\log\left(\frac{C}{C_0}\right) = \log K - N \log t \tag{2}$$

$$t_{0.5} = 10^{(1/N \log(K/0.5))} \tag{3}$$

The degradation of DEHP is directly related to releasable TOCw, perhaps the presence of TOCw improved the solubility and microbial degradability under thermophilic conditions in several studies [61, 62]. Bauer and Herrmann [18] revealed that the elution of DEHP from MSW was facilitated by the presence of dissolved organic matter (DOM) even under anaerobic conditions.

7 Antibiotics in the Environment

Pharmaceutical antibiotics are widely used for the medical treatment of microbial infective diseases. Consequently, tons of antibiotics are annually administered to humans and animals [64]. It is estimated that there are approximately 200,000 pharmaceuticals on the world market. At present, the antibiotics use ranges from 100,000 to 200,000 t annually in the world of which 50–70% is used in the veterinary prophylaxis and/or as growth promoters in animal breeding. Recently, there has been an increasing and alarming concern about penetration of pharmaceuticals into the water and wastewater systems [65–67]. Most pharmaceutical antibiotics are designed to be quickly excreted from the treated body and are consequently entering into sewerage system either unaltered or as metabolites. Some of the excreted antibiotics at higher dosages are bioactive and recalcitrant [68]. On the other hand, antibiotics in the concentration below the lethal level are a

serious risk to health and to the environment due to the generation of drug-resistance of pathogenic bacteria and bacterial resistance to disinfection products. A recent study reported that almost 100% samples taken from various parts of the hydrosphere contained trace concentrations of antibiotics [65].

The classification of antibiotics and the most popular antibiotic in its class are given in Table 6. Usually, the antibiotics excreted are partially metabolized and end up in sewage system. Some of the most commonly used antibiotics (e.g., sulfa drugs and β-lactam antibiotics) are difficult to biodegrade because of their complex structure, which protect them from the attack of wastewater biocoenosis. Moreover, the difficulty in biodegradation is also due to the lack of significant microorganism

Table 6 Biodegradation of DEHP in composting processes

Class of antibiotics	Name of the most commonly used antibiotic	Use
Aminoglycoside	Amikacin and gentamycin	Infections caused by Gram-negative bacteria such as *E. coli* and *Klebsiella*
Ansamycins	Geldanamycin	Antitumor antibiotics
Carbapenems	Ertapenam, doripenam, and meropenam	Bactericidal for both Gram-positive and Gram-negative organisms
Cephalosporin	Ceftiofur and cephapirin	Gastrointestinal upset, diarrhea, nausea and allergic reactions
Licosamides	Clindamycin and lincomycin	Serious staph-, pneumo-, and streptococcal infections in penicillin-allergic patients, also anaerobic infections
Lipopeptide	Daptomycin	Gram-positive organisms
Macrolide	Azithromycin, erithromycin, and telithromycin	Streptococcal infections, lower and upper respiratory tract infections and pneumonia
Nitrofurans	Furazolidone	Bacterial or protozoal diarrhea or enteritis
β-Lactam and penicillins	*Amoxicillin, novamax*	*Wide range of streptococcal infections, syphilis and Lyme diseases*
Polypeptides	Bacitracin, colistin, and polymyxin B	Eye, ear, or bladder infections
Quinolones	Ciprofloxacin, enoxacin, and levofloxacin	Urinary tract infections
Sulfanomides	*Sulfadimethaoxine, sulfamethazine, sulfamethoxazole, sulfadiazine, and sulfathiazole*	*Urinary tract infections*
Tetracycline	*Demeclocycline, minocycline, and tetracycline*	*Syphilis, chlamydial infections and Lyme disease, mycoplasmal diseases and acne rickettsial infections*
Others (against mycobacteria)	Clofazimine, ethionamide, and rifampicin	Gram-positive and mycobacteria

in the corresponding environment where the antibiotics are present. Therefore, a great portion of these compounds are not removed by classical sewage and wastewater treatment plants and are eventually accumulate either in sewage sludge due to high hydrophobicity or discharged into receiving waters. These effects lead to significant persistency of several antibiotics in various environments [69].

On the other hand, significant quantity of antibiotics are used as feed supplements to promote the growth of domestic animals and their dosage ranging from 3 to 220 g/kg of feed depending on type and size of animals and the nature of antibiotics. Several investigations pointed out that as much as 70–90% of some antibiotics maybe excreted as the parent compound in manures [70–72]. Generally, manures from various sources are rich in nutrients (e.g., animal wastes from livestock forming); so, the land application of manures without intensive treatment is a common practice in many parts of the world. It has been shown that antibiotics generally remain stable during manure storage and end up in agricultural fields in manure applications. Due to this, residues of the antibiotics were found in soils and adjacent areas [73]. Therefore, agricultural application of manures containing antibiotics is also one of the dominating pathways for the release of antibiotics into the terrestrial environment (e.g., soil).

8 Biodegradation of Antibiotics in Composting Processes

At present, the antibiotics principally originating from either human pharmaceutical entering the environment via wastewater treatment plans and/or from veterinary antibiotics (VAs) entering the environment via application of animal manures and manure-based composts to agricultural lands are generating serious threat to the environment. Composting process involves diverse variety of microorganisms, which eventually is the most preferable process for removing antibiotics from soils, sediments, and manures. Some of the composting investigations carried out in the recent past are shown in Table 7. Commercial compost generally consists of 30–50% animal manures with the remaining material being composed of organic material such as sawdust, husks, and oil cake [77]. Some researchers pointed out that the concentrations of VAs decreased significantly during the incubation of manures. For instance, the concentration of chlortetracycline (CTC) was reduced by 92% in poultry manure and 27% in pig manure during incubation under aerobic conditions [70]. The observed decreases were due to the adsorption and/or complexation of VAs to organic compounds and divalent cations present in manures. During composting more VA adsorption sites are generated (as part of the process and due to the feed matrix) [60], and consequently, extractable concentrations of VAs and their metabolites decline with time in high organic matrix [78]. Kim et al. [65] investigated the decline in extractable tetracycline (CTC), sulfonamides (SMZ), and macrolides (TYL) during composting. After composting for 5–6 weeks in batch reactors, the concentration of all three VAs declined below the relevant Korean guideline values. The mineralization of antibiotics during

Table 7 Recent investigations on biodegradation of antibiotics in composting process

Reference	Composting method	Feeding materials	Target antibiotics	Operating condition	Outcomes
Dolliver et al. [74]	Managed pile (12 m^3) and also in vessel composting units (rotating drum type)	Turkey manure	Chlortetracycline (CTC), monensin, sulfamethazine (SMZ) and tylosin (TYL)	Initial extractable concentration of targets: CTC—1.5 mg/kg; monensin—11.9 mg/kg; TYL—3.7 mg/kg; SMZ—10.8 mg/kg	1. Total-extractable CTC concentration declined rapidly compared to other antibiotics 2. CTC decline over 99% in 35 days 3. First-order decay of the antibiotics was observed 4. Control treatment was better than managed pile and in-vessel composting 5. Low-level manure management after an initial moisture adjustment was suggested as the practical option for manure management
Bao et al. [70]	Lab-scale in-vessel composting (20–25 l identical lab vessels)	Broiler manure, layer-hen manure, and hog manure	Chlortetracycline (CTC)	CTC initial concentration: 94.71 mg/kg in broiler manure and 879.6 mg/kg in hog manure; Manure turned every day for first 28 and 4 days once subsequently	1. More than 90% CTC depleted in 42 days of composting 2. Removal is less in hog manure (only 27%) 3. Half-lives of CTC: 11, 86.6 and 12.2 days in broiler manure, hog manure, and layer-hen manure, respectively, according to first-order kinetics

Ramaswamy et al. [75]	Lab-scale in-vessel composting (120 l)	Commercial poultry manure	Salinomycin	C:N ratio – 25:1; salinomycin concentration – 22 mg/kg	1. Salinomycin decreased to 2×10^{-5} µg/kg in 38 days of composting 2. TKN and carbon losses from composting reactor were 36% and 10%, respectively
Kim et al. [76]	Lab-scale in-vessel composting (60 l)	Pig manure and saw dust	Chlortetracycline (CTC), sulfamethazine (SMZ), and tylosin (TYL)	Applied at three different concentrations, i.e., 2, 10, and 20 mg/kg	1. Three antibiotics concentration decline below 1 mg/kg 2. CTC and SMZ decline highly dependent on the presence of saw dust

composting is due to the release of different hydrolytic enzymes (protease, lipase, and cellulose) by microorganisms [79].

Antibiotics removal during composting may occur due to hydrolysis, biodegradation, and sorption to composting feed matrix. Sorption to the composting feed matrix leads to an overestimation of the efficiency of the system to antibiotics removal and to the release of persistent molecules after biomass death [80]. The "carbon dioxide (CO_2)-evolution test," formerly known as "Modified Sturm test" allows the evolution of the biodegradability of the antibiotics via the measure of the produced CO_2 [81]. In this test, the CO_2 produced by microbial activity was trapped in a solution of barium hydroxide ($Ba(OH)_2$) which precipitate as barium carbonate in the presence of CO_2. The remaining barium hydroxide is titrated with standard HCl, and the mass of CO_2 produced ($m_{CO_2,P}$) during composting is estimated (4). In the similar way, the mass of CO_2 produced from the control composting runs can also be calculated ($m_{CO_2,PE}$). In addition, the theoretical mass of carbon ($m_{CO_2,theo}$) which can be produced from the target antibiotic(s) (m_{CiTC}) and composting feedstock (m_{CiRC}) can be calculated from (5). From (4) and (5), the biodegradation percentage of the antibiotics can be calculated from (6).

$$m_{CO_2,P} = \left(C_{Ba(OH)_2} V_{(Ba(OH)_2)} - C_{HCl} V_{HCl}\right) \times 44 \tag{4}$$

$$m_{CO_2,theo} = \left(\frac{\left(m_{CiTC} + m_{CiRC}\right) \times 44}{12}\right) \tag{5}$$

$$\text{Biod}\ (\%) = \left(\frac{m_{CO_2,P} - m_{CO_2,PE}}{m_{CO_2,theo}}\right) \times 100 \tag{6}$$

9 Summary and Conclusion

The presence of DOM can alter the partitioning behavior and the solubility of DEHP in the dissolved phase. Moreover, DEHP has the tendency to adsorb quantitatively onto solid particles especially when particulate matter is present. In composting, the rate of DEHP degradation was more rapid in the thermophilic phase compared to mesophilic phase. However, the degradation of DEHP was not directly related to releasable TOCw due to the presence of TOCw improving the solubility and microbial degradability under thermophilic conditions. Moreover, DEHP degradation was in good correlation with VS, TC, and TN in most of the studies. DEHP degradation could be well fitted using two-phase degradation kinetics, i.e., rapid initial phase expressed using first-order kinetics and subsequent slow phase could be explained by fractional power kinetics. However, active composting microorganisms involved in DEHP degradation can be useful resources for the biodegradation of DEHP, and similar compounds are present in industrial wastewater and sediment.

Antibiotics from human pharmaceutical and from VAs pose serious threat to the environment. Composting is an effective process for the mineralization/treatment of antibiotics, and it is mainly due to the release of different hydrolytic enzymes (protease, lipase, and cellulose) by microorganisms. The quantification of total organic carbon utilized during composting is essential to exactly identify the degree of treatment achieved especially in case of hydrophobic HOMs and antibiotics. Further research is required to identify the microorganisms, the enzymes, and other specific factors such as degradation, mineralization, or sorption that are involved in the mitigation of HOMs and antibiotics concentrations in soils/sediments/manure during composting.

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Fungal-Mediated Degradation of Emerging Pollutants in Sewage Sludge

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Abstract This chapter summarizes the novel approaches employed for the removal of emerging pollutants from sludge by means of fungal processes. The first part of the chapter introduces the potential application of fungi in bioremediation, which sets the background for the next chapters of the book. This includes a description of the fungal enzymatic systems usually involved in the oxidation of pollutants, as well as a brief review of the wide range of contaminants so far known to be degraded by fungi. The second half of the chapter presents the state of the art in application of fungi to remove emerging pollutants in sludge which, due to its novelty, is reduced to only a few reports performed in solid-phase and slurry reactors with *Trametes versicolor*, mostly under sterile conditions but with references of removal of pharmaceuticals under non-sterile conditions. Data available include several emerging pollutants which were grouped according to their function or activity: pharmaceutical compounds (26 drugs), brominated flame retardants (6), UV filters (7) and estrogens (3). Finally some considerations about the residual toxicity of the processes are discussed.

Keywords Biodegradation, Emerging pollutants, Sewage sludge, *Trametes versicolor*, White-rot fungi

Contents

Abbreviations and Symbols

4DHB	4,4′-Dihydroxybenzophenone
4-MBC	3-(4-Methylbenzylidene) camphor
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AS	Activated sludge
BFR	Brominated flame retardants
BP1	Benzophenone-1
BP3	Benzophenone-3
COD	Chemical oxygen demand
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
Deca-BDE-209	Deca-bromodiphenyl ether
DecaBDEthane	Deca-bromodiphenyl ethane

DGGE	Denaturing gradient gel electrophoresis
DMP	2,6-Dimethoxyphenol
DNT	2,4-Dinitrotoluene
dw	Dry weight
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EDC	Endocrine disrupting compound
EE2	17-α-Ethinylestradiol
EHMC	Ethylhexyl-methoxycinnamate
HBT	1-Hydroxybenzotriazole
Hepta-BDE-183	2,2′,3,4,4′,5′,6-Hepta-bromodiphenyl ether
Hexa-BDE-154	2,2′,4,4′,5,6′-Hexa-bromodiphenyl ether
LiP	Lignin peroxidase
LMEs	Lignin-modifying enzymes
LMPs	Lignin-modifying peroxidases
MBR	Membrane bioreactor
MnP	Manganese peroxidase
n.d.	Not detected
NCPA	*N*-(4-Cyanophenyl)acetohydroxamic acid
ND24	Naproxen degradation test
NHA	*N*-Hydroxyacetanilide
OC	Octocrylene
OD-PABA	Octyl-dimethyl-*p*-aminobenzoic acid
PAH	Polycyclic aromatic hydrocarbon
PBDEs	Polybrominated diphenyl ethers
Penta-BDE-99	2,2′,4,4′,5-Penta-bromodiphenyl ether
PhC	Pharmaceutical compounds
RDX	Cyclotrimethylenetrinitramine
RSD	Relative standard deviation
Tetra-BDE-47	2,2′,4,4′-Tetra-bromodiphenyl ether
TNT	2,4,6-Trinitrotoluene
U	Activity units
UV-F	Ultra-violet filters
VP	Versatile peroxidase
WRF	White-rot fungi
WSP	Wheat straw pellets
WWTP	Wastewater treatment plant

1 Introduction

The concept of developing a technology for the environmental application of fungi, particularly white-rot fungi (WRF) appeared in the 1980s [1]. Since then, the development of biotechnologies using WRF has been studied to treat a wide variety

of wastes and their role in the bioremediation of hazardous compounds in soils has been established. Most studies on bioremediation have focused on bacteria as degraders because of their rapid growth, and their usual ability to employ the pollutants as only substrates. Bacterial degradation of xenobiotics differs from WRF-mediated degradation: while bacteria usually employ the pollutants as nutrient sources (C and N), in WRF oxidation by means of their lignin-modifying enzymes (LMEs) yields no net energy, and the degradation becomes a co-metabolic process in which additional C and N sources are required [2]. This capacity represents an advantage respect bacteria-mediated approaches as it prevents the need to internalize the pollutant into the cell, therefore permitting to attack low soluble compounds and avoiding toxicity problems.

The capacity of WRF to transform and mineralize a wide range of pollutants without a preconditioning period via co-metabolic pathways makes them interesting for the degradation of recalcitrant xenobiotics. The use of WRF and their LMEs for the removal of xenobiotics has been reviewed elsewhere [1–7].

Chemicals degraded by WRF include pesticides such as organochlorines DDT and its very toxic metabolite DDE [8, 9] and organophosphate pesticides such as chlorpyrifos, fonofos and terbufos [10]; polychlorinated biphenyls (PCBs) of different degrees of chlorine substitution [11–13], some even to mineralization [14, 15]; diverse polycyclic aromatic hydrocarbons (PAHs) in liquid media and from contaminated soils or in complex mixtures such as creosote [16–18]; components of munition wastes including TNT and its metabolites DNT [19–23], nitroglycerin [24] and RDX [25].

Specific effluents have also been subjected to WRF-mediated remediation studies. Decolourization, dechlorination and detoxification of highly toxic bleach plant effluents derived from the pulp and paper industry have been reported [26–28], while degradation and decolourization of synthetic dyes due to the non-specificity of the LMEs have been widely documented [29, 30]. Likewise, treatment of the acidic, phenolic-rich olive oil mill wastewater has shown COD reduction, decolourization and dephenolization [31–34].

More recently, research moved towards the application of WRF to remove the so-called emerging pollutants. From these organic contaminants, the endocrine disrupting compounds (EDC) comprise the most studied group. Removal of EDC has been demonstrated in aqueous phase and soil with WRF and LMEs [5, 35, 36], and even some work has been conducted at different reactor scale configurations [37–40]. On the contrary, approaches on the removal of pharmaceutical compounds (PhC) are incipient, and most of the work has been performed in liquid media, aiming at demonstrating the ability of WRF to degrade the xenobiotics. Reports include the degradation of analgesics [41–46], antibiotics [47–49], psychiatric drugs [50–52] and lipid regulators [45, 46]. Similar is the panorama of brominated flame retardants (BFR) [53–55] and UV-filters (UV-F) [56], whose degradation by WRF has been barely studied.

2 White-Rot Fungi

WRF comprise an ecophysiological group of fungi capable to biodegrade lignin. Taxonomically WRF include mostly basidiomycetes and some ascomycetes [57]. As the most significant lignin degraders among the wood-inhabiting microorganisms, WRF present an extracellular oxidative system employed in the primary attack of lignin and its posterior mineralization [58] in a non-specific and non-selective mechanism. This enzymatic system includes LMEs, which are extracellular and metal-containing oxidoreductases, especially peroxidases and laccases. The extracellular enzymes generate diffusible oxidizing agents that attack the molecules, many of these being activated oxygen species [59, 60]. The reactions they catalyze include lignin polymerization and dimethoxylation, decarboxylation, hydroxylation and breakdown of aromatic rings.

WRF show several features which make them interesting agents for potential use in bioremediation: (1) their enzymatic system is highly non-specific and therefore able to oxidize a wide range of pollutants; (2) the main enzymes are produced constitutively, thus reducing the need to adaptation at polluted sites or matrices; (3) most oxidative enzymes are extracellular, which permits the degradation of low solubility contaminants; (4) the wide distribution and hyphae-growth facilitate colonization and access to sequestered pollutants; (5) inexpensive lignocellulosic wastes can be employed as substrate/carrier for growth/inoculation of WRF, as they are also necessary as nutrient source during co-metabolic removal of organopollutants.

2.1 Enzymatic System of WRF

2.1.1 Lignin-Modifying Enzymes

Decomposition of lignin is possible thanks to the production of LMEs. WRF secrete mainly two different groups of LMEs, laccases and lignin-modifying peroxidases (LMPs), particularly lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP), which act synergistically during lignin degradation [61]. The main difference is the electron acceptor, O_2 for laccases and H_2O_2 for peroxidases. Besides the fungal oxidative enzymes, the reactions of lignin biodegradation also involve secreted fungal metabolites like phenolic and other aromatic compounds, peptides, organic acids and lignocellulose-derived compounds and metal ions [61, 62].

The secretion pattern is species dependent; different WRF species produce various combinations of the main lignin degrading enzymes (LiP, MnP and laccase). According to their production, WRF can be grouped in three categories [63]: LiP–MnP group, like *Phanerochaete chrysosporium*; MnP-laccase group,

including *Trametes versicolor*, *Dichomitus squalens*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus, Lentinus edodes* and *Panus tigrinus*; LiP-laccase group, like *Phlebia ochraceofulva*. An additional group, the laccase-aryl alcoholoxidase group, has also been proposed.

The production of ligninolytic enzymes takes place during the secondary metabolism of several WRF. The lignin degrading system is induced when starvation of C or N occurs; moreover, agitation and temperature can significantly affect the levels of these enzymes; factors affecting LMEs are reviewed by Gao et al. [1].

Laccase

Fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper blue phenoloxidases. They comprise glycosylated proteins expressed in multiple forms and variable molecular weight, ranging from 59 to 110 kDa. Laccase is expressed as multiple constitutive and induced isoenzymes [30, 64]. The enzyme contains four copper atoms (Cu), in different states of oxidation (I, II, III) [65], which play an important role in the catalytic mechanism. Laccase oxidizes different compounds while reducing O_2 to H_2O, a total reduction of four electrons.

The low specificity of electron-donating substrates is remarkable for laccases. These enzymes have high redox potential, making them able to oxidize a broad range of aromatic compounds (e.g. phenols, polyphenols, methoxy-substituted phenols, aromatic amines, benzenethiols) through the use of oxygen as electron acceptor. Other enzymatic reactions they catalyze include decarboxylations and demethylations [66].

The catalytic cycle of laccase includes several one-electron transfers between a suitable substrate and the copper atoms, with the concomitant reduction of an oxygen molecule to water during the sequential oxidation of four substrate molecules [66]. With this mechanism, laccases generate phenoxy radicals that undergo non-enzymatic reactions [65]. Multiple reactions lead finally to polymerization, alkyl-aryl cleavage, quinone formation, C_{α}-oxidation or demethoxylation of the phenolic reductant [67].

Reported redox potentials of laccases are lower than those of non-phenolic compounds, and therefore these enzymes cannot oxidize such substances [7]. However, it has been shown that in the presence of small molecules capable to act as electron transfer mediators, laccases are also able to oxidize non-phenolic structures [68, 69]. As part of their metabolism, WRF can produce several metabolites that play this role of laccase mediators. They include compounds such as *N*-hydroxyacetanilide (NHA), *N*-(4-cyanophenyl)acetohydroxamic acid (NCPA), 3-hydroxyanthranilate, syringaldehyde, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), violuric acid, 1-hydroxybenzotriazole (HBT), 2,2,6,6-tetramethylpipperidin-*N*-oxide radical and acetovanillone, and by expanding the range of compounds that can be oxidized, their presence enhances the degradation of pollutants [3].

Peroxidases

The class II secreted fungal heme peroxidases include the LMPs LiP, MnP and VP [70]. All of these enzymes are extracellular and contain protoporphyrin IX (heme) as prosthetic group. They use H_2O_2 or organic hydroperoxides as electron accepting cosubstrates during the oxidation of diverse compounds. They are secreted as glycosilated, 35–38 kDa size proteins.

The classical peroxidative catalytic cycle involves the formation of a so-called compound I intermediate product of the binding of the hydrogen peroxide to the heme group of the enzyme and the subsequent release of a water molecule. The cycle operates through a second intermediate, compound II, to the resting state enzyme by two individual one-electron withdrawals from the reducing substrates [70].

LiP (EC 1.11.1.14) and MnP (EC 1.11.1.13) were first described in the WRF *P. chrysosporium* in the 1980s [71].

MnP is the most commonly widespread of the class II peroxidases [72, 73]. It catalyzes a H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}. The catalytic cycle is initiated by binding of H_2O_2 or an organic peroxide to the native ferric enzyme and formation of an iron-peroxide complex; the Mn^{3+} ions finally produced after subsequent electron transfers are stabilized via chelation with organic acids like oxalate, malonate, malate, tartrate or lactate [74]. The chelates of Mn^{3+} with carboxylic acids cause one-electron oxidation of various substrates; thus, chelates and carboxylic acids can react with each other to form alkyl radicals, which after several reactions result in the production of other radicals. These final radicals are the source of autocatalytically produced peroxides and are used by MnP in the absence of H_2O_2. The versatile oxidative capacity of MnP is apparently due to the chelated Mn^{3+} ions, which act as diffusible redox-mediator and attacking, non-specifically, phenolic compounds such as biopolymers, milled wood, humic substances and several xenobiotics [72, 75, 76].

LiPs are secreted as multiple isozymes and isoforms. They are catalytically the most powerful fungal peroxidase and have the ability to directly oxidize dimeric lignin model compounds. In the presence of H_2O_2, LiP catalyzes oxidation of an endogenously generated low-molecular-mass redox mediator veratryl alcohol (the preferred aromatic electron donor [77]), which subsequently generates aryl cation radicals through one-electron oxidations of non-phenolic aromatic nuclei in lignin. These are then degraded to aromatic and aliphatic products, which are mineralized intracellularly. The produced radicals can participate in diverse reactions, including benzylic alcohol oxidation, carbon–carbon bond cleavage, hydroxylation, phenol dimerization/polymerization and demethylation [2]. The substrate oxidation capacity of LiP includes the bleaching of coloured compounds, depolymerization of synthetic lignin and transformation of organopollutants such as PAHs, chlorophenols and explosives [78–81].

VP (E.C. 1.11.1.46) was first described in liquid cultures of *Pleurotus eryngii* growing on peptone as nitrogen source [82, 83] and *Bjerkandera* sp. [84]. VP is a heme containing structural hybrid between MnP and LiP, as it is able to oxidize Mn^{2+}, veratryl alcohol, simple amines, phenolic, nonphenolic and high molecular

weight aromatic compounds and high-redox potential dyes in reactions which are of manganese-independent character [85]. Therefore this enzyme has a wider catalytic versatility for electron donors as compared to LiP and MnP, and it has been postulated that its catalytic cycle is constituted by the sum of the catalytic cycles of those enzymes [70].

2.1.2 Cytochrome P450 System

Ability of WRF to degrade pollutants was first ascribed to the LMEs in nitrogen-limiting conditions, particularly LiP and MnP in *P. chrysosporium*, one of the first WRF employed as potential bioremediation agent. However, it was demonstrated afterwards that some xenobiotics such as 2,4-dichlorophenoxiacetic acid and 2,4,5-trichlorophenoxyacetic acid could be degraded in nitrogen-rich media, without the expression of LiP and MnP [86, 87], thus suggesting the role of additional enzymatic systems. Posterior research demonstrated that the intracellular cytochrome P450 system exerts a leading role in the degradation of organic contaminants by WRF, as reviewed by Cerniglia [88]. Evidence is based on the induction pattern of cytochrome P450 codifying genes in response to exposure to organic pollutants [89] and also on the important reduction in the extent of degradation in the presence of cytochrome P450 inhibitors, observed in the metabolism of PAHs by *P. ostreatus* [90], dibenzyl sulphide [91], aromatic sulphur compounds such as dibenzothiophene [92], chlorinated hydrocarbons such as trichloro- and perchloroethylene by *T. versicolor* [93, 94]. Moreover, the complete sequencing of *P. chrysosporium*'s genome revealed an estimated of 148 genes corresponding to P450 monooxygeneses, the highest number of sequenced fungi so far [95, 96]. The intracellular cytochrome P450 monooxygenases catalyze a broad range of reactions, which include hydroxylation, heteroatom oxygenation, dealkylation, epoxidation of C=C bonds, reduction and dehalogenation [97]. Its role in lignin degradation has been related to the intracellular depletion of metabolites released after the depolymerization of lignin by extracellular peroxidases [89].

3 Treatment of Sludge by Fungi

Sludge is an inevitable by-product of the wastewater treatment process. The terms sewage sludge and biosolids refer to the insoluble residue produced during biological wastewater treatment and subsequent sludge stabilization procedures [98]. Usually sludge resulting from these treatment operations is mainly liquid, with a solid content ranging from 0.5% to 15%. In this respect, treatment processes are aimed at increasing the solid content in order to reduce the sludge volume, or decreasing volatile solids and stabilizing the fraction of organic matter. The increase in solids involves sludge thickening and dewatering, a requisite

prior to the application of subsequent processes such as composting, incineration and landfilling. Dewatering techniques include centrifugation, filtration, water evaporation and percolation (wetlands) [99]. On the other hand, common stabilization approaches include biological anaerobic and aerobic digestion, lime stabilization, composting and heat drying, or new techniques such as radiation [98, 100, 101]. Nonetheless, recent research of the fate of emerging pollutants in WWTPs by environmental chemists has revealed that those treatments are not efficient enough in the removal of organic micropollutants [102–107], as it is reviewed in the chapter by Hernandez-Raquet [108] of this book.

In this context, the search for alternative green technology approaches becomes an imperative task. Among the potential biotechnological possibilities, fungal feasible bioremediation approaches for removal of organic pollutants from sludge are circumscribed to those in which the fungus is able to survive and colonize, and include slurry phase bioremediation and solid phase, compost-like biotreatment with dewatered sludge. Although not employed for the treatment of sludge, fungal-mediated slurries have been applied for the ex situ bioremediation, at bench or reactor scale, of soils contaminated with PAHs [109–112], hexachlorocyclohexane [113], pentachlorophenol [114] or dioxin-like compounds [115]. Similarly, several reports deal with the application of fungal bioaugmentation for the treatment of PAH-, pentachlorophenol- and DDT-contaminated soil with dissimilar results in the efficiency of removal in composting [116–118] and biopile-processes [119–121], including field-scale trials for the removal of pentachlorophenol with *T. versicolor* [122]. Moreover, More et al. [123] reviewed potential applications of filamentous fungi for wastewater sludge treatment (other than biodegradation of pollutants), such as organic solids reduction, bioflocculation and biocoagulation and enhanced dewaterability/filterability.

Despite the high potential described for *T. versicolor* and WRF in general in terms of degrading ability, most of the reports refer to liquid media and soils; however research concerning their application in complex matrixes such as sludge is limited. Moreover, as degradation of many hazardous materials has been demonstrated mostly at laboratory scale, and especially under sterile conditions, the technical challenges including bacterial competition and the scale-up of the processes remain before considering a real application.

The next sections discuss the removal of several groups of emerging pollutants at pre-existent concentrations from sewage sludge by means of fungal treatments in solid-phase biopiles, both under sterile and non-sterile conditions and in bioslurry reactors under sterile conditions.

3.1 Solid-Phase Treatment

The use of solid-phase techniques has especially increased in the last decades in the case of fungal-mediated bioprocesses, since they reproduce the natural habitat and growth conditions for filamentous fungi. Moreover, solid-phase bioreactors

constitute an inexpensive treatment method for remediation of solids, since they require minimum maintenance and small amounts of energy for aeration and mixing, thus making these systems cost-effective when long treatments are required [124]. This feature is especially important in the potential treatment of sludge, as the spectrum of pollutants with variable degrees of recalcitrance it contains is clearly wide. As good fungal colonization of the solid matrix (i.e. dry sludge) is necessary in bioremediation, the fungal inoculum should facilitate the delivery of the biomass and provide a lignocellulosic nutrient source for growth [125]. In this respect, WRF are produced on organic lignocellulosic wastes such as wood chips, wheat straw, corn cobs, grape stalks or barely bran which, in addition, promote the production of LMEs [126] for the posterior application in the target matrix.

In the cases here described, an agricultural semi-processed residue, wheat-straw pellets (WSP), was employed as a component of the solid-phase system [127]. Besides acting as a substrate and carrier, WSP plays also a role as a bulking material in the sludge-system. Moreover, *T. versicolor* grown on this substrate showed a better degrading performance, though less biomass and laccase production, when compared to other wastes [128]. The biopiles consisted of sewage sludge (obtained after stabilization by thermal dewatering, ~90% solids) supplemented with 38% (w/w, dry basis) *T. versicolor* inoculums previously grown on WSP [129].

Treatment time was set in 42 days, similar to that employed in typical sludge-composting processes. The determination of ergosterol content, a specific component of fungal cell membranes and a biomarker of viable mycelium [130] demonstrated fungal growth and colonization of the system (Fig. 1). Likewise, important laccase activity was achieved during the treatment process. Moreover, the results from the ND24 test (based on the degradation of spiked naproxen after 24 h, [131]), employed to evaluate degrading ability in solid matrices, suggested that the pollutant-removal potential of the fungus persisted in the sewage sludge biopiles. Overall biological parameters indicated that *T. versicolor* had been active throughout the treatment period.

Under non-sterile conditions, microbial community analysis by denaturing gradient gel electrophoresis (DGGE) revealed the prevalence of *T. versicolor* as the main organism in the system up to 21 days (half of the process); however by day 42, other fungi took over as the most abundant taxons. Remarkably, comparison of fungal bioaugmented with non-bioaugmented sludge-biopiles showed a marked inhibitory effect of *T. versicolor* over bacterial populations (unpublished results), which is a promising finding, regarding the usual limitations and challenges pointed to the use of WRF in real applications [1].

3.1.1 Removal of PhC

Pharmaceuticals comprise one of the most common groups of organic microcontaminants present in biosolids, given that conventional biological wastewater treatment processes are inadequate for their complete removal [132].

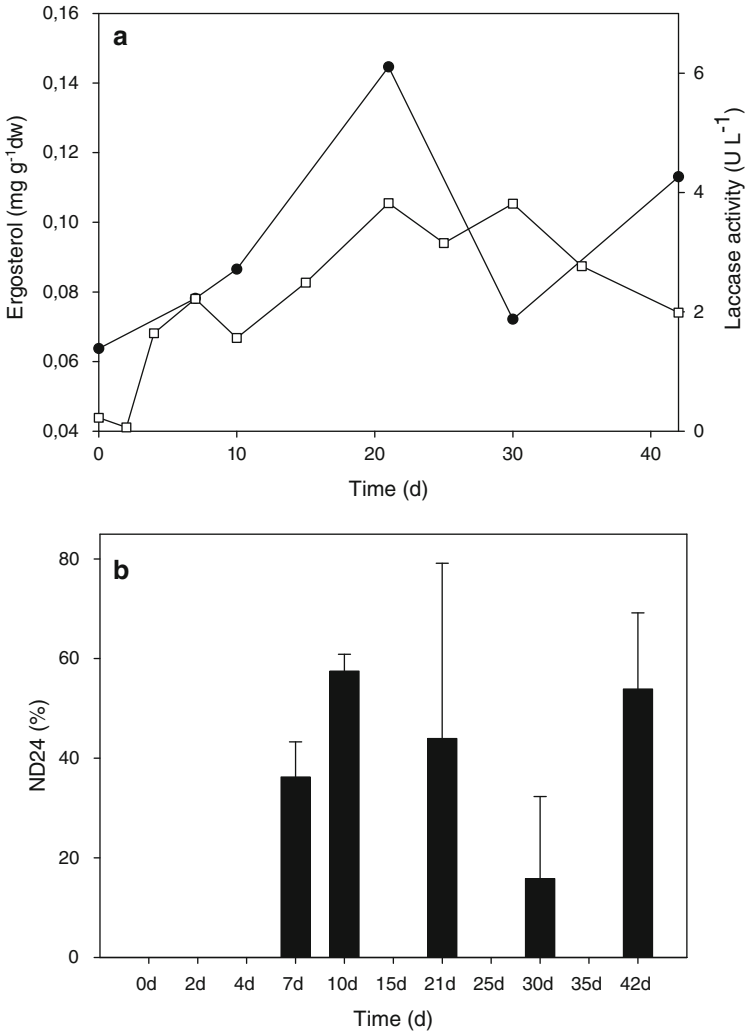

Fig. 1 Growth and activity of *T. versicolor* during a solid-phase treatment of sewage sludge. (**a**) Ergosterol content (*circle*) and laccase activity (*square*); (**b**) degrading ability measured as ND24 (adapted from [129])

For fungal degradation of PhC some data are also available from non-sterile biopiles. From 43 PhC analyzed, 14 were detected in the thermally dehydrated sterile sludge and 9 in non-sterile sludge (Table 1, concentrations reported on a dw basis). Considering that the biosolids had previously received conventional activated sludge (AS) and anaerobic digestion treatments, the occurrence of PhC indicates that many removal values described in the literature for WWTPs refer to elimination from the aqueous phase more than biodegradation or transformation, since they do not take into account the amounts sorbed to biosolids.

Table 1 Occurrence and removal of emerging pollutants in thermally dehydrated sewage sludge after solid-phase treatment with *T. versicolor*

Group of emerging contaminants		Compounds	Raw sludge		Treated sludge			
			Concentration	RSD (%)	Concentration	RSD (%)	Removal (%)	References
			(ng g^{-1})		(ng g^{-1})			
PhC	Analgesics/anti-inflammatories	Ibuprofen	85.9 (161.0)	10.7 (13.3)	13.1 (74.6)	9.2 (8.6)	75 (54)	[129, 133]
		Diclofenac	60.3 (53.6)	15.9 (9.0)	13.6 (24.7)	11.0 (6.1)	64 (54)	
		Mefenamic acid	17.9	11.7	3.1	16.1	72	
	Phenazone type drugs	Phenazone	9.6	22.9	n.d.	–	100	
	Lipid regulators and cholesterol lowering statin drugs	Bezafibrate	4.5	2.2	n.d.	–	100	
		Fenofibrate	4.2 (13.7)	14.3 (13.1)	n.d. (n.d.)	–	100 (100)	
		Atorvastatin	37.8 (19.3)	9.3 (13.5)	4.7 (6.7)	23.4 (23.9)	80 (65)	
	Psyquiatric drugs	Diazepam	19.3	14.5	6.8	2.9	43	
		Carbamazepine	25.6 (10.5)	19.5 (6.7)	9.1 (11.1)	1.1 (11.7)	43 (−4.9)	
	Histamine H2-receptor antagonists	Cimetidine	11.4	5.3	n.d.	–	100	
		Ranitidine	(17.5)	(7.4)	(n.d.)	–	(100)	
	Diuretics	Hydrochlorothiazide	26.7 (10.8)	11.6 (15.7)	8.0 (3.7)	10.0 (8.1)	52 (65)	
		Furosemide	(23.4)	(7.3)	(n.d.)	–	(100)	
	β-blockers	Atenolol	13.6	16.2	n.d.	–	100	
	Antibiotics	Clarithromycin	21.0 (35.8)	10.5 (19.0)	n.d. (6.5)	− (18.5)	100 (82)	
		Sulfamethazine	19.1	25.4	n.d.	–	100	
		Sulfapyridine	29.4	15.6	n.d.	–	100	
		Sulfathiazole	71.1	9.0	n.d.	–	100	

BFR		($ng\ g^{-1}$)		($ng\ g^{-1}$)			[134]
	Deca-BDE-209	285	5	41.0	14	86	
UV-F		($\mu g\ g^{-1}$)		($\mu g\ g^{-1}$)			[135]
	4DHB	0.07	6	n.d	–	100	
	BP3	0.06	11	n.d	–	100	
	4-MBC	3.10	9	0.40	11	87	
	OC	8.00	11	0.92	10	89	
	EHMC	2.20	5	0.16	5	93	
Estrogens		($ng\ g^{-1}$)		($ng\ g^{-1}$)			Unpublished data
	E1	19.66	–	n.d.	–	100	
	E2	3.70	16.21	n.d.	–	100	
	E3	3.40	20.23	n.d.	–	100	

Values corresponding to biopiles containing non-sterile sludge are presented in parenthesis. Removal values consider dilution effect by WSP; all concentration are referred to a dw basis

The most abundant pollutants belonged to the group of analgesic/anti-inflammatory compounds: ibuprofen (85.9 ng g^{-1}) and diclofenac (60.3 ng g^{-1}). Important removal of both compounds occurred after the fungal treatment in sterile conditions, around 75% and 64%, respectively, while in the non-sterile process the removal was 54% for both compounds. A removal of 41% was reported for ibuprofen in a study which considered both the dissolved and the adsorbed fraction during anaerobic digestion of sewage sludge [102]. On the other hand, dissimilar results from no elimination to removal values up to 69% have been described for diclofenac in anaerobic digestion processes [102, 106]. Mefenamic acid, another analgesic, was also detected at a concentration of 17.9 ng g^{-1} and subsequently reduced in 72% after treatment; meanwhile complete removal of the analgesic/antipyretic phenazone was accomplished. Degradation of mefenamic acid by *P. chrysosporium* in defined medium has been reported by Hata et al. [136]. Complete elimination of another analgesic, naproxen, was achieved after 72 h in sewage sludge cultures inoculated with *T. versicolor*; however, in that case the pharmaceutical was spiked instead of pre-existent [137].

Two psychiatric drugs were detected and partially degraded in the sludge: diazepam (only in sterile sludge) and carbamazepine. The findings regarding the antiepileptic carbamazepine are highly remarkable, as its almost negligible removal has been widely documented in conventional AS and membrane bioreactor (MBR) treatments [102, 138]. Furthermore, given its persistence, carbamazepine has been proposed as a molecular marker of sewage [103]. Removal of 43% was achieved after applying the fungal treatment. However, the non-sterile process failed to significantly reduce the concentration of the drug. Previously, elimination of 48% was only reported for high concentrations of carbamazepine spiked in solid-phase sludge treatments with *T. versicolor* in sterile conditions [137]. Similarly, the anxiolytic diazepam, found at 19.3 ng g^{-1}, presented a removal of 43%, similar to the performance showed by anaerobic digestion processes [102].

The lipid regulator drugs bezafibrate and fenofibrate at initial concentrations of 4.5 and 4.2 ng g^{-1} in sterile sludge were completely removed after the fungal treatment. The fate of fenofibrate, also present in non-sterile sludge, was the same. Meanwhile, the most abundant atorvastatin, a cholesterol lowering statin, decreased its concentration in 80% and 65% in sterile and non-sterile conditions, respectively.

Antibiotics exhibited a highly efficient elimination. The macrolide clarithromycin (initial concentration 21.0 ng g^{-1}) and the sulfonamides (sulfamethazine, sulfapyridine and sulfathiazole, initial concentrations ranging from 19.1 to 71.1 ng g^{-1}) were completely removed in sterile sludge. The degradation of clarithromycin was only slightly reduced in non-sterile biopiles (82%). Reports of analogous treatments applied to solid wastes for the removal of antibiotics are scarce, with different levels of success depending on the sulfonamides. Thus, in an anaerobic fermentation process of swine manure, some sulfonamides including sulfadiazine, sulfamerazine, sulfamethoxypyridazine, sulfamethoxazole and sulfamethoxine were eliminated at rates over 70%; however, sulfamethazine and sulfathiazole were not removed at all after 34 days [139]. Similarly, in a process of natural attenuation in biosolids,

sulfamethoxazole was completely degraded, whereas sulfamethazine was reduced in only 40% [140].

Other PhC were completely removed, including atenolol (β-blocker) and cimetidine (histamine H2-receptor antagonist), found in sterile sludge, and furosemide (diuretic) and ranitidine (histamine H2-receptor antagonist), found in non-sterile sludge. On the other hand, the diuretic hydrochlorothiazide was eliminated with an efficiency of 52% and 65% in sterile and non-sterile biopiles, respectively, which suggests a potentiated effect of the fungus with the sludge microbiota.

In a recent study, Walters et al. [141] described the occurrence and loss of several PhC from biosolid-soil mixtures exposed at ambient outdoor conditions for 3 years. Some compounds showed no detectable loss over the monitoring period, including diphenhydramine, fluoxetine, thiabendazole and triclosan, while half-life estimates ranging from 182 to 3,466 days were determined for others such as azithromycin, carbamazepine, ciprofloxacin, doxycycline, tetracycline, 4-epitetracycline, gemfibrozil, norfloxacin and triclosan. These findings highlight the potential use of *T. versicolor* to reduce the impact of biosolids once released to the environment, which could reduce the concentrations of PhC in much shorter periods of treatment.

3.1.2 Removal of Deca-BDE-209

Deca-BDE-209 is considered as one of the major sources of pollution by polybrominated diphenyl ethers (PBDEs) [142], and it is usually the one found at higher concentrations in sewage sludge samples. Characterization of the raw sludge showed an initial deca-BDE-209 concentration of 285 ng g^{-1}, which decreased, after the fungal treatment to 25.4 ng g^{-1}, equivalent to a reduction in 86%. The scarce reports of analogous treatments focused towards the elimination of PBDEs in solid wastes refer to anaerobic digestion. In this respect Gerecke et al. [143] achieved a reduction in the deca-BDE-209 concentration of 30% in sewage sludge under bench-scale anaerobic conditions, while Shin et al. [107] obtained a removal of 39.8% for deca-BDE-209 at bench-scale and around 45% in pilot-scale anaerobic digesters. No additional studies with fungi were found in the literature.

3.1.3 Removal of UV-F

Characterization of the raw sewage sludge in terms of UV-F occurrence and removal is shown in Table 1. The most abundant UV-F was OC at 8.00 μg g^{-1}, followed by 4-MBC (3.10 μg g^{-1}) and EHMC, also known as OMC, (2.20 μg g^{-1}), while lower concentrations were obtained for 4DHB (0.07 μg g^{-1}) and BP3 (0.06 μg g^{-1}). These results are not strange considering the nature of the sludge employed (dehydrated) and the fact that 4-MBC, OC and EHMC present the lowest water solubilities and highest log K_{ow} values.

The fungal treatment with *T. versicolor* resulted in an important reduction in the concentration of UV-F. 4DHB and BP3 were completely removed, while the

remaining compounds were depleted from 87% to 93%. The slightly lower removal efficiency of 4-MBC, OC and EHMC could be partially explained by the higher log K_{ow} of these compounds, which may translate in a reduced bioavailability for the fungus. Reports on fate of UV-F are also scarce, and in the case of WWTPs they are usually based on the removal from the aqueous phase, which does not reflect a real elimination due to the important fraction of those highly hydrophobic compounds that remains adsorbed onto the sludge. The behavior of BP3, 4-MBC and OC was evaluated at the different stages of a wastewater reclamation plant, showing reductions ranging from 7.6% to 21% in the coagulation-flocculation treatment, 3.6–7.3% during continuous microfiltration and 16–25% in the ozonation process [144].

3.1.4 Removal of Estrogens

Raw sludge presented concentrations of E1, E2 and E3 ranging from 3.4 to 19.7 ng g^{-1}, E1 being the most abundant (Table 1). The fungal treatment showed a high efficiency by completely removing all of the estrogens. Other studies in solid matrices include the use of free laccase for the remediation of contaminated sand [40]. The sand was spiked with EDC and treated first in test tubes with laccases from *Trametes* sp. and *Pycnoporus coccineus* and then in a sand-laccase solution (800 U L^{-1}) slurry in a rotating reactor. In tubes, removal of EE2 ranged from 70% to >90%, while the elimination in the rotating reactor reached 85% after 8 h. The same group obtained also a removal of 20% and 60% for E1 after 1 day and 3 days, respectively, in an analogous test tube system with spiked sand and treated with laccase from *P. coccineus* [36].

3.1.5 Reduction of Sludge Toxicity

As the mere disappearance of the parent pollutants cannot be considered as complete degradation and in some opportunities degradation-intermediates can be even more toxic than their parent compounds, a global estimation of toxicity was necessary to determine the reduction of toxic potential in the fungal-treated sludge. The standardized tests of acute immobilization of *Daphnia magna* and bioluminescence inhibition of *Vibrio fischeri* were accordingly applied.

The raw sludge presented high toxicity values in both tests, but especially for the marine bacteria test. Untreated sludge was less toxic than raw sludge, but this effect can be ascribed merely to the dilution of the sludge when mixed with non-toxic substrates (e.g. WSP). However, comparing the values obtained for the untreated sludge with those for the treated sludge, a decrease in toxicity was observed. In the *D. magna* test 56% of toxicity was removed; meanwhile in the *V. fischeri*-based test, a strong decrease in toxicity, from a high value (593 TU) in the untreated sludge to no inhibition, was achieved after the treatment with *T. versicolor*.

Considering that the primary use of biosolids is land application, and particularly on agricultural soils, a reduction in global phytotoxicity is desired and tests focused on seed germination were also performed. The results of the germination index and root elongation [145] clearly demonstrated that the fungal-treated sludge is more suitable for the germination and growth of the tested vegetable-seeds compared to the untreated sludge, in which the germination of crops such as lettuce, pepper and tomato was completely suppressed.

3.2 Bioslurry Treatment

A bioslurry phase system consists of the suspension of a solid phase in water or other liquid medium to a concentration typically between 5% and 40% (w/v) and kept under agitation conditions to allow the microbial growth of the indigenous microbiota or a particular inoculated microorganism [114]. Bioslurry systems for bioremediation purposes have been mostly conducted with bacterial cultures [146, 147], although in the last few years WRF were also successfully applied to soil bioremediation of PAHs, hexachlorocyclohexane and pentachlorophenol [110, 113, 114].

In this case, the bioslurry employed for the degradation of emerging pollutants consisted of a stirred tank bioreactor containing sewage sludge obtained from the outlet of an anaerobic digestor (3.6% w/w in solid content) from a WWTP, and inoculated with *T. versicolor* pellets [148]. The system operated at 25°C in batch mode for 26 days, similar to the residence time of the sludge in the anaerobic reactor. The growth and activity of *T. versicolor* are shown in Fig. 2. Ergosterol profiles revealed a continuous increase in fungal biomass; however, laccase was detected in the reactor only between days 14 and 18. Although the pH was set at 4.5, near the optimum for maximum laccase production [149], enzymatic activity was lower compared to higher solid-content sludge slurries without pH control [137]. The shear stress exerted by the stirrer may have contributed to the reduced laccase production, as described for ligninolytic enzymes in other WRF [110, 150]. Despite the absence of laccase activity in the last stage of the treatment, the steep increase in ergosterol during this period indicated the presence of metabolically active biomass.

3.2.1 Removal of PhC

For therapeutic drugs, the highest concentrations in the raw sludge corresponded to the analgesics diclofenac (209 ng g^{-1}) and ibuprofen (135 ng g^{-1}), and the sulfonamide antibiotic sulfathiazole (143.0 ng g^{-1}). Next in abundance were the diuretic compounds furosemide (79.9 ng g^{-1}) and hydrochlorothiazide (41.3 ng g^{-1}), and the analgesic ketoprofen (42.4 ng g^{-1}). The remaining PhC were found at concentrations below 40 ng g^{-1}. The list of the 24 detected

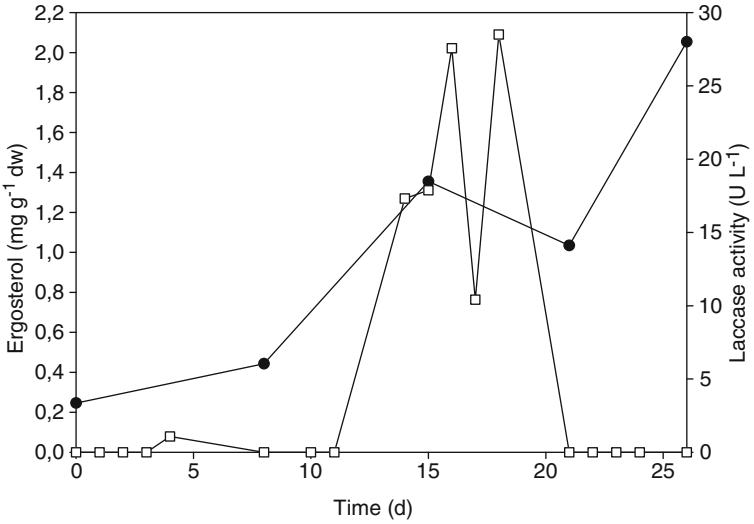

Fig. 2 Time course of ergosterol content (*circle*) and laccase activity (*square*) during a bioslurry reactor treatment of sewage sludge with *T. versicolor* (adapted from [148])

compounds and their removal values after fungal treatment is shown in Table 2 (concentrations reported on a dw basis).

Removal of analgesics varied from 40% to 92%. Ibuprofen was the most efficiently removed (91.9%), however the degradation of this compound by *T. versicolor* in liquid medium results in the production of 1,2-hydroxy ibuprofen, a final and more toxic metabolite [45], as it is discussed by Cruz-Morató et al. in this volume [151]. The isomers 1-hydroxy ibuprofen and 2-hydroxy ibuprofen, also described in the early stages of fungal-mediated degradation [45], have been identified in raw wastewater [152]. On the other hand, indomethacin and diclofenac were removed at around 60%, while elimination of ketoprofen, naproxen and mefenamic acid ranged between 40% and 50%. The removal of naproxen (80–90%) and ibuprofen (30–60%) has been described in anaerobic digestion of sewage sludge [102]; nonetheless, the adaptation of sludge was necessary to achieve the degradation of diclofenac (80%). Similar fungal slurry systems at smaller scale and containing 25% sludge have resulted in the partial elimination of spiked naproxen (47%) [137]. Degradation of some of these analgesics has been described with *T. versicolor* in liquid phase, in most cases with a reduction in the toxicity [42–45]; further details in this respect are reviewed by Cruz-Morató et al. in this volume [151].

The bioslurry treatment successfully removed several of the PhC to non-detectable levels after 26 days: three histamine H2-receptor antagonists (ranitidine, famotidine, cimetidine), two β-blockers (atenolol, sotalol), one barbiturate (butalbital) and one antidiabetic compound (glibenclamide). The elimination of the sulfonamide antibiotics sulfapyridine (100%), sulfamethazine (91.0%) and

Table 2 Occurrence and removal of emerging pollutants in sewage sludge after treatment in a bioslurry reactor with *T. versicolor*

Group of emerging contaminants		Compounds	Raw sludge		Treated sludge			
			Concentration	RSD (%)	Concentration	RSD (%)	Removal (%)	References
PhC			(ng g^{-1})		(ng g^{-1})			[148]
	Analgesics/anti-inflammatories	Ketoprofen	42.4	6.83	21.3	1.01	49.8	
		Naproxen	6.17	0.91	3.38	0.68	45.3	
		Ibuprofen	135	14.1	10.9	0.88	91.9	
		Indomethacine	9.60	1.00	3.65	0.29	61.9	
		Diclofenac	209	5.86	84.0	1.37	59.8	
		Mefenamic acid	14.2	3.05	8.37	0.20	41.1	
	Phenazone type drugs	Phenazone	36.7	10.9	20.3	3.64	44.7	
	Lipid regulators and cholesterol lowering statin drugs	Bezafibrate	11.8	1.21	6.43	0.26	45.3	
		Gemfibrozil	14.2	3.05	8.37	0.20	41.1	
		Atorvastatin	38.0	3.36	19.1	0.57	49.8	
	Psychiatric drugs	Diazepam	7.71	0.49	5.70	0.72	26.1	
		Carbamazepine	29.2	2.57	20.2	3.49	30.7	
	Histamine H2-receptor antagonists	Ranitidine	7.92	0.78	n.d.	–	100	
		Famotidine	12.0	0.78	n.d.	–	100	
		Cimetidine	10.4	1.31	n.d.	–	100	
	Sulfonamide antibiotics	Sulfamethazine	6.1	22.0	0.5	–	91.0	
		Sulfapyridine	21.4	2.6	n.d.	–	100	
		Sulfathiazole	143.0		20.1	26.4	85.9	
	β-blockers	Atenolol	1.70	0.17	n.d.	–	100	
		Sotalol	4.88	0.89	n.d.	–	100	
	Barbiturates	Butalbital	16.3	2.30	n.d.	–	100	
	Diuretics	Hydrochlorothiazide	41.3	0.50	6.89	0.11	83.3	
		Furosemide	79.9	11.3	27.7	2.30	65.3	
	Antidiabetics	Glibenclamide	17.4	1.94	n.d.	–	100	

(continued)

Table 2 (continued)

Group of emerging contaminants		Compounds	Raw sludge		Treated sludge			
			Concentration	RSD (%)	Concentration	RSD (%)	Removal (%)	References
BFR			(ng g^{-1})		(ng g^{-1})			
	PBDEs	Tetra-BDE-47	5.39	13	4.11	10	23.7	
		Penta-BDE-99	6.05	23	3.24	25	46.4	
		Hexa-BDE-154	5.48	35	2.56	15	53.3	
		Hepta-BDE-183	0.61	13	0.62	12	0	
		Deca-BDE-209	232	5.5	145	16	37.5	
	Emerging BFRs	DecaBDEthane	26.3	22	22.1	18	16.0	
UV-F			(μg g^{-1})		(μg g^{-1})			
		BP1	0.08	7	n.d	–	100	
		4DHB	0.051	7	0.050	9	1	
		BP3	0.034	10	0.019	7	22	
		4-MBC	0.520	11	0.205	11	61	
		OC	7.71	11	3.214	8	58	
		OD-PABA	0.012	11	0.004	12	70	
		EHMC	1.031	7	0.211	8	79	
Estrogens			(ng g^{-1})		(ng g^{-1})			Unpublished data
		E1	21.33		n.d.	–	100	
		E2	14.79	6.39	n.d.	–	100	
		E3	10.15	11.07	n.d.	–	100	

Concentrations are referred to a dw basis

sulfathiazole (85.9%) and the diuretic drugs (furosemide and hydrochlorothiazide at 65–83%) was also high but incomplete. The elimination of those sulfonamides has been described in liquid medium with *T. versicolor* pellets, with the participation of laccase and cytochrome P450 [133, 153].

Other groups of PhC showed lower removal values, although some degree of elimination was achieved in every case. These groups included the phenazone-type drugs (phenazone, 44.7%) and the lipid regulators and cholesterol lowering statin compounds (bezafibrate, gemfibrozil, atorvastatin, 41.1–49.8%). Similar to findings from solid-phase treatment, the psychiatric drug carbamazepine showed the lowest elimination value at 30.7% together with diazepam (26.1%). An analogous sludge slurry system with *T. versicolor* produced a degradation of 57% when carbamazepine was spiked [137]. Removal up to 60% has been described for diazepam in anaerobic digestion of sludge [102].

3.2.2 Removal of BFR

As it is usually reported, the most abundant BFR detected in the sludge was deca-BDE-209 at a concentration of 232 ng g^{-1}. The other congeners were present at concentrations below 10 ng g^{-1}, except the so-called "emerging BFR" decaBDEthane, detected at 26.3 ng g^{-1}. Removal after the fungal slurry was rather low and ranged from 16% to 53%; however, only hepta-BDE-183 remained unchanged after the treatment (Table 2). The most degradable congener was hexa-BDE-154 (53%), while deca-BDE-209 was removed at only 38%, much less compared to the high removal obtained in solid-phase systems. Remarkably, no correlation in the elimination was observed according to the bromination degree of the detected congeners. Bacterial biotransformation of BFR has been recently reported in aerobic and anaerobic conditions in diverse matrices such as soil, sediments and anaerobic treatment of sewage sludge [104, 154–156]. On the other hand, only two reports deal with BFR degradation by WRF in liquid medium [55, 134] (see [157], this volume). They include the use of surfactants, as the high log K_{ow} values of BFR have been considered as limiting factors for their biologically mediated transformation processes. However, the results from the slurry demonstrate that transformation can still be achieved in a system without additional surfactants.

3.2.3 Removal of UV-F

OC (7.71 μg g^{-1}), EHMC (1.03 μg g^{-1}) and 4-MBC (0.520 μg g^{-1}) were the most abundant UV-F detected in the raw sludge. The remaining sunscreens were found at concentrations below 0.1 μg g^{-1}. Removal values were higher than 58% for most of the compounds (Table 2), including the complete elimination of BP1. Only BP3 was poorly removed (22%), while 4DHB showed complete recalcitrance and could not be transformed in the process. Higher removals were expected for the least

hydrophobic UV-F (such as 4DHB and BP3, log K_{ow} 2.55 and 3.79) and lower for the most hydrophobic (log K_{ow}: 5.8, 6.15 and 7.35 for EHMC, OD-PABA and OC, respectively), which would strongly adsorb to the solid fraction of the sludge. Nonetheless, the removal profiles did not show this pattern. This finding could suggest that the fungus was able to colonize the solid particles in the slurry or that degradation occurred in the aqueous phase after desorption due to equilibrium displacement. The degradation of 4-MBC and BP3, described with *T. versicolor* in liquid medium [56, 135], is discussed by Badia-Fabregat in this volume[158].

3.2.4 Removal of Estrogens

E1, E2 and E3 were detected at values ranging from 10 to 21 ng g^{-1} (Table 2). In this case, the fungal treatment was extremely efficient, with complete removal for the three chemical species. Degradation of endocrine disrupting chemicals by WRF and their ligninolyitic enzymes has been reviewed elsewhere [4], but never reported in this matrix.

3.2.5 Toxicity

Contrary to the solid-phase biopiles, results from toxicological assays (*D. magna* and *V. fischeri*) indicate an increase in the toxicity of residual sludge after the fungal treatment in the bioslurry. These findings suggest that micropollutants could be more bioavailable in this system than in solid phase, and that aqueous media could favor abiotic reactions among the metabolic intermediates, not so common in solid phase. This is supported by the increase in the toxicity observed in the aqueous-phase degradation for some of the organic pollutants detected in the sludge, including ibuprofen [45], carbamazepine [51] and some BFR [134]. The use of HCl to adjust the pH of sludge to the optimum for *T. versicolor* growth could have led to the formation of higher polarity and higher toxicity intermediates, which contributed to the global increase of toxicity.

4 Concluding Remarks

The fungal-mediated degradation of emerging pollutants from sewage sludge by WRF has been recently reported through the application of solid-phase and bioslurry systems with *T. versicolor*. Solid-phase biopiles have in common the use of a lignocellulosic residue and thermally dehydrated sludge, and included removal values from sterile and non-sterile conditions. Slurry treatments, on the other hand, employed sludge from the effluent of an anaerobic digester and the reported removals refer only to sterile conditions.

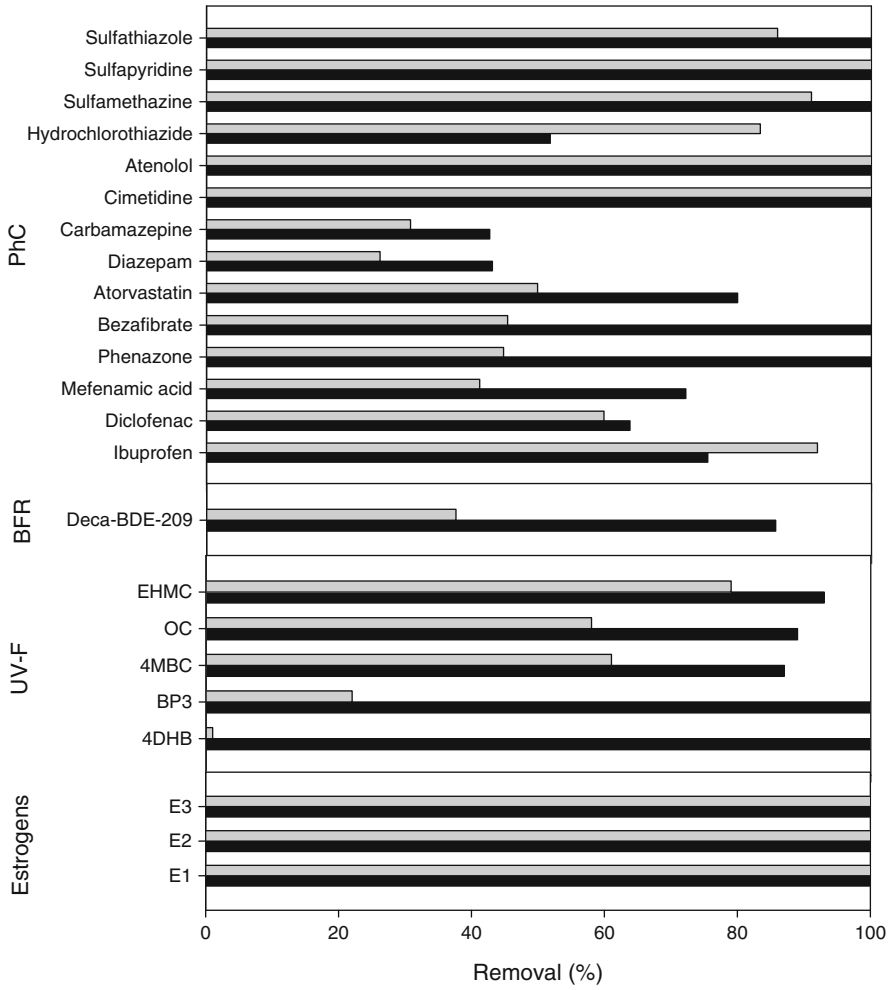

Fig. 3 Comparison of solid-phase biopiles (*black bars*) and bioslurry reactor (*gray bars*) in the removal of several emerging pollutants from sewage sludge with *T. versicolor*. Only compounds found during both kinds of treatment are included

Comparison of the efficiency removal (which includes only the pollutants found in both kinds of sludge before fungal treatment, Fig. 3) indicates that a solid-phase treatment is more efficient than the bioslurry process, as only two compounds (ibuprofen and hydrochlorothiazide) were removed at a higher extent in the latter system. In the case of a few compounds (cimetidine, atenolol and sulfapyridine), complete degradation was accomplished regardless of the treatment employed, but in most of the cases the elimination was significantly higher in solid-phase. That was especially highlighted in the case of some UV-F (4DHB and BP3), several PhC (phenazone and bezafibrate) and the deca-DBE-209, as the removal in solid-phase

was more than twice the obtained in bioslurry. The first step to overcome one of the biggest challenges in bioaugmentation, i.e. colonization of the matrix, was done, resulting in the removal of PhC in non-sterile sludge, at efficiencies close to those under sterile conditions in most of the cases. The remarkable difference in the final toxicity of the sludge obtained from both strategies, increased in the slurry and dramatically decreased in the biopiles, also supports the possibility of employing a solid-phase treatment with WRF as an eco-friendly strategy to reduce the release of toxic contaminants into the environment.

Further research should focus on: (1) the description of the mechanisms involved in the degradation in situ; (2) the identification of the metabolites released during the process to better estimate the toxicity of the residues; (3) the relationship between the WRF and the microbiota, which may counteract the degrading capacity; and clearly, (4) the optimization of the process and (5) the scale-up necessary to implement a possible real application. Given the challenge of structural elucidation studies with complex environmental matrices such as sludge, the work performed so far respect point (2) in liquid media is reviewed in the next chapters of the book for PhC [151], UV-F [158] and BFR [157].

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Biodegradation of Pharmaceuticals by Fungi and Metabolites Identification

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Abstract Pharmaceutical compounds comprise a widely employed group of therapeutic agents now considered as emerging micropollutants. This chapter summarizes the state of the art in the degradation of pharmaceuticals by fungi in liquid matrices (with emphasis on white-rot fungi), including the use of both whole cells and fungal enzymes. The identification of the metabolites produced as well as the proposed degradation pathways available for some drugs are discussed. The information is organized according to the activity of the pharmaceutical compounds, grouped in: anti-inflammatory/analgesic drugs, psychiatric drugs, lipid regulators, antibiotics and other antimicrobial agents, β-blockers, estrogens, and iodinated contrast media. Considering the interest in potential application of fungal treatments in future real scale bioremediation of effluents, the ecotoxicology of the process is included when available.

Keywords Degradation, Pharmaceuticals, Toxicity, Transformation products, White-rot fungi

Contents

Abbreviations and Symbols

ABTS	2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt
BSTR	Batch stirred tank reactor
CLEAs	Cross-linked enzyme aggregates
DMHAP	3,5-Dimethoxy-4-hydroxyacetophenol
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EC_{50}	Half maximal effective concentration
EE2	17α-Ethinyl estradiol
FBR	Fluidized bed reactor
HBT	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time

LAC	Laccase
LiP	Lignin peroxidase
LME	Lignin modifying enzymes
MnP	Manganese peroxidase
PBR	Packed-bed reactor
SYD	Syringaldehyde
TP	Transformation products
VA	Violuric acid
VP	Versatile peroxidase
WRF	White-rot fungi
WWTP	Wastewater treatment plant

1 Introduction

The most important removal pathways of organic compounds during wastewater treatment are biotransformation/biodegradation, adsorption to the sludge, and stripping by aeration (volatilization). Also, abiotic removal from the aqueous phase by hydrolytic degradation and/or isomerization/epimerization can occur. In most of the studies, abiotic (adsorption) and biotic degradation (transformation by microorganisms) processes cannot be distinguished, and the term "removal" is usually indistinctly employed to refer to these phenomena. Moreover, without stoichiometric accounting for the transformation products (TPs) of biodegradation one cannot conclude whether the compound was completely destroyed (mineralized) or only structurally altered. Therefore, during the biological treatment of wastewater, microbial enzymatic activity may lead to the formation of TPs that preserved or even increased persistency and/or activity. Considering the potential persistency and toxicity of TPs generated during drinking and wastewater treatment, their identification and quantification, as well as elucidation of main reaction mechanisms are necessary for safe application of such processes. These products may have preserved the mode of action of the parent compound, or even be biologically more active, thus the disappearance of the parent drug does not necessarily equate to its detoxification. Quantitative evaluation of all the TPs formed, as well as their rate constants would afford kinetic and mechanistic data for the evaluation of efficiency in removing pharmaceuticals from real waters. More importantly, for a complete risk assessment study on the TPs of pharmaceuticals formed during drinking and wastewater treatment, determination of their ecotoxicity is fundamental and a prerequisite for a comprehensive protection of the environment.

However, studies dealing with identification of biodegradation products of drugs in wastewater treatment plants (WWTPs) are very scarce, possibly due to the complexity of screening and structural elucidation studies in environmental matrices such as wastewater and sludge.

Biotechnological approaches have emerged as promising tools for the elimination of micropollutants including pharmaceuticals. Some of them rely on the use of

specific microorganisms with marked degrading ability, especially bacteria and less frequently fungi. The broad degrading ability of fungi and their enzymes and their implication in the transformation and mineralization of several organic pollutants make them potential agents for bioremediation processes, as it was reviewed by Rodríguez-Rodríguez et al. in this volume [1]. The present chapter summarizes the work done to date on the fungal-mediated degradation of pharmaceuticals and the identification of their TPs in liquid media. For the reasons above described, most of the research found in the literature focuses on the degradation in liquid media, since the easy control of parameters such as addition of nutrients, pH, and the contaminant concentration facilitates the study of the degradation and the identification of TPs. On the other hand, many works presented in the next sections are referred to as degradation regardless whether the target compound is mineralized (completely removed) or transformed to another compound. As mineralization is hardly reported, in the discussion the degradation described refers to transformation, unless explicit mineralization is indicated.

The information is organized in two tables, comprising the degradation of pharmaceuticals by whole fungal cells (Table 1) or by fungal enzymes (Table 2); the discussion is grouped according to the therapeutic function of the pharmaceuticals in anti-inflammatory/analgesic drugs, psychiatric drugs, lipid regulators, antibiotics and other antimicrobial agents, β-blockers, estrogens, and iodinated contrast media. Many of the studies were performed as preliminary approaches in order to determine the feasibility of applying fungal treatments to the biodegradation of pharmaceuticals from real liquid matrices. Therefore, when data is available, the toxicological characteristics of the residual effluent are discussed to assess the suitability of the treatment proposed.

2 Pharmaceuticals

2.1 Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory agents are used extensively as nonprescription drugs, and residues of these compounds have been detected ubiquitously in WWTP effluents at the μg L^{-1} levels, and they also frequently occur at the ng L^{-1} level in the aquatic environment (see [2], this volume). As a result, many researchers have focused on the degradation of these pharmaceuticals, which are one of the most studied groups of therapeutic agents in terms of fungal transformation.

One of the most widely used anti-inflammatory drugs is ibuprofen. Marco-Urrea et al. [3] performed a degradation screening using four white-rot fungi (WRF) (*Trametes versicolor*, *Ganoderma lucidum*, *Irpex lacteus*, and *Phanerochaete chrysosporium*) for the removal of 10 mg L^{-1} of ibuprofen after 7 days of incubation in serum bottles and observed that the contaminant was degraded by all of the

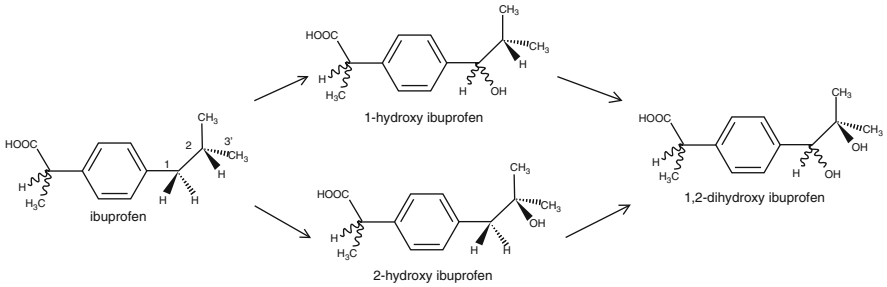

Fig. 1 Suggested degradation pathway of ibuprofen by *T. versicolor* (adapted from Marco-Urrea et al. [3])

fungi. However, they noticed that *T. versicolor* was able to degrade the contaminant in only 1 h, while more time was needed to achieve the complete depletion with the other WRF. 1-Hydroxy ibuprofen and 2-hydroxy ibuprofen were identified as TPs during the early stages of the degradation by *T. versicolor,* which were subsequently degraded to 1,2-dihydroxy ibuprofen (Fig. 1). In addition, Microtox bioassay experiments performed at different incubation times revealed an increase in the toxicity at the final time point (7 days), when only 1,2-hydroxy ibuprofen was detected (8.3 Equitox m^{-3} vs 0.05 Equitox m^{-3} in the controls with ibuprofen). This finding remarks the importance of the identification of metabolites in any treatment, because they may be more toxic than the parental compound. Rodarte-Morales et al. [4] confirmed the total elimination of ibuprofen by *P. chrysosporium* before 4 days, which agrees with the results by Marco-Urrea aforementioned. In addition, they reported complete degradation of this contaminant by *Bjerkandera* sp. R1 and *Bjerkandera adusta* before 4 and 7 days, respectively. However, neither identification of metabolites nor toxicological analysis were performed in order to evaluate the feasibility of the treatment.

Since it is known that WRF produce highly oxidative enzymes, several studies have also focused on the determination of which of these enzymes are involved in the degradation of contaminants. Marco-Urrea et al. [3] reported that laccase (LAC) from *T. versicolor* and manganese peroxidase (MnP) from *Bjerkandera* sp. are not involved in the degradation of ibuprofen, according to the negligible levels (<10%) of degradation after 24 h. They also tested the degradation of ibuprofen by LAC adding different mediators, such as 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), 1-hydroxybenzotriazol (HBT), violuric acid (VA), and 3,5-dimethoxy-4-hydroxyacetophenol (DMHAP), but their addition failed to oxidize ibuprofen as well. Tran et al. [5] confirmed those results reporting low degradation values (35%) of ibuprofen (10 μg L^{-1}) when mediators were added. Marco-Urrea et al. [3] also published that the addition of cytochrome P450 inhibitors did not affect the degradation of that contaminant, thus concluding that an alternate enzyme system different from LACs, MnPs, and cytochrome P450 monooxygenases is involved in the first step of ibuprofen degradation. Rodarte-Morales et al., [6] carried out experiments for the biotransformation of three

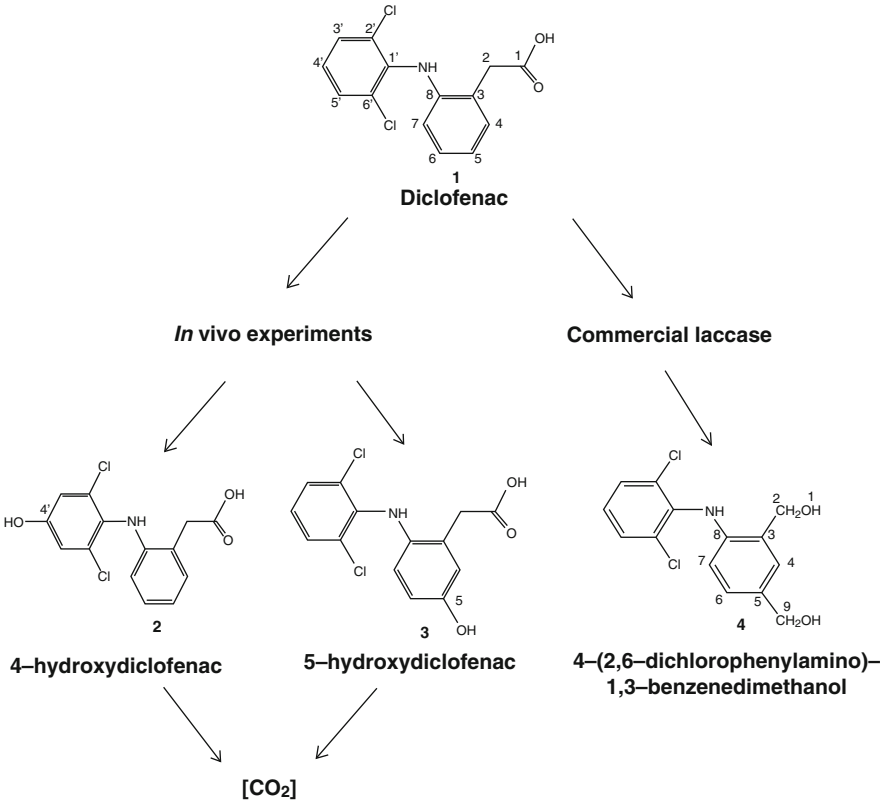

Fig. 2 Suggested degradation pathway of diclofenac by *T. versicolor* and its enzyme LAC (adapted from Marco-Urrea et al. [7])

pharmaceutical active compounds (diclofenac, naproxen, and ibuprofen) by *P. chrysosporium* in a fed batch stirred reactor under air and oxygen supply. They observed a complete removal of the contaminant before 24 h after the addition of ibuprofen pulses every 2 days and during 30 days. To sum up, ibuprofen was degraded for all the WRF so far studied. However, a more toxic transformation product was identified in the unique case where subproducts were elucidated. This raises the question whether the treatment by fungi would be a good strategy for the removal of this drug.

The capability of diclofenac degradation by *T. versicolor* was demonstrated by Marco-Urrea et al. [7]. In flask-scale experiments, they observed that almost complete diclofenac removal (>94%) occurred within the first hour with fungal pellets when the drug was added both at relatively high (10 mg L^{-1}) and environmentally relevant low (45 μg L^{-1}) concentrations in a defined liquid medium. In that treatment, 4′-hydroxydiclofenac and 5-hydroxydiclofenac were found as TPs (Fig. 2), which in 24 h disappeared leading to a decrease in ecotoxicity calculated by the Microtox test, thus suggesting the complete mineralization of

the drug. In addition, the authors carried out experiments to determine the possible role of some enzymes in the degradation of diclofenac. They concluded that the cytochrome P450 system plays a key role in the first step of diclofenac degradation, because the addition of inhibitors of this enzyme resulted in a marked inhibition of diclofenac removal. Purified LAC catalyzes the transformation of diclofenac (>95% degradation) to 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol but it was not the only enzyme responsible for diclofenac degradation in *T. versicolor* pellets, as different subproducts were detected when the whole fungus was employed. Tran et al. [5] confirmed the degradation of diclofenac by purified LAC, reaching more than 90% in 30 min. Likewise, Lloret et al. 8] reported complete degradation of diclofenac by purified LAC from *Myceliophthora thermophila* using mediators, and 83% removal without them. Other enzymes such as VP from *B. adusta* [9] and lignin peroxidase (LiP) from *P. chrysosporim* [10] have also shown the ability to remove this micropollutant, but none of those reports refers to the identification of the TPs. Hata et al. [11] reported the degradation of diclofenac with other WRF, in particular *Phanerochaete sordida*. The target compound, in a concentration of 30 mg L^{-1}, completely disappeared after 4 days, indicating a slower degradation rate than *T .versicolor* (totally removed after 1 h). *P. sordida* also produced the hydroxylated metabolites found in the degradation by *T. versicolor*, but in addition 4,5-dihydroxydiclofenac was identified as a metabolite. The ability of *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium* to degrade diclofenac was studied by Rodarte-Morales et al. [4]. Their results revealed a complete degradation with the three strains after 7 days, except by *Bjerkandera*. sp. R1, which accomplished the elimination in 4 days. More recently research was focused on the scale-up of the degradation process by *P. chrysosporium* in a fed batch stirred reactor under air and oxygen supply [6]. In that work, diclofenac was added into the reactor as pulses every 2 days during 30 days, resulting in complete removal, but metabolites were not analyzed. All these studies have demonstrated that diclofenac can be completely removed by WRF. The degradation of diclofenac by whole fungal cells produced hydroxylated subproducts, which at the end of the treatment had disappeared leading to a decrease in toxicity, suggesting diclofenac mineralization. In addition, researchers observed that many enzymatic systems are involved in the first step of the diclofenac transformation, which could also participate in different steps of its possible mineralization.

Naproxen, is an arylpropionic acid, widely used to relieve mild to moderate pain and in the treatment of osteo- and rheumatoid arthritis. Rodarte-Morales et al. [4] reported complete removal of naproxen (1 mg L^{-1}) in 7 days by *Bjerkandera* sp. R1 and *B. adusta,* and in 4 days by *P. chrysosporium*. On the other hand, Marco-Urrea et al. [12] performed degradation experiments with *T. versicolor* and the contaminant at 10 mg L^{-1} and 55 μg L^{-1}. In both cases naproxen was completely degraded in a few hours (approx. 6 h), with a higher removal rate for *T. versicolor*. 2-(6-Hydroxynaphtalen-2-yl)propanoic acid and 1-(6-methoxynaphthalen-2-yl) ethanone were identified as the main metabolites of naproxen, but after 6 h they disappeared, resulting in a final nontoxic medium, as determined by Microtox. On the other hand, Marco-Urrea et al. [12] and Tran et al. [5] reported the possible

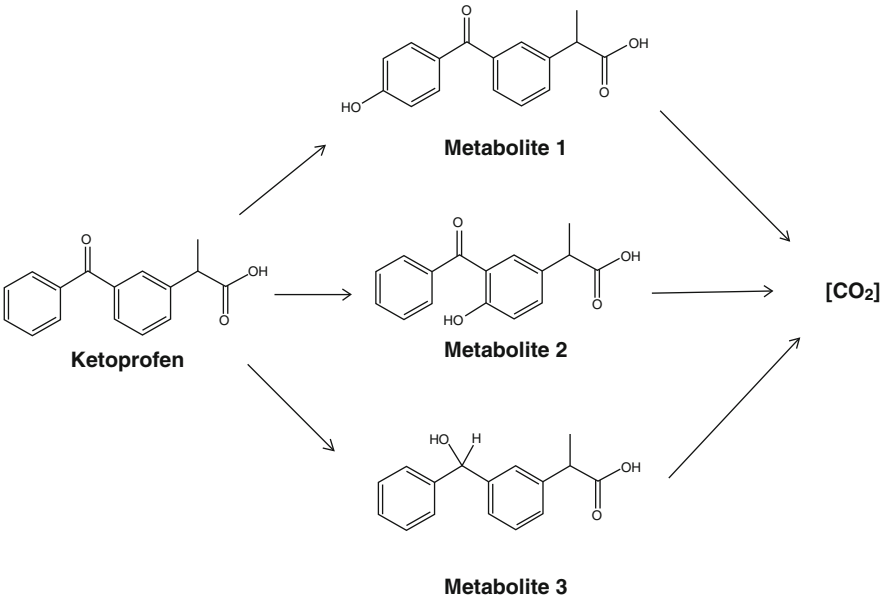

Fig. 3 Suggested degradation pathway of ketoprofen by *T. versicolor* (adapted from Marco-Urrea et al. [13])

implication of cytochrome P450 in the degradation, and the negligible elimination by purified LAC from *T. versicolor,* which was complete (95% after 30 min) when a mediator was added. The same behavior was observed by Lloret et al. [8] when LAC from *M. thermophila* was in contact with the contaminant. Similarly Eibes et al. [9] reported an 80% degradation of naproxen with versatile peroxidase (VP) from *B. adusta* after 7 h.

Ketoprofen is a therapeutic agent with analgesic and antipyretic effects, generally prescribed for toothaches that result in the inflammation of the gums. The fungal degradation of that drug has only been studied with *T. versicolor* by Marco-Urrea et al. [13], who reported the removal of ketoprofen to non-detectable levels in 24 h when it was added at 10 mg L^{-1}, whereas at low concentration of 40 μg L^{-1} it was almost completely removed after 5 h. During time-course degradation experiments, the metabolites 2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid (1), 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoic (2) acid and 2-(3-benzyl-4-hydroxyphenyl)-propanoic acid were identified (3) (Fig. 3). However, none of these intermediates was detected at the end of the experiment (7 days) except small amounts of 2-(3-benzyl-4-hydroxyphenyl)-propanoic acid (0.08 mg), which was lower in comparison with the amount detected at 24 h (0.53 mg), suggesting a possible mineralization of ketoprofen. The Microtox test showed minimal toxicity, with 15 min EC_{50} higher than 90%. Regarding the enzymatic degradation, LAC from *T. versicolor* is not involved in the first step of the degradation, as the addition of the LAC-mediator system failed to oxidize ketoprofen. In contrast, the cytochrome P450 inhibitor 1-aminobenzotriazole produced a reduction in the

ketoprofen degradation rate, suggesting that the first oxidation step of the ketoprofen is cytochrome P450 mediated [5, 13].

Mefenamic is a nonsteroidal anti-inflammatory drug used to treat pain, including menstrual pain. Hata et al. [11] treated that drug with *P. sordida*, and obtained a 90% reduction in mefenamic acid concentration (initial concentration 24 mg L^{-1}) after 6 days. The system produced four metabolites, identified as 3′-hydroxymethyl-mefenamic acid, 3′-hydroxymethyl-5-hydroxymefenamic acid, 3′-hydroxmethyl-6′-hydroxymefenamic acid, and 3′-carboxymefenamic acid. Moreover, the authors confirmed that the fungus almost completely removed the acute lethal toxicity of mefenamic towards the freshwater crustacean *Thamnocephalus platyurus* after 6 days of treatment, suggesting that the metabolites are less toxic than the parental compound.

Other anti-inflammatory drugs whose degradation by fungi has been studied include fenoprofen, indomethacin, and propyphenazone. Tran et al. [5] evaluated the degradability of these pharmaceuticals (10 μg L^{-1} of each) by cultures of *T. versicolor*. After 48 h of incubation, they observed complete degradation of fenoprofen and indomethacin and approximately 75% for propyphenazone. In addition, enzymatic assays with LAC from *T. versicolor* resulted in almost complete degradation for fenoprofen (>90% after 3 h) and indomethacin (>90% after 30 min), but negligible levels of degradation (25% after 3 h) were achieved in the case of propyphenazone. However, neither analysis of the TPs nor toxicity assays were performed.

To summarize, the anti-inflammatory drugs studied are degraded at high rates by fungi (about some hours). Studies carried out with inhibitors of intracellular enzyme system (cytochrome p450) and purified extracellular enzymes like LAC, LiP, MnP, and VP revealed that diverse enzymatic systems can act simultaneously to degrade anti-inflammatory drugs when whole fungal cells are used. In general, the first step in the degradation of these drugs involves the production of hydroxylated metabolites. However, in some cases such as ibuprofen, the TPs cannot be subsequently degraded, producing an increase in the toxicity, but in other cases (ketoprofen, diclofenac) the hydroxylated products are degraded leading to a decrease in toxicity, suggesting the mineralization of the compound.

2.2 Psychiatric Drugs

Carbamazepine is one of the most widely prescribed drugs for the treatment of epilepsy, trigeminal neuralgia, and some psychiatric diseases. The degradation of carbamazepine has concerned the scientific community during the last years due to its barely or nondegradability in conventional WWTPs (see [2], this volume). In a degradation screening with four WRF (*T. versicolor*, *G. lucidum*, *I. lacteus*, and *P. chrysosporium*), Marco-Urrea et al. [3] reported the elimination of this pollutant at 10 mg L^{-1} after 7 days only with *T. versicolor* and *G. lucidum*, achieving removals of 58% and 47%, respectively. The application of enzymatic degradation

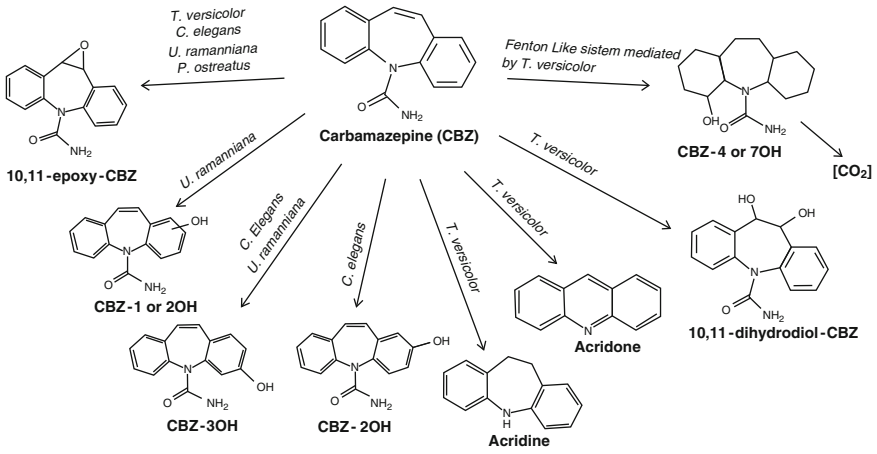

Fig. 4 TPs in carbamazepine degradation by different fungi

using MnP and commercial LAC-mediator system failed to improve the degradation, suggesting that the extracellular ligninolytic enzyme system did not play a role in the first step of carbamazepine degradation [5, 11]. However, Hata et al. [11] increased the extent of carbamazepine degradation by pure LAC performing repeated treatments with the addition of LAC-mediator pulses every 8 h, achieving a removal of 60% after 48 h. During this process they found 10,11-dihydro-10,11-epoxycarbamazepine and 9(10H)-acridone as degradation products at the end of the experiment (48 h). On the other hand, experiments using the cytochrome P450 inhibitors 1-aminobenzotriazole and piperonyl butoxide produced a reduction in the removal, thus indicating a possible role of this enzymatic complex in the oxidation of carbamazepine by *T. versicolor* and *Pleurotus ostreatus* [5, 14]. Jelić et al. [15] confirmed the degradation of this psychiatric drug by *T. versicolor* pellets obtaining a removal of 94% after 6 days when the contaminant was added at 9 mg L^{-1}, while at close environmentally relevant concentrations of 50 μg L^{-1}, 61% of the contaminant was degraded in 7 days. In experiments at 9 mg L^{-1} the compounds 10,11-dihydro-10,11-epoxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, acridone, and acridine were found as TPs (Fig. 4). Kang et al. [16] reported 10,11-dihydro-10,11-epoxycarbamazepine as the major by-product in the transformation of carbamazepine by *Umbelopsis ramanniana* and *Cunninghamella elegans,* but also detected two hydroxylated by-products (2-and 3-hydroxy carbamazepine). Other experiments with *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium* were carried out by Rodarte-Morales et al. [4]. Complete degradation was achieved by all the fungi after 14 days. Nevertheless, a slow removal was observed during the first week of assay (<33%), which agrees with previous works from Marco-Urrea et al. [3] with *P. chrysosporium*. A study by Golan-Rozen et al. [14] revealed 99% removal of carbamazepine by *P. ostreatus* with subsequent transformation to 10,11-epoxycarbamazepine. In addition, the use of inhibitors of MnP resulted in lower elimination levels (10–30%), which suggests MnP

participation in the transformation process. The same authors also suggested that the carbamazepine removal might be partially attributed to the activity of VP, contrary to studies with VP from *B. adusta*, which have shown undetectable removal yields [9]. Low removal yields (<10%) were also obtained with LiP from *P. chrysosporium* [10].

A biological Fenton-like system mediated by *T. versicolor* was used as a new treatment to degrade carbamazepine [17]. That treatment consisted in producing extracellular oxidizing species responsible for the degradation of the pollutants, through a quinone redox cycling mechanism catalyzed by an intracellular quinone reductase and any of the ligninolytic enzymes of *T. versicolor*, after addition of the lignin-derived quinone 2,6-dimethoxy-1,4-benzoquinone and Fe^{3+}-oxalate in the medium. In that treatment, 80% of the contaminant was degraded. In addition, researchers found two hydroxylated isomers of carbamazepine, named 4- and 7-hydroxycarbamazepine, which completely disappeared at the end of the incubation period, regarded by the authors as a possible mineralization indicator.

To summarize, carbamazepine can be completely degraded by some fungi like *T. versicolor*, *P. ostreatus*, *U. ramanniana,* and *C. elegans*. The intracellular enzyme system cytochrome P450 seems to play a key role in the first step of the process. It is worth mentioning that the first step of the oxidative breakdown of carbamazepine in the cytochrome P450-mediated metabolism in humans is the oxidation to 10,11-epoxycarbamazepine [18] and consecutively to 10,11-dihydro-10,11-dihydroxycarbamazepine and other hydroxylated compounds [19]. The identification of these metabolites in the studies above mentioned might serve as an additional proof of the involvement of cytochrome P450 in the degradation of carbamazepine. Experiments with MnP and VP indicated that the same enzymes in different fungi can or not participate in the degradation of carbamazepine. Finally, the reports indicate the accumulation of TPs in the medium; however, no analyses of toxicity were carried out, and subsequently the suitability of the treatment is in doubt.

On the other hand, the continuous treatment of carbamazepine has been described by Jelić et al. [15] in an air-pulsed fluidized bed bioreactor (FBR) with *T. versicolor* pellets. The system achieved a reduction of 54% in the outflow respect to the inflow concentration (approximately 200 $\mu g\ L^{-1}$) at the steady state (day 25) with a hydraulic retention time (HRT) of 3 days, corresponding to a degradation rate of 11.9 $\mu g\ g^{-1}$ dry weight fungal pellets day^{-1}. By-products were not found during the process, probably due to the low concentration of the contaminant. Parallel experiments in a batch operated bioreactor resulted in high degradation (96%) after 2 days with the release of 10,11-dihydro-10,11-epoxycarbamazepine as the major transformation product. The authors reported low toxicity values (Microtox test) in both bioreactor treatments. However, in both continuous and batch operation the values of toxicity were higher than those in the control (containing only carmabazepine and medium) indicating that TPs may be more toxic than the parent compound.

Citalopram and fluoxetine are antidepressant drugs widely used in human medicine and very persistent in WWTPs. However, there are few studies about the degradation of these pharmaceuticals by fungi and none of them identifies TPs.

Rodarte-Morales et al. [4] observed the complete degradation of citalopram by the WRF strains *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium* after 14 days. Nevertheless, the degradation rate was higher with *B. adusta,* as 58% of the citalopram was degraded after 4 days, while the other fungi removed less than 10% after 4 days. Regarding degradation of fluoxetine, the authors reported partial degradation by all the fungi tested, showing removal values ranging from 23 to 46% after 14 days. Eibes et al. [9] studied the degradation of these two pharmaceuticals by VP from *B. adusta* with poor success: elimination of 18% and 10% for citalopram and fluoxacin, respectively.

The degradation of diazepam, a tranquilizer drug, has also been assessed with *Bjerkandera* sp. R1, *B. adusta* and *P. chrysosporium* [4]; however, none of the WRF was able to completely degrade this compound, obtaining degradation yields between 39% and 57% after 14 days of incubation.

All the aforementioned psychiatric drugs are partially degraded by WRF in long periods (days) in comparison with previous anti-inflammatory drugs described (some hours). The major metabolite from carbamazepine was consistently 10,11-epoxycarbamazepine; however, other TPs, especially hydroxylated compounds, were identified during the fungal treatments. In the only study where toxicity was assessed, the TPs of carbamazepine seemed to be more toxic than the predecessor.

2.3 Lipid Regulators

Clofibric acid is the main pharmacologically active metabolite of the lipid-lowering drugs, clofibrate, etofibrate, and etofylline clofibrate. This compound shows low removal efficiency, approx. 28%, in conventional wastewater treatment technologies [20], and its ubiquitous presence has been demonstrated in the environment [21]. The first evidences for clofibric degradation by fungi were described by Marco-Urrea et al. [3] and Tran et al. [5]. Their results showed that clofibric acid (10 mg L^{-1}) was almost totally degraded by *T. versicolor* after 7 days. Other WRF (*G. lucidum, I. lacteus* and *P. chrysosporium*) failed to degrade the contaminant. The failure of MnP and a LAC-mediator system to degrade clofibric acid led to rule out the participation of these extracellular fungal enzymes in the first step of clofibric acid degradation. In contrast, experiments with active fungal cultures and cytochrome P450 inhibitors suggested a key role of this enzymatic complex in the oxidation of clofibric acid by *T. versicolor*. This fungus was also applied in the treatment of clofibric acid by the induction of oxidizing agents via quinone redox cycling [17]. This approach resulted in the elimination of more than 80% of the drug after 6 h when added at an initial concentration of 10 mg L^{-1}. The authors found a clofibric acid hydroxylated derivative as the main TP of the degradation process, which totally disappeared by the end of the experiment, thus suggesting the possible mineralization of the drug. Anyhow, toxicity was not assessed to evaluate the global applicability of the treatment.

Continuous bioreactor treatment was developed for the removal of clofibric acid by *T. versicolor* (data not yet published). That bioreactor was operated in

continuous mode with a HRT of 4 days and a clofibric acid concentration in the inflow close to real concentrations (160 μg L^{-1}). The reactor achieved an 80% reduction in the concentration in the outflow at the steady state, indicating a clofibric acid degradation rate of 16.5 μg g^{-1} dry weight fungal pellets day^{-1}. During the treatment only 2-(4-chlorophenozy)-2-(hydroxymethyl)propanoic acid was detected as a major metabolite, confirming the degradation of the contaminant. However, that metabolite was not degraded by the fungus. In addition, results in the standard toxicity bioassay (Microtox test) indicated that the treated effluent was more toxic than the initial inflow, suggesting the production of a more toxic metabolite, which casts doubts on the suitability of the treatment.

Gemfibrozil also belongs to the group of drugs known as fibrates, employed to lower lipid levels. Tran et al. [5] studied gemfibrozil degradation by *T. versicolor* active cultures and its LAC. The researchers obtained a removal of 75% after 7 days when the whole fungus was inoculated. In addition, less than 30% was degraded in experiments with crude and commercial LAC, indicating that these extracellular enzymes are not involved in the first step of gemfibrozil degradation and suggesting that the oxidation of the target compound is done by intracellular enzymes.

Summarizing, intracellular enzyme cytochrome P450 seems to play an important role in the oxidation of lipid regulators while extracellular ligninolytic enzymes are not involved in their degradation. In addition, for clofibric acid hydroxylation products of higher toxicity were obtained after the treatment with fungal cells and also in fungal-mediated Fenton-like process, which in the former case was accumulated leading to an increase in the toxicity, but disappeared in the latter, suggesting mineralization.

2.4 Antibiotics

2.4.1 Sulfonamides

Among antibiotics, the group of the sulfonamides is, comparatively, one of the most studied in terms of fungal degradation. Enzymatic-mediated transformation has been demonstrated with LAC and VP. Schwarz and co-authors [22] reported transformations of 10%, 75%, and 96% for sulfanilamide, sulfadimethoxine, and sulfapyridine, respectively, after 15 days with commercial LAC from *T. versicolor* (48,000 U L^{-1}). Aniline was confirmed as a breakdown product of sulfapyridine, while the SO_2 extrusion products 4-(2-imino-1-pyridyl)aniline and 4-(6-imino-2,4-dimethoxypyrimidin-1-yl)aniline were determined for sulfapyridine and sulfadimethoxine, respectively. An additional metabolite with elemental composition $C_{12}H_{11}N_3O$ was assigned to sulfapyridine transformation; however, only tentative structures were proposed: 4-(2,3-diaminophenyl)imino-cyclohexa-2,5-dien-1-one and *N*-(3-pyridyl)pyridine-3-sulfonamide. Degradation was also achieved in shorter periods (hours) in lower LAC activity systems (50–350 U L^{-1}) in the case of sulfamethazine [23], sulfapyridine, and sulfathiazole [24], though it took place in

the presence of LAC mediators. The desulfonated was a common metabolite obtained from the three sulfonamides. In addition, desamino-sulfamethazine and hydroxy-sulfamethazine were identified for sulfamethazine, while a formyl intermediate obtained after the loss of the pyrimidine/thiazole group was recognized from the transformation of sulfapyridine and sulfathiazole. Similarly, the use of VP from *B. adusta* (200 U L^{-1}) resulted in 80% transformation of sulfamethoxazole in 7 h [9]. Intermediate metabolites included 3-amino-5-methylisoxazole and a possible dimerization product of sulfamethoxazole, while anions such as nitrate, nitrite, and sulfate were detected as well.

Degradation of sulfonamides has also involved the use of whole cell fungal systems. García-Galán et al. [23] studied the degradation of sulfamethazine by *T. versicolor* pellets in liquid medium, obtaining almost complete removal after 20 h. Metabolites from in vivo experiments included the formylated (*N*-(4,6-dimethylpyrimidin-2-yl)-4-(formylamino)benzenesulfonamide) and desulfonated (*N*-(4,6-dimethylpyrimidin-2-yl)benzene-1,4-diamine) intermediates, the latter being also identified from enzymatic degradation. Similar work with *T. versicolor* by Rodríguez-Rodríguez et al. [24] led to the elucidation of several degradation intermediates of sulfapyridine and sulfathiazole. From the former sulfonamide, the recognized metabolites included the desulfonated, formyl and hydroxyl intermediates, formyl-desulfonated sulfapyridine, and a formyl-hydroxylated metabolite produced after the loss of the pyridine ring (Fig. 5). As in the case of sulfamethazine, the desulfonated was the only intermediate found both in cell-mediated and enzymatic degradation. For sulfathiazole the formyl intermediate was found, as well as a more transformed metabolite result of the hydroxylation of the desaminated and desulfonated parental compound. In those studies the possible role of the cytochrome P450 was also demonstrated in the transformation of sulfamethazine and sulfathiazole, but it was unclear in the case of sulfapyridine. In addition, the simultaneous removal of the three latter sulfonamides was achieved under continuous operation in a 1.5 L FBR containing *T. versicolor* pellets. Elimination was >94% for each sulfonamide at a HRT of 72 h [24].

The complete degradation of sulfamethoxazole was also reported within 14 days with *P. chrysosporium*, *Bjerkandera* sp. R1 and *B. adusta* [4], although, contrary to the reports of enzymatic transformation, metabolites were not identified. Partial removal (from 30% to 55%) of sulfamethoxazole from activated-sludge-mixed liquor and the effluent of a WWTP was demonstrated at bench scale within 5 days with *P. chrysosporium* propagules entrapped in a granular bioplastic formulation [25]. This approach was also successful in the partial elimination of other kinds of antibiotics, e.g., ciprofloxacin (see below) and the macrolide erythromycin.

Both enzymatic and whole cell-mediated transformation of sulfonamides has been described, usually with high removal efficiencies and relatively short treatments. Although different metabolites have been elucidated, no clear pathways have been defined; however, the desulfonated metabolites have been widely identified (with LAC and fungal cells) along with the products of hydroxylation, formylation, and deamination reactions, and combinations of them.

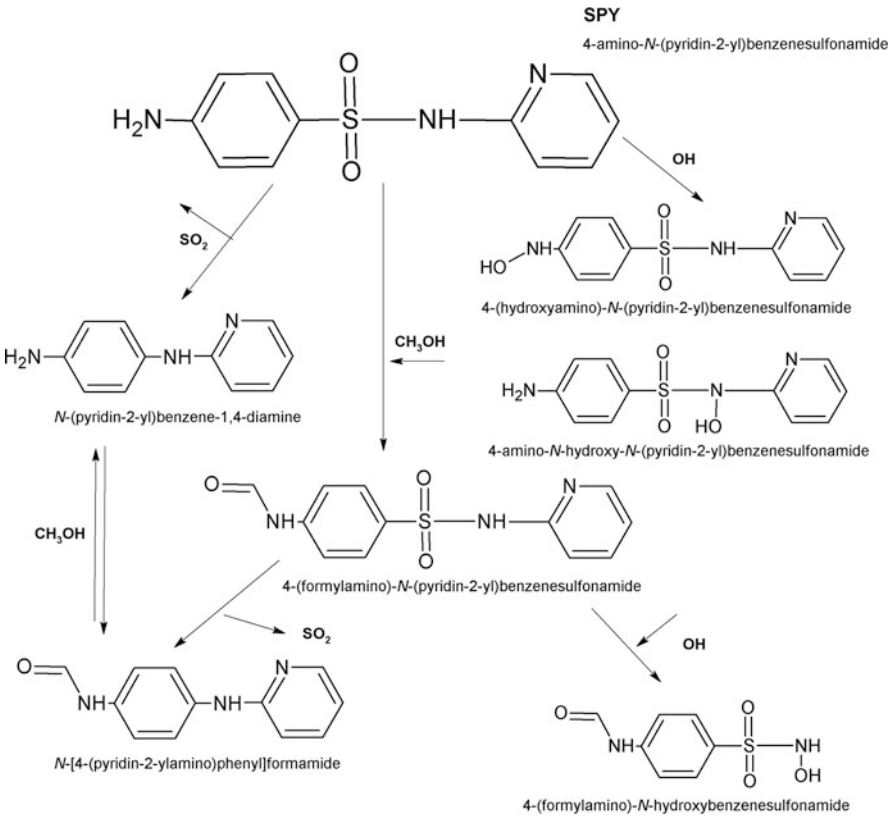

Fig. 5 TPs of sulfapyridine after degradation by *T. versicolor* (adapted from Rodríguez-Rodríguez et al. [24])

2.4.2 Tetracyclines

Fungal degradation of tetracyclines has been only described by enzymatic means but not with fungal cells. Wen and co-workers [26] described the use of crude lignin peroxidase from *P. chrysosporium* (40 U L^{-1}) which produced a 95% removal of tetracycline and oxytetracycline in 5 min; the degradation was pH dependant and was enhanced by increasing concentrations of veratryl alcohol and H_2O_2. Similarly, Suda et al. [27] reported the complete elimination of tetracycline and chlortetracycline in 15 min, and doxycycline and oxytetracycline in 1 h by LAC from *T. versicolor* in the presence of HBT. Transformation resulted in the complete loss of inhibitory effect towards *Escherichia coli*, *Bacillus subtilis,* and the green algae *Pseudokirchneriella subcapitata*. Nonetheless, the identification of TPs of these therapeutic drugs by fungal enzymes has not been yet reported.

2.4.3 Quinolones

Transformation of quinolones by fungi, especially fluoroquinolones, has received some attention. In this respect the most widely studied antibiotic is ciprofloxacin. Wetzstein et al. [28] described the transformation of >50% ciprofloxacin by *Gloeophyllum striatum* after 90 h, with some production of $^{14}CO_2$ from ^{14}C-labeled structures and reduction in antibacterial activity. Eleven metabolites were elucidated, including mono- and dihydroxylated congeners, an isatin-type compound (proving elimination of C-2) and metabolites indicating both elimination and degradation of the piperazinyl moiety. In a similar study with *T. versicolor*, Prieto et al. [29] achieved >90% elimination after 7 days and 98% after 20 h with whole cells and LAC/mediators, respectively. The role of the cytochrome P450 enzymatic complex was also implied in the transformation, and six intermediates were elucidated: three previously described by Wetzstein et al. [28], plus a new product of the piperazinyl moiety breakdown. The other two metabolites corresponded to dimeric products formed by a C–C covalent bond and followed by several transformations including the breakdown of the piperazinyl group, removal of a cyclopropyl group and hydroxylation.

Metabolism of ciprofloxacin by *Pestalotiopsis guepini* yielded *N*-acetylciprofloxacin (the most abundant metabolite), desethylene-*N*-acetylciprofloxacin, *N*-formylciprofloxacin, and 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxilic acid [30]. Other transformation reports include the regioselective production of *N*-acetylciprofloxacin by *Mucor ramannianus* [31] and the optically active 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl ciprofloxacin by *Trichoderma viride* [32].

Partial mineralization of enrofloxacin, ranging from 5% to 53%, was demonstrated by different wood-rotting fungi grown on wheat straw after eight weeks, including several strains of the brown-rot fungus *G. striatum* (the most efficient degrader) and the white-rot fungi *I. lacteus*, *P. chrysosporium*, and *Stropharia rugosoannulata* [33]. Further work with *G. striatum* by Wetzstein and co-workers [34] led to the identification of several metabolites, including 3-, 6-, and 8-hydroxylated congeners of enrofloxacin (with replacing of the fluorine by a hydroxyl group in one case); 5,6- (or 6,8-), 5,8-, and 7,8-dihydroxylated congeners, prone to autoxidative transformation; products of the cleavage of the heterocyclic core of enrofloxacin (an isatin-type compound and an anthranilic acid derivative) and products of both elimination and degradation of the piperazinyl moiety (1-ethylpiperazine, the 7-amino congener and desethylene-enrofloxacin). Four different degradation routes were proposed (Fig. 6), initiated by either oxidative descarboxylation, defluorination, hydroxylation at C-8 or oxidation of the piperazinyl moiety. Wetzstein et al. [35] subsequently described the patterns of metabolites produced from enrofloxacin by seven basidiomycetes indigenous to agricultural sites. Their findings showed similar patterns of major metabolites, but differed considerably from those obtained from *G. striatum*, due particularly to the absence of monohydroxylated congeners and a greater variety of metabolites derived from the modification of the piperazine moiety. Metabolites comprised

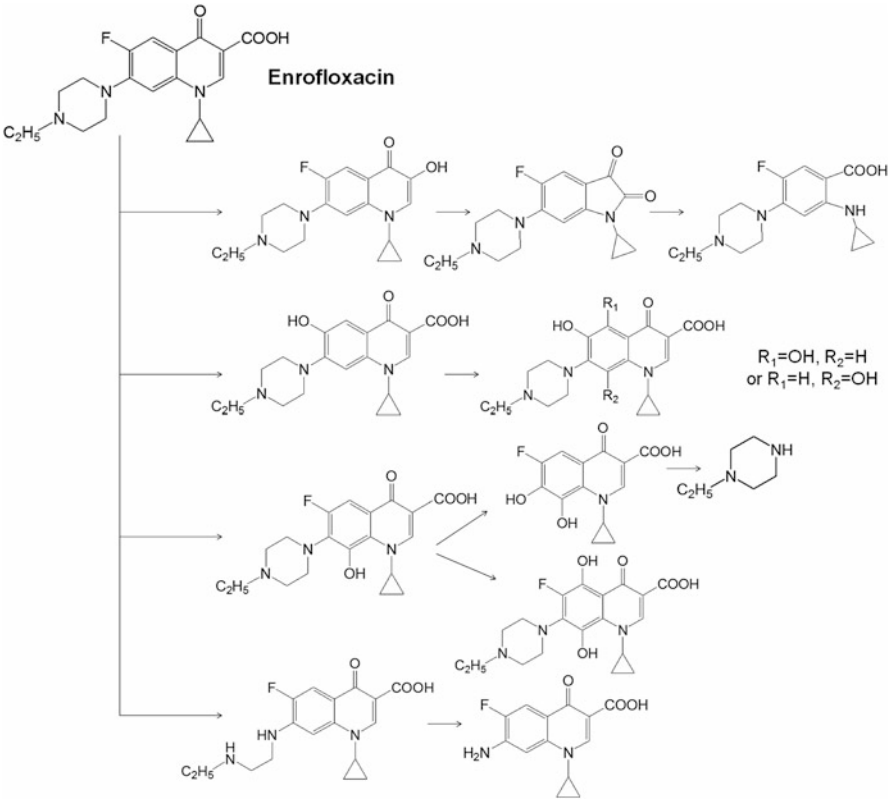

Fig. 6 Main routes of degradation of enrofloxacin employed by *G. striatum* (adapted from Wetzstein et al. [34])

ethylpiperazine moieties with oxido-, hydroxyl-, oxo-, and acetoxy groups, or showing partial degradation, linked to the unmodified, oxidatively decarboxylated, or multiply hydroxylated core of enrofloxacin and to isatin- and anthranilic acid-type enrofloxacin congeners. Metabolites with hydroxylated aromatic rings likely suffered additional ring cleavage to form four potential oxidizable *o*-aminophenol and one catechol-type intermediates. The transformation of this fluoroquinolone was also studied with *M. ramannianus*, with 78% removal of the parent compound after 21 days and the identification of enrofloxacin *N*-oxide, *N*-acetylciprofloxacin, and desethylene-enrofloxacin as metabolites, the former being the most abundant [36].

Norfloxacin transformation has been demonstrated by *P. guepini* [30] and *T. viride* [32]. In the first case, the metabolites identified included *N*-acetylnorfloxacin as the major intermediate, desethylene-*N*-acetylnorfloxacin, *N*-formylnorfloxacin, and 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. For *T. viride*, 4-hydroxy-3-oxo-4-vinylcyclopent-1-enyl norfloxacin was elucidated as an intermediate. In both reports, the intermediates were analogous

to those derived from ciprofloxacin by the same fungi. Similarly, Prieto et al. [29] showed the degradation of norfloxacin by enzymatic means (LAC with mediators, 33% after 20 h) and whole cells of *T. versicolor* (>90% after 7 days). Three metabolites resulting from the transformation of the piperazinyl moiety were identified, with the accumulation of one of them, 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxilic acid.

Transformation of the fluoroquinolones sarafloxacin to *N*-acetylsarafloxacin and desethylene-*N*-acetylsarafloxacin by *M. ramannianus* [37] and flumequine to two diasteroisomers of 7-hydroxyflumequine and 7-oxoflumequine by *C. elegans* [38] was also reported. Meanwhile, the quinolone-like antibiotic cinoxacin produced 1-ethyl-1,4-dihydro-3-(hydroxymethyl)[1,3]dioxolo[4,5-*g*]cinnolin-4-one and 1-ethyl-1,4-dihydro-6,7-dihydroxy-3-(hydroxymethyl)cinnolin-4-one when transformed by *Beauveria bassiana* [39].

Transformation of quinolones has been reported especially with fungal cells of diverse species, even obtaining partial mineralization, though LAC also proved relative efficiency in the removal of some of them. The abundance of metabolites described, many times analogous from one compound to another, has led to the proposal of several degradation pathways, which usually involve breakdown of the parental molecules and reactions of hydroxylation, acetylation, or formylation, among others.

2.5 Other Antimicrobial Agents

Triclosan is a broad spectrum antibacterial agent with antifungal and antiviral properties, widely employed in personal care products such as soaps, shampoos, toothpastes, and cosmetics [40]. Fungal-mediated degradation studies have been mainly performed by means of enzymatic processes, although a couple of whole cell transformation reports are available.

Inoue et al. [41] studied the elimination of triclosan by MnP from *P. chrysosporium*, and LAC from *T. versicolor*. MnP (0.5 nkat mL^{-1}) was the most efficient, achieving a removal of 94% after 30 min and almost complete after 60 min, while LAC (2.0 nkat mL^{-1}) and LAC-HBT removed triclosan at 51% and 66%, respectively. Moreover, the treatment with MnP resulted in the complete loss of bacterial inhibition activity after 30 min and reduced the algal growth inhibition by 90% after 60 min. Similar results were obtained by Kim and Nicell [40] with LAC from *T. versicolor* after 6 h, with concomitant reduction in toxicity even without mediators.

Immobilized enzymes, particularly LAC, have been employed in the degradation of triclosan. The immobilization of LAC from *Coriolopsis polyzona* through the formation of cross-linked enzyme aggregates (CLEAs) and their subsequent use in an FBR for the removal of endocrine disrupting compounds [42] produced the complete removal of triclosan, nonylphenol, and bisphenol A (5 mg L^{-1} each) at a HRT of 150 min. The application of CLEAs in a perfusion basket reactor [43]

resulted in the continuous elimination of the above-mentioned mixture at 85% with a HRT of 325 min during 7 days. Moreover, the system was able to eliminate >95% triclosan and nonylphenol at 100 mg L^{-1} with a HRT of 400 min. The immobilization of VP from *B. adusta* in the form of CLEAs, although successful in the removal of other endocrine disrupting compounds, could only eliminate 26% of triclosan after 10 min in batch experiments [44]. Immobilized LAC from *C. polyzona* on the diatomaceous earth support Celite® R-633 [45] was employed as a catalyst for the removal of triclosan (5 mg L^{-1}) in a packed-bed reactor (PBR). Its operation in repeated batch treatments resulted in the complete elimination of the antimicrobial agent at a contact time of less than 200 min during five cycles. Similarly, the conjugation of LAC from *T. versicolor* with the biopolymer chitosan [46] produced a high-stable solid biocatalyst which degraded triclosan from aqueous solutions (100% after 6 h) with a higher efficiency than free LAC (60% after 6 h).

The identification of TPs derived from triclosan degradation has been determined through experiments with LACs from *G. lucidum* and *C. polyzona* and whole cell cultures of *T. versicolor* and *Pycnoporus cinnabarinus*. Enzymatic degradation with LAC from *G. lucidum* (5,000 U L^{-1}) removed 57% triclosan within 24 h and produced dimers and trimers of the parental compound. The degradation was enhanced by the addition of LAC mediators (HBT or syringaldehyde, SYD), reaching 90% removal, and resulted in the formation of different intermediates: 2,4-dichlorophenol and dechlorinated forms of 2,4-dichlorophenol [47], thus suggesting two mechanisms of triclosan removal by LAC, oligomerization in the absence of mediators, and ether bond cleavage with subsequent dechlorination in the presence of mediators (Fig. 7). The production of high-molecular-weight metabolites through a radical polymerization mechanism was also demonstrated by Cabana et al. [48] who identified dimers, trimers, and tetramers, formed through C–C and C–O bonds in enzymatic degradation with LAC from *C. polyzona*. In this study, the removal of triclosan was 65% with a LAC activity of 100 U L^{-1}, either at 4 or 8 h of treatment. The same authors also described the production of triclosan oligomers as a result of removal with LAC from *T. versicolor* [46].

Hundt et al. [49] described the transformation of triclosan by whole cell cultures of *T. versicolor* and identified the conjugates 2-*O*-(2,4,4′-trichlorodiphenyl ether)-β-D-xylopyranoside and 2-*O*-(2,4,4′-trichlorodiphenyl ether)-β-D-glucopyranoside, and in coincidence with the enzymatic treatment described by Murugesan et al. [47], 2,4-dichlorophenol. Under the same cultivation conditions, *P. cinnabarinus* also produced the glucoside conjugate determined for *T. versicolor* and an additional product corresponding to the methylation of triclosan, identified as 2,4,4′-trichloro-2′-methoxydiphenyl ether. The conjugates exhibited reduced cytotoxicity and antimicrobial activity than triclosan. Elimination of triclosan by fungal cells was also evaluated by Cajthalm et al. [50], although the identification of metabolites was not performed. From seven WRF tested, all but one (*B. adusta*) significantly removed the antimicrobial agent within 14 days, with a progressive reduction in the estrogenic activity throughout the process.

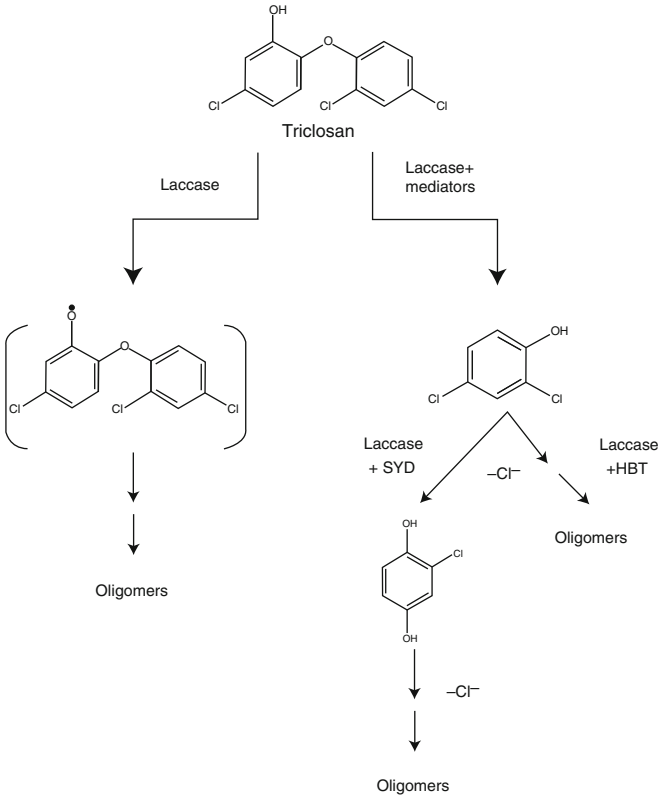

Fig. 7 Proposed pathways for the degradation of triclosan by *G. lucidum* (adapted from Murugesan et al. [47])

Either by enzymatic means or with fungal cells, the transformation of triclosan seems to elapse through oligomerization and the production of 2,4-dichlorophenol. Other different metabolites have been identified depending on the process.

Phenothiazine and its derivates have been used as anthelmintic agents, as well as antiarrhythmic drugs, coronary vasodilators, and antidepressants. Parshikov et al. [51] reported the biotransformation of *N*-acetylphenothiazine, a phenothiazine derivate with an *N*-caronyl substituent, with three fungi, *Aspergillus niger*, *Cunninghamella verticillata*, and *Penicillium simplicissimum*, usually employed as model organisms in studies of drug bioconversion processes in mammalian systems. In the first step of the degradation all the fungi produced *N*-acetylphenothiazine sulfoxide and phenothiazine, while in a second step the last metabolite was transformed to phenothiazine sulfoxide. Furthermore, *C. verticillata* also could degrade phenothiazine to phenothiazin-3-one and phenothiazine-*N*-glucoside.

Oseltamivir, better known as Tamiflu, is the antiviral worldwide employed for the treatment of influenza (flu). Due to the importance of that drug for treating regular seasonal flu and its potential use in the case of pandemic flu scenarios, it is

not strange that oseltamir has also been detected in high concentrations in the environment during flu season. In novel fungal-mediated approaches, Accinelli et al. [25] evaluated the efficiency of a granular bioplastic formulation which entraps propagules of *P. chrysosporium* for the removal of oseltamivir from wastewater samples. Their results showed a significant increase in the removal, obtaining two times more elimination values after 30 days in the bioremediated wastewater compared to the controls. This work demonstrated the success of the bioplastic matrix to facilitate the adaptation of the fungus to unusual environments such as wastewater. However, the identification of TPs was not carried out and the toxicity was not assessed; hence, the suitability of the treatment could not be evaluated.

Artemisinin is a naturally occurring sesquiterpene lactone that shows promising attributes as the basis of an anti-malarial agent. However its toxicity and water insolubility limit the application of the drug. This issue could be overcome with the production of semisynthetic derivatives of artemisinin, such as 7β-hydroxyartemisinin, which increase the antimalarial activity. For this reason, Parshikov et al. [52–54] carried out experiments to examine the transformation of artemisinin to 7β-hydroxyartemisinin with different fungi. The authors achieved a 79% conversion with *C. elegans* but secondary TPs were also found: 7β-hydroxy-9α-artemisinin (6%), 4α-hydroxy-1-deoxoartemisinin (5.4%), and 6β-hydroxyartemisinin (7%). Similarly, three strains of *M. ramannianus* were able to metabolize artemisinin into significant yields of hydroxylated metabolites, in particular 7β-hydroxyartemisinin (88%) and 6β-hydroxyartemisinin (1%), 4α-hydroxy-1-deoxoartemisinin (6%), and 7α-hydroxyartemisinin (5%). The same research group also assessed the transformation of artemisinin by the fungi *Eurotium amstelodami* and *A. niger* and identified two major TPs, 5β-hydroxyartemisinin and 7β-hydroxyartemisinin from both organisms: 63% and 32% yields, respectively, from the extract of *E. amstelodami*, and 80% and 19%, respectively, from the extract of *A. niger*. These results suggest that the fungal transformation of artemisinin takes place through the production of hydroxylated compounds.

2.6 β-Blockers

β-Blockers comprise a group of therapeutic agents employed for the treatment of cardiac arrhythmias, cardioprotection after myocardial infarction, and hypertension. Propranolol, first successful β-blocker developed, and atenolol are among the most commonly used β-blockers for cardiovascular diseases. Due to their long-term use in Europe and North America and their subsequent occurrence in the aquatic environment, they were selected by Marco-Urrea et al. [17] to be degraded by biological Fenton-like system mediated by *T. versicolor* (see Sect. 2.2), developed to degrade emerging contaminants. With an initial pharmaceutical concentration of 10 mg L^{-1}, they achieved degradations above 80% after 6 h of incubation for atenolol and propranolol. The main degradation metabolites produced in the redox

cycling treatment were identified as hydroxylated derivatives for both compounds. These metabolites were accumulated in the medium and the toxicity was not assessed.

2.7 Estrogens

Degradation of estrogens has not only been studied especially by enzymatic means, but there are also some reports of transformation by whole fungal cells.

VP from *B. adusta* was shown to completely degrade estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2) after 5–25 min, even at low VP activity (10 U L^{-1}) [9]. MnP from *P. chrysosporium* and LAC from *T. versicolor* in the presence of HBT degraded E2 and EE2 within 1 h as well, with an 80% reduction in their estrogenic activity. Extending the treatment to 8 h resulted in the total removal of the estrogenic activity [55]. Similar results were obtained with MnP from *P. sordida* and LAC from *T. versicolor* in the degradation of E1, as the estrogen was removed after 1 h of treatment with the complete elimination of estrogenic activity within 2 h [56].

The oxidation of E2 was described by Nicotra and co-workers [57] in two different LAC-mediated systems: in organic solvents with enzyme from *Myceliophthora* sp. (previously adsorbed on glass beads) and a biphasic system with enzyme from *Trametes pubescens*. In both cases, the production of C–C and C–O dimers occurred, attributed to the generation of oxygen radicals that can delocalize to carbon-located radicals, thus producing reactive monomer intermediates. The dimers could suffer further oxidation leading to the generation of oligomers and polymers (Fig. 8).

Enzymatic degradation was tested with commercial LAC from *M. thermophila* (2,000 U L^{-1}). E2 and EE2 were completely degraded even in the absence of mediators after 3 and 5 h, respectively, and after 1 h in the presence of some mediators. For E1 total removal was achieved in 8 h in the presence of VA and >70% for the other mediators after 24 h, whereas elimination reached 65% in the absence of mediators [8]. The immobilization of this enzyme by encapsulation in a sol–gel matrix [58] was employed for the treatment of a mixture of E1, E2, and EE2 both in a batch stirred tank reactor (BSTR) operating in cycles and a continuous PBR. Removal of estrogens was >85% in the BSTR and 55%, 75%, and 60% for E1, E2, and EE2, respectively, in the PBR. Both systems were able to reduce the estrogenic activity of the mixture in 63%. Likewise, the immobilization of VP in the form of CLEAs completely removed E2 and EE2 within 10 min from batch experiments, with a concomitant reduction of estrogenic activity, higher than 60% for both compounds [44].

The application of LAC for the removal of estrogens from municipal wastewater was assessed by Auriol and co-authors [59]. Different enzyme levels were tested and a LAC activity of 20,000 U L^{-1} was enough to achieve complete removal of E1, E2, estriol (E3), and EE2 in 1 h from both the wastewater and the synthetic

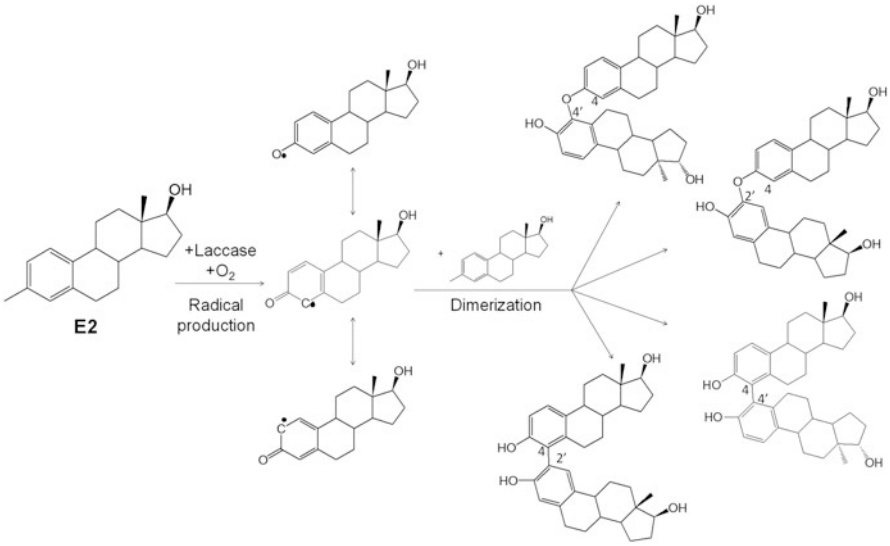

Fig. 8 Pathway of LAC-mediated oxidation of E2 (adapted from Cabana et al. [42] and Nicotra et al. [57])

water in 1 L batch reactors. This work also aimed at evaluating the wastewater matrix effect on the enzymatic process. By comparing with synthetic water, it was concluded that the wastewater constituents did not have a significant effect on the conversion of the estrogens. The same group evaluated the removal of estrogenic activity from the LAC-catalyzed process and compared it with a horseradish peroxidase treatment (8,000–10,000 U L^{-1}), which was also able to remove the hormones in 1 h. According to results from the recombinant yeast assay, the LAC process yielded a residue with a slightly lower estrogenic activity (elimination of 97%), compared to horseradish peroxidase (removal of 88%) [60].

Degradation of estrogens has been also demonstrated with whole fungal cells. E2 and EE2 were removed by *T. versicolor* pellets in batch (flasks) and in a continuous FBR [61]. Removal of both compounds was >97% within 24 h in batch cultures. The continuous bioreactor was operated for 26 days at a HRT of 120 h, and achieved complete removal of E2, added at different concentrations ranging from 3 to 18.8 mg L^{-1}, and simultaneous complete removal of E2 and EE2 added after biomass renovation (day 19). Degradation was ascribed to extracellular LAC. Likewise, Tamagawa et al. [56] reported 98% removal of E1 after 5 days by *P. sordida* and attributed the process to the effect of the extracellular ligninolytic enzymes released during the treatment. Cajthalm et al. [50] studied the degradation of EE2 (among other endocrine disrupting compounds) by eight ligninolytic fungal strains. *I. lacteus*, *P. cinnabarinus,* and *P. ostreatus* were the most efficient degraders, as they reduced EE2 below the detection limit within 3 days, followed

by *T. versicolor* within 7 days and *B. adusta* and *Dichomitus squalens* within 14 days. Only *P. chrysosporium* and *Phanerochaete magnoliae* failed to completely remove the synthetic estrogen. EE2 degradation was accompanied by a decrease in the estrogenic activity of the solution, except in the case of *P. magnoliae*.

The fungal transformation of estrogens, by enzymes or cells, seems to produce metabolites with reduced estrogenic activity in short periods, usually days or even few hours. However, only oligomeric products have been identified in enzymatic processes and a lack in the identification of metabolites derived from whole cell fungal systems is still observed.

2.8 Iodinated Contrast Media

X-ray contrast agents, as triiodinated benzoates, permit visualization of the details of the internal structure of organs that would otherwise not be apparent. Given that iodinated X-ray contrast agents are very stable and chemically inert, they are excreted untransformed by humans and are not degraded in their subsequent pass through WWTPs.

The degradation of triiodinated benzoates by *T. versicolor* was investigated by Rode and Müeller [62].The authors reported a removal of diatrizoate of approximately an 80% after 14 days. During the time course experiments, they found three TPs. In a first step, diatrizoate was degraded to 3,5-di(acetamido)-2,6-diiodobenzoate (metabolite 1) and 3,5-di(acetamido)-4,6-diiodobenzoate (metabolite 2), which indicates that the degradation evolves through the successive release of iodide ions (Fig. 9). A third by-product identified as 3,5-di(acetamido)-2-monoiodobenzoate (metabolite 3) was formed by a second deiodination of the former metabolites. Degradation yields of 58%, 65%, and 80% were also reported after 14 days when aminotrizoate, acetrizoate, and iodipamide were incubated with *T. versicolor*, respectively. During the degradation of iodipamide five TPs were detected, while in the case of acetrizoate, which has one acetamido group in the aromatic ring, the HPLC chromatograms showed only one metabolite. No aromatic metabolites were detected in the experiments with aminotrizoate, maybe due to the polymerization of the highly reactive amino group, which generates undetectable products in HPLC, or the direct cleavage of the ring structure. Nevertheless, the TPs of the three triiodinated compounds were not identified.

Degradation of iopromide, a nonionic X-ray contrast agent, and its precursor aminotriiodoisophthalic acid were also assessed with *T. versicolor*. Rode and Müller [63] reported almost complete degradation (90%) of iopromide over 15 days and detected 14 metabolites from iopromide. From all the TPs, 5-methoxyacetylamino-4-monoiodoisophthalic acid (2,3-dihydroxy-propyl) diamide (metabolite 1), 5-methoxyacetylamino-4(6)-monoiodoisophthalic acid [(2,3-dihydroxy-propyl)-methyl)] diamide (metabolite 2) and 5-methoxyacetylamino-2,6-diiodoisophthalic acid [(2,3dihydroxy-*N*-methyl-propyl)-2,3-dihydroxy-propyl)] diamide (metabolite 3) were recognized as the main metabolites (Fig. 10). Metabolite 1 was deiodinated

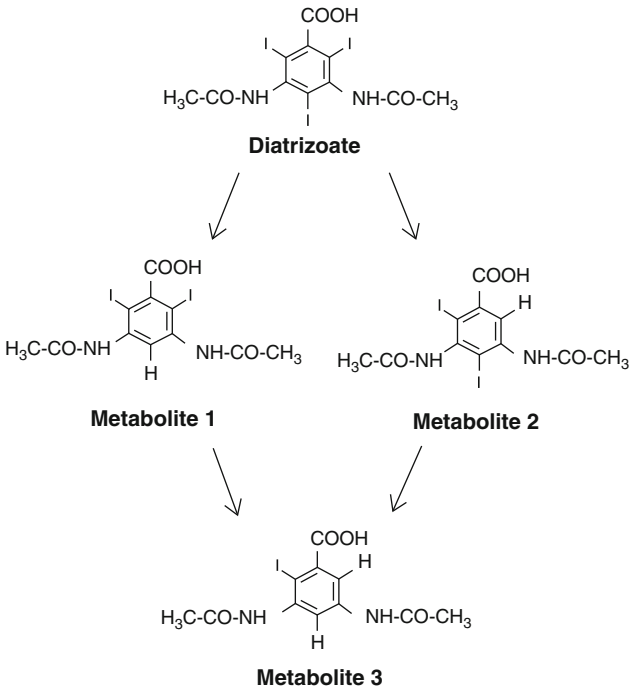

Fig. 9 Proposed transformation of diatrizoate by *T. versicolor* (adapted from Rode and Müller [62])

at the 2 and 6 positions of the aromatic ring and demethylated on one side chain. Metabolite 2 was twice deiodinated and demethylated, like metabolite 1, and additionally depropylated. The authors observed that when metabolite 2 appeared, the increase in concentration of metabolite 1 slowed down. For this reason, they suggested that the metabolite 2 was formed by depropylation of metabolite 1. Metabolite 3 was monodeiodinated at the 4 position on the aromatic ring. Regarding aminotriiodoisophthalate, which is the matrix moiety of iopromide without the side chains, they observed a degradation of only 50% after 14 days, with the subsequent production of only one metabolite. Finally, Rode and Müller indicate that the reductive deiodination of iodinated X-ray contrast agents is a general biodegradative pathway of *T. versicolor* and seems to occur prior to the cleavage of the ring structure.

The role of extracellular enzymatic systems in the degradation of triiodinated aromatics compounds was demonstrated by the same authors. Degradation yields between 87% and 93% for diatrizoate, iodipamide, and acetrizoate, and between 68% and 73% for aminotrizoate and aminotriiodoisophthalate were observed in in vitro experiments with extracellular enzyme concentrate of *T. versicolor* in the

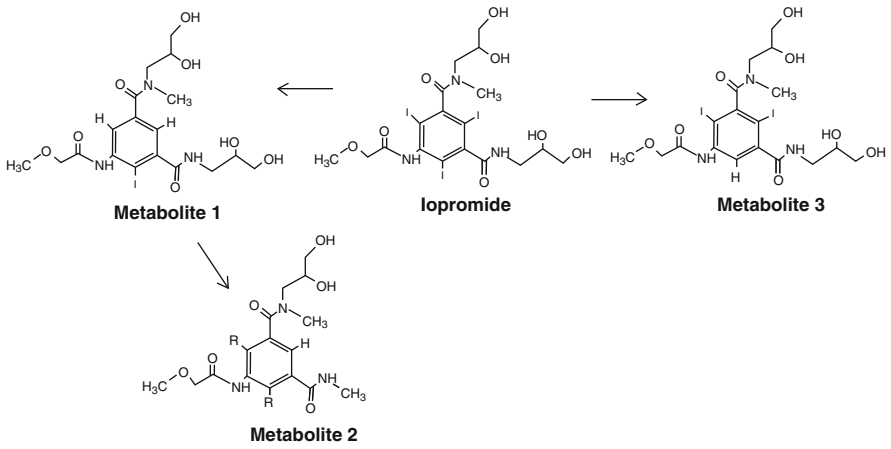

Fig. 10 Identified metabolites from iopromide degradation by *T. versicolor* and proposed degradation scheme (adapted from Rode and Müller [63])

presence of $MnSO_4$ and malonate. The identification of the same metabolites in the whole cell cultures supports the evidence that their production from the triiodinated aromatic compounds is caused by the extracellular enzymes of *T. versicolor*. Rode and Müller [63] carried out similar experiments with commercial peroxidase, LAC, tyrosinase, LiP, and MnP from *P. chrysosporium*. The three former enzymes failed to degrade all the tested compounds, whereas LiP achieved the polymerization of the amino compounds aminotrizoate and aminotriiodoisophthalic acid. Only MnP was able to degrade all the target drugs, remarking its possible participation in the transformation of triiodinated aromatic compounds by WRF.

3 Concluding Remarks

Fungi are attractive candidates for designing effective bioremediation strategies of pharmaceuticals because of the unspecificity of their oxidative enzymatic system that includes not only LME but also intracellular enzymes such as cytochrome P450. In particular, fast degradation, from minutes to few days, has been demonstrated for estrogens, β-blockers, some anti-inflammatory drugs, antibiotics, and other antimicrobial agents, while iodinated contrast agents and psychiatric drugs are removed at slower rates. The participation of LME and cytochrome P450 has been shown in the transformation of members from every family of drugs, except in lipid regulators, in which the LME were not involved in the process. According to the metabolites identified, the most commonly reactions

involved in the transformation of pharmaceuticals by fungi include hydroxylations, formylations, deaminations, and their combinations (anti-inflammatory drugs, psychiatric drugs, antibiotics, β-blockers, lipid regulators, and other anti-microbial agents); dehalogenations (iodinated contrast media and triclosan) and oligomerizations (estrogens and triclosan). Analogous TPs identified for members of the same families (quinolones and sulfonamides) suggest common degradation pathways for similar molecules. Mineralization has been barely demonstrated, only suggested for some anti-inflammatory drugs (diclofenac and ketoprofen) and quinolones. In some cases, the accumulation of TPs has led to an increase of the toxicity (ibuprofen, carbamazepine, and clofibric acid). Therefore, the assessment of this parameter is essential to evaluate the suitability for the potential application of these processes in the treatment of effluents. Moreover, the fungal treatments have resulted in the reduction of estrogenic activity (estrogens and triclosan) and antimicrobial activity (triclosan). The use of different reactor configurations (continuous and batch fluidized bed, packed bed, perfusion basket reactors) for anti-inflammatory, psychiatric drugs, lipid regulators, and triclosan removal has proved the possibility of scaling up the pharmaceutical degradation process. However, most of the published studies to date on removal of pharmaceuticals by fungi were carried out in synthetic liquid media under controlled conditions of pH, temperature, and absence of competitors that allow demonstrating the ability of the tested fungi to degrade the target pharmaceutical. Despite great promise of fungi as removal agents, a number of challenges remain to be surmounted in using them at larger field scale, i.e., competition with autochthonous microorganisms, nonoptimal pH, presence of inhibitors in wastewaters, etc. To date, these aspects have been barely studied and future research efforts on fungal bioremediation technology should shed light on them to translate our basic knowledge on fungi into cost-effective practical bioremediation applications.

Table 1 Summary of pharmaceutical degradation by whole cell fungi and their TP

Family drug	Drug	Fungus	Treatment	Initial concentration	Removal rate	Metabolites	References
Anti-inflammatories/ analgesic drugs	Diclofenac	*B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	1 mg L^{-1}	Total degradation in 7 days		[4]
		P. chrysosporium			Completely disappeared in 4 days		
		Bjerkandera sp.			Total degradation in 4 days		
		P. chrysosporium	Degradation in an aerated fed-batch bioreactor	1 mg L^{-1}	Diclofenac was added every 2 days. Every pulse was completely removed after 23 h		[6]
		P. sordida	Mycelium incubation in flask shaked at 150 rpm and 30°C	30 mg L^{-1}	Completely disappeared after 4 days	4-Hydroxydiclofenac; 5-hydroxydiclofenac; 4′,5-dihydroxydiclofenac	[11]
		T. versicolor	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25°C	10 mg L^{-1} and 45 μg L^{-1}	Complete degradation after 1 h in both cases	4′-Hydroxydiclofenac; 5-hydroxydiclofenac	[7]
	Fenoprofen	*T. versicolor*	Incubation at 30°C in shaken condition for 48 h	10 μg L^{-1}	100% removed		[5]
	Ibuprofen	*B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	1 mg L^{-1}	Total degradation in 7 days		[4]
		P. chrysosporium			Total degradation in 4 days		
		Bjerkandera sp.			Total degradation in 4 days		
		P. chrysosporium	Degradation in an aerated fed-batch bioreactor	1 mg L^{-1}	Diclofenac was added every 2 days. Every pulse was completely removed after 23 h		[6]
		I. lacteus	Cultures were incubated in serum bottles shaked at 135 rpm and 25°C during 7 days	10 mg L^{-1}	100% before 7 days		[3]
		G. lucidum			100% before 7 days		
		P. chrysosporium			70–88% after 7 days		
		T. versicolor			Complete degradation after 1 h	1-Hydroxy ibuprofen; 2-hydroxy ibuprofen; 1,2-dihydroxy ibuprofen	
	Indomethacin	*T. versicolor*	Incubation at 30°C in shaken condition for 48 h	10 μg L^{-1}	100% removed		[5]

Ketoprofen	*T. versicolor*	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25°C	10 mg L^{-1} and 40 μg L^{-1}	100% removed after 24 h and 5 h, respectively	2-[3-(4-Hydroxybenzoyl) phenyl]-propanoic acid; 2-[(3-hydroxy(phenyl)methyl) phenyl]-propanoic acid; 2-(3-benzoyl-4-hydroxyphenyl)-propanoic acid	[13]
Mefenamic	*P. sordida*	Mycelium incubation in flask shaked at 150 rpm and 30°C	10^{-4} M	90% after 6 days of treatment.	3′-Hydroxymethyl mefenamic acid; 3′-hydroxymethyl-5-hydroxymefenamic acid; 3′-hydroxymethyl-6′-hydroxymefenamic acid; 3′-carboxy mefenamic acid	[11]
Naproxen	*B. adusta* *P. chrysosporium* *Bjerkandera* sp.	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	1 mg L^{-1}	100% after 7 days 100% after 4 days 100% after 7 days		[4]
	P. chrysosporium	Degradation in an aerated fed-batch bioreactor	1 mg L^{-1}	Diclofenac was added every 2 days. Every pulse was completely removed after 23 h		[6]
	T. versicolor	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25°C	10 mg L^{-1} and 55 μg L^{-1}	Complete degradation after 6 h and 5 h, respectively	2-(6-Hydroxynaphthalen-2-yl) propanoic acid; 1-(6-methoxynaphthalen-2-yl)ethanone	[12]
Propyphenazone	*T. versicolor*	Incubation at 30°C in shaken condition for 48 h	10 μg L^{-1}	75% removed at 2 days		[5]

(continued)

Table 1 (continued)

Family drug	Drug	Fungus	Treatment	Initial concentration	Removal rate	Metabolites	References
Antibiotics	Cinoxacin	*B. bassiana*	Experimental cultures in flasks were incubated at 28°C with rotary shaking at 180 rpm during 20 days.	20 mM	52.7% removed after 20 days	1-Ethyl-1,4-dihydro-3-(hydroxymethyl)-[1,3] dioxolo[4,5-g]cinnolin-4-one 1-Ethyl-1, 4-dihydro-6,7-dihydroxy-3-(hydroxymethyl)cinnolin-4-one	[32]
	Ciprofloxacin	*G. striatum*	Erlenmeyer flasks containing 30 ml of medium and fungi were incubated at 150 rpm at room temperature for 13 weeks	10 mg L^{-1}	33% after 13 weeks.	Monohydroxylated congeners; dihydroxylated congeners	[30]
		M. Ramannianus	Cultured incubated for 14 days at 28°C with shaking at 200 rpm	300 μM	89.9 ± 0.4% removed after 14 days	1-Cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(4-acetyl-1-piperazinyl)-3-quinolinecarboxylic acid	[31]
		P. guepini	Incubation of mycelium for 18 days	300 μM	67.8% removed after 18 days	*N*-acetylciprofloxacin (52%) Desethylene-*N*-acetylciprofloxacin (9.2%); *N*-formylciprofloxacin (4.1%); 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid (2.3%)	[30]
		T. viride	Incubation of mycelium for 16 days	300 μM	31% had been transformed to the product	4-Hydroxy-3-oxo-4-vinylcyclopent-1-enyl ciprofloxacin	[39]
		P. chrysosporium	Inoculation of granular bioplastic formulation entrapping propagules of *P. chrysosporium* in wastewater	10 μg mL^{-1}	80% after 30 days incubation		[25]

Ciprofloxacin	*T. versicolor*	Pellets incubated at 30°C under orbital agitation	2 mg L^{-1}	>90% after 7 days	7-((2-Aminoethyl)amino)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; 1-cyclopropyl-6-fluoro-8-hydroxy-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid; 7-((2-acetamidoethyl) amino)-1-cyclopropyl-6-fluoro-8-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; two dimeric products formed by C–C covalent bond and followed by several transformations	[29]
Enrofloxacin	*G. striatum* *S. rugosoannulata* *P. chrysosporium* *I. lacteus*	Wood-rotting fungi grown on wetted wheat straw	3.3 µg mL^{-1}	53.3% ± 1.2% in 8 weeks 5.1% ± 0.6% in 8 weeks 25.6% ± 0.6% in 8 weeks 13.7% ± 0.8% in 8 weeks		[33]
	M. ramannianus	Cultures grown in sucrose-peptone broth were dosed with enrofloxacin	253 µM	22% of the enrofloxacin remained after 21 days	Enrofloxacin *N*-oxide (62%) *N*-acetylciprofloxacin (8%); desethylene-enrofloxacin (3.5%)	[36]
	Several basydiomycetes	Static cultures inoculated with mycelium	28.5 mg L^{-1}	n.d.	61 different compounds; see text for more details	[35]
	G. striatum	Mycelia suspended in a defined liquid medium with contaminant were shaked at 150 rpm during 8 weeks	10 mg L^{-1}	Production of 27.3% $^{14}CO_2$ from [^{14}C] enrofloxacion	3-, 6-, and 8-hydrolated congeners of enrofloxacin 5,6-(or 6,8-), 5,8-, and 7,8-dihydroxylated congeners Isatin-type compound Anthranilic acid derivative 1-Ethylpiperazine; desethylene-enrofloxacin	[34]

(continued)

Table 1 (continued)

Family drug	Drug	Fungus	Treatment	Initial concentration	Removal rate	Metabolites	References
	Erythromycin	*P. chrysosporium*	Inoculation of granular bioplastic formulation entrapping propagules of *P. chrysosporium* in wastewater	10 μg mL^{-1}	98% after 30 days.		[25]
	Norfloxacin	*P. guepini*	Incubation of mycelium for 18 days	313 μM	68.9% removed after 18 days	*N*-Acetylnorfloxacin (55.4%); desethylene-*N*-acetylnorfloxacin (8.8%); *N*-for-mylnorfloxacin (3.6%); 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (2.1%)	[30]
		T. viride	Incubation of mycelium for 16 days	313 μM	42% had been transformed to the product	4-Hydroxy-3-oxo-4-vinylcyclopent-1-enyl norfloxacin	[39]
		T. versicolor	Pellets incubated at 30°C under orbital agitation	2 mg L^{-1}	>90% after 7 days	7-((2-Aminoethyl)amino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; 7-((2-scetamidoethyl)amino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid	[29]
	Flumequine	*C. elegans*	Cultures at 28°C and shaking	308 μM	n.d.	7-Hydroxyflumequine (two diastereomers); 7-oxoflumequine	[38]
	Sarafloxacin	*M. ramannianus*	Cultures grown in sucrose-peptone broth were dosed with sarafloxacin	260 μM	59% of the starting material remained.	*N*-acetylsarafloxacin; desethylene-*N*-acetylsarafloxacin	[37]

Sulfamethazine	*T. versicolor*	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25°C	9 mg L^{-1}	>95% after 20 h	*N*-(4,6-dimethylpyrimidin-2-yl) benzene-1,4-diamine (desulfonated sulfamethazine); *N*-(4,6-dimethylpyrimidin-2-yl)-4-(formylamino) benzenesulfonamide (formylated sulfamethazine)	[23]
	T. versicolor	Continuous degradation in an air pulsed fluidized bed bioreactor	5 mg L^{-1}	94% at HRT 72 h		[24]
Sulfathiazole	*T. versicolor*	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25°C	11 mg L^{-1}	100% after 7 days	Formylated sulfathiazole; desulfonated-desaminated-hydroxylated sulfathiazole	[24]
		Continuous degradation in an air-pulsed fluidized bed bioreactor	5 mg L^{-1}	>95% at HRT 72 h		
Sulfapyridine	*T. versicolor*	Pellets incubation in Erlenmeyer flask shaked at 135 rpm and 25°C	9 mg L^{-1}	100% after 48 h	Desulfonated sulfapyridine; formylated sulfapyridine; formylated-desulfonated sulfapyridine; hydroxylated sulfapyridine; a hydroxy-formyl intermediate after the loss of the pyridine ring	[24]
		Continuous degradation in an air-pulsed fluidized bed bioreactor	5 mg L^{-1}	>99% at HRT 72 h		
Sulfamethoxazole	*P. chrysosporium*	Inoculation of granular bioplastic formulation entrapping propagules of *P. chrysosporium* in wastewater	10 µg mL^{-1}	98% after 30 days.		[25]

(continued)

Table 1 (continued)

Family drug	Drug	Fungus	Treatment	Initial concentration	Removal rate	Metabolites	References
		B. adusta	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	1 mg L^{-1}	Complete degraded before 14 days		[4]
		P. chrysosporium			80% degradation after 7 days		
		Bjerkandera sp.			Complete degraded before 14 days		
Other antimicrobial agents	*N*-acetylphenothiazine	*A. nigen*	Cultures were dosed and then incubated for 72 h at 28°C with shaking at 200 rpm.	20 mg ml^{-1}	Residual *N*-acetylphenothiazine	*N*-acetylphenothiazine; sulfoxide Phenothiazine sulfoxide	[51]
		C. verticillata				*N*-acetylphenothiazine sulfoxide; phenothiazine sulfoxide; phenothiazin-3-one; phenothiazine *N*-glucoside;	
		P. simplicissimum				*N*-acetylphenothiazine sulfoxide; phenothiazine sulfoxide; phenothiazine	
	Triclosan	*I. lacteus*	Static culture inoculated with mycelium	2.5 mg L^{-1}	>95% after 14 days		[50]
		B. adusta			>95% after 14 days		
		P. chrysosporium			>95% after 14 days		
		P. magnolia	Static culture inoculated with mycelium	2.5 mg L^{-1}	90% after 14 days		
		P. ostreatus			95% after 14 days		
		T. versicolor			91% after 14 days		
		P. cinnabarinus	Static culture inoculated with mycelium	2.5 mg L^{-1}	>95% after 14 days		
		D. squalens			>95% after 14 days		
		T. versicolor			>95% after 14 days	2,4-Dichlorophenol; the conjugates 2-*O*-(2,4,4′-trichlorodiphenyl ether)-β-D-xylopyranoside and 2-*O*-(2,4,4′-trichlorodiphenyl ether)-β-D-glucopyranoside	[49]
		P. cinnabarinus	Mycelium inoculation and shaking at 30°C	0.25 mM	n.d.	2,4,4′-Trichloro-2′-methoxydiphenyl ether; the conjugate 2-*O*-(2,4,4′-trichlorodiphenyl ether)-β-D-glucopyranoside	

	Artemisinin	*E. amstelodami*	Cultures were incubated at 28°C in environmental shakers during 14 days.	500 mg L^{-1}	Residual artemisinin after 14 days	5β-Hydroxyartemisinin; 7β-hydroxyartemisinin	[52]
		Aspergillus nigen			Residual artemisinin after 14 days	5β-Hydroxyartemisinin; 7β-hydroxyartemisinin	
		M. Ramannianus			Completely degraded after 14 days	6β-Hydroxyartemisinin; 7β-hydroxyartemisinin; 7α-hydroxyartemisinin; 4α-hydroxy-1-deoxoartemisinin	[53]
		C. elegans			96.5% degradation after 14 days	6β-Hydroxyartemisinin; 7β-hydroxyartemisinin; 7α-hydroxy-9α-artemisinin 4α-Hydroxy-1-deoxoartemisinin	[54]
	Oseltamivir	*P. chrysosporium*	Inoculation of granular bioplastic formulation entrapping propagules of *P. chrysosporium* in wastewater	10 μg mL^{-1}	50% after 16 days incubation		[25]
B-blockers	Atenolol	*T. versicolor*	Degradation of contaminant by induction of oxidizing agents in *T. versicolor* via quinone redox cycling.	10 mg L^{-1}	80% reached after 6 h of incubation.	Atenolol hydroxylated derivative (P282)	[17]
	Propanolol	*T. versicolor*	Degradation of contaminant by induction of oxidizing agents in *T. versicolor* via quinone redox cycling.	10 mg L^{-1}	50% in 1 h and 80% after 6 h incubation	Hydroxy propranolol (P275)	[17]
Lipid regulator	Clofibric acid	*I. lacteus*	Cultures were incubated in serum bottles shaked at 135 rpm and 25°C during 7 days	10 mg L^{-1}	Low degradation		[3]
		P. chrysosporium			Low degradation		
		G. lucidum			Low degradation		
		T. versicolor			91% after 7 days of the treatment		
		T. versicolor	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	30 μg L^{-1}	Completely degraded after 4 days		Not yet published
		T. versicolor		160 μg mL^{-1}	80% of the inflow concentration was	2-(4-Chlorophenoxy)-2-(hydroxymethyl) propanoic	

(continued)

Table 1 (continued)

Family drug	Drug	Fungus	Treatment	Initial concentration	Removal rate	Metabolites	References
			Continuous degradation in an air-pulsed fluidized bed bioreactor		reduced at the steady state. 16.5 µg removed g^{-1} d.w. day^{-1}		
		T. versicolor	Degradation of contaminant by induction of oxidizing agents in *T. versicolor* via quinone redox cycling.	10 mg L^{-1}	80% reached after 6 h of incubation.	Clofibric hydroxylated derivative	[17]
	Gemfibrozil	*T. versicolor*	Incubation at 30°C in shaken condition for 48 h	10 µg L^{-1}	70% degraded after 2 days		[5]
Psychiatric drugs	Carbamazepine	*P. chrysosporium*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	1 mg L^{-1}	Completely removed before 14 days		[4]
		Bjerkandera sp.			Completely removed before 14 days		
		B. adusta			Completely removed at 14 days		
		T. versicolor	Incubation at 30°C in shaken condition for 48 h	10 µg L^{-1}	75% degraded after 2 days		[5]
		P. chrysosporium	Cultures were incubated in serum bottles shaked at 135 rpm and 25°C during 7 days	10 mg L^{-1}	<10% after 7 days incubation		[3]
		G. lucidum			47% after 7 days incubation		
		T. versicolor			58% after 7 days treatment		
		I. lacteus			<10% after 7 days incubation		
		P. ostreatus	Incubation in Erlenmeyer flasks at 28°C in the dark	10 mg L^{-1}	60% degraded after 17 days		[14]
		P. ostreatus			48% after 17 days		
	Carbamazepine	*U. ramanniana*			25% removed after 25 days	11-Epoxy-carbamazepine; 2-hydroxy-carbamazepine; 3-hydroxy-carbamazepine	[16]
		C. elegans			45% of degradation after 25 days	11-Epoxy-carbamazepine; 2-hydroxy-carbamazepine; 3-hydroxy-carbamazepine	
		P. ostreatus			Complete degradation after 10 days		

		T. versicolor	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	10 mg L^{-1} and 50 μg L^{-1}	High concentrations 94% elimination after 6 days and in low 61% after 7 days.	Acridine; acridone; 10,11-dihydro-10,11-dihydroxy-carbamazepine; 11-epoxy-carbamazepine	[15]
		T. versicolor	Degradation in an air-pulsed fluidized bed bioreactor operated in batch and continuous	200 μg mL^{-1}	Completely degraded after 2 days in batch mode. In continuous operation, 54% of the inflow concentration was reduced at the steady state. 11.9 μg removed g^{-1} d. w day^{-1}	11-Epoxy-carbamazepine	
		T. versicolor	Induction of oxidizing agents in *T. versicolor* via quinone redox cycling.	10 mg L^{-1}	50% in 1 h and 80% after 6 h incubation	Two hydroxylated isomers of CBZ (P254A and B)	[17]
	Diazepam	*P. chrysosporium* *Bjerkandera* sp. *B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	1 mg L^{-1}	57% removed in 5 days 54% removed in 5 days 56% removed in 5 days		[4]
	Fluoxetine	*P. chrysosporium* *Bjerkandera* sp. *B. adusta*	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	1 mg L^{-1}	<10% in 2 weeks 23% after 14 days <10% in 2 weeks		[4]
	Citalopram	*B. adusta* *P. chrysosporium* *Bjerkandera* sp.	Erlenmeyers flasks containing defined medium and contaminant were statically incubated at 30°C for 2 weeks	1 mg L^{-1}	Complete degradation before 14 days Complete degradation before 14 days 58% removal after 4 days		[4]
Estrogens	17B-estradiol	*T. versicolor*	Continuous degradation in an bioreactor	18.8 mg L^{-1}	100% at a HRT 120 h		[61]
	17a-ethinylestradiol	*T. versicolor*		7.3 mg L^{-1}	100% at a HRT 120 h		[61]
		I. lacteus *B. adusta* *P. chrysosporium*	Static culture inoculated with mycelium	2.5 mg L^{-1}	100% after 14 days 100% after 14 days 30% after 14 days		[60]

(continued)

Table 1 (continued)

Family drug	Drug	Fungus	Treatment	Initial concentration	Removal rate	Metabolites	References
		P. magnoliae			70% after 14 days		
		P. ostreatus			100% after 14 days		
		T. versicolor			100% after 14 days		
		P. cinnabarinus			100% after 14 days		
		D. squalens			100% after 14 days		
X-ray contrast media	Diatrizoate	*T. versicolor*	Flasks scale at 30°C	1 mM	80% after 14 days	3,5-Di(acetamido)-2,6-diiodobenzoate; 3,5-di(acetamido)-4,6-diiodobenzoate; 3,5-di(acetamido)-2-monoiodobenzoate	[62]
	Iodipamide	*T. versicolor*			80% after 14 days	Five metabolites detected	
	Aminotrizoate	*T. versicolor*			58% after 14 days	No metabolites detected	
	Acetrizoate	*T. versicolor*			65% after 14 days	1 metabolite detected	
	Aminotriiodoisophthalic acid	*T. versicolor*			50% after 14 days	One metabolite detected	
	Aminotriiodoisophthalic acid	*T. versicolor*			50% after 14 days	One metabolite detected	[63]
	Iopromide	*T. versicolor*		1 mM	90% after 15 days	5-Methoxyacetylamino-4-monoiodoisophthalic acid (2,3-dihydroxy-propyl) diamide; 5-methoxyacetylamino-4(6)-monoiodoisophthalic acid [(2,3-dihydroxy-propyl)-methyl)] diamide; 5-methoxyacetylamino-2,6-diiodoisophthalic acid [(2,3dihydroxy-*N*-methyl-propyl)-2,3-dihydroxy-propyl)] diamide.	

Table 2 Summary of pharmaceutical degradation by fungal enzyme and their TP

Family drug	Drug	Fungal enzyme	Treatment	Initial concentration	Removal rate	Metabolites	References
Anti-inflammatories/ analgesic drugs	Diclofenac	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	100% after 25 min	4-(2,6-Dichlorophenyl amino)-1,3-benzenedimethanol	[9]
		LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	83–100% after 24 h with mediators, 83% without mediators		[58]
		LiP from *P. chrysosporium*	Flask scale	5 mg L^{-1}	100% after 2 h		[10]
		LAC from *T. versicolor*	Flask scale	40 mg L^{-1}	>95% after 4.5 h		[7]
		LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	>90% in 30 min		[5]
	Fenoprofen	LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	>90% in 3 h		[5]
	Ibuprofen	LAC from *T. versicolor*/ MnP from *Bjerkandera sp*	Flask scale	10 mg L^{-1}	Negligible after 24 h, even with mediators		[3]
		LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	~35% in 3 h		[5]
	Indomethacin	LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	>90% in 30 min		[5]
	Ketoprofen	LAC from *T. versicolor*	Flask scale	10 mg L^{-1}	Negligible after 20 h, even with mediators		[13]
		LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	~50% in 3 h		[5]
	Naproxen	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	80% after 7 h		[9]
		LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	36–68% after 24 h with mediators		[8]
		LAC from *T. versicolor*	Flask scale	20 mg L^{-1}	>95% after 30 h with HOBT, <10% without mediator		[12]
		LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	>90% in 30 min		[5]
	Propyphenazone	LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	~25% in 3 h		[5]
Antibiotics	Tetracycline	LiP from *P. chrysosporium*	Flask scale	50 mg L^{-1}	>99% after 30 min, only in the presence of veratryl alcohol		[26]
		LAC from *T. versicolor*	Flask scale	10^{-4} M	100% after 1 h with mediators		[27]

(continued)

Table 2 (continued)

Family drug	Drug	Fungal enzyme	Treatment	Initial concentration	Removal rate	Metabolites	References
	Oxytetracycline	LiP from *P. chrysosporium*	Flask scale	50 mg L^{-1}	>99% after 30 min, only in the presence of veratryl alcohol		[26]
		LAC from *T. versicolor*	Flask scale	10^{-4} M	100% after 1 h with mediators		[27]
	Chlortetracline	LAC from *T. versicolor*	Flask scale	10^{-4} M	100% after 15 min with mediators		[27]
	Doxycycline	LAC from *T. versicolor*	Flask scale	10^{-4} M	100% after 15 min with mediators		[27]
	Sulfadimethoxine	LAC from *T. versicolor*	Flask scale	10^{-3} M	75.1% after 15 days	4-(6-Imino-2, 4-dimethoxy pyrimidin-1-yl) aniline; additional metabolites were preliminary assigned but not confirmed	[22]
	Sulfamethazine	LAC from *T. versicolor*	Flask scale	20 mg L^{-1}	22% after 50 h without mediators, 93–100% with mediators	*N*-(4,6-dimethyl pyrimidin-2-yl)benzene-1,4-diamine (desulfonated sulfamethazine); *N*-(4,6-dimethyl pyrimidin-2-yl) benzenesulfonamide (desaminated sulfamethazine); N_1-hydroxysulfa methazine or N_4-hydroxysulfa methazine (hydroylated sulfamethazine)	[23]
	Sulfamethoxazole	VP from *B. adusta*	Flask scale	25 mg L^{-1}	80% after 7 h	3-Amino-5-methyli soazole	[9]
	Sulfanilamide	LAC from *T. versicolor*	Flask scale	10^{-3} M	10.0% after 15 days	Aniline was preliminary assigned but not confirmed	[22]
	Sulfapyridine	LAC from *T. versicolor*	Flask scale	10^{-3} M	95.6% after 15 days	Aniline; 4-(2-imino-1-pyridyl)aniline; additional metabolites were preliminary assigned but not confirmed	[22]

		LAC from *T. versicolor*	Flask scale	20 mg L^{-1}	75–98% with mediators	Desulfonated sulfapyridine; a formyl intermediate after the loss of the pyrimidine ring	[24]
	Sulfathiazole	LAC from *T. versicolor*	Flask scale	16 mg L^{-1}	82–100% with mediators	Desulfonated sulfapyridine; a formyl intermediate after the loss of the thiazole ring	[24]
	Ciprofloxacin	LAC from *T. versicolor*	Flask scale	10 mg L^{-1}	16% after 20 h; 97.7% with mediators		[29]
	Norfloxacin	LAC from *T. versicolor*	Flask scale	10 mg L^{-1}	0% after 20 h, 33.7% with mediators		[29]
Other antimicrobial agents	Triclosan	MnP from *P. chrysosporium*	Flask scale	28.95 mg L^{-1}	100% after 90 min		[41]
		LAC from *T. versicolor*	Flask scale	28.95 mg L^{-1}	66% after 90 min with mediators, 10–51% without mediators		[41]
		LAC from *C. polyzona*	PBR with immobilized LAC; repeated batch cycles	5 mg L^{-1}	>90% after 200 min		[43]
		LAC from *C. polyzona*	Continuous perfusion basket reactor with CLEAs	5 mg L^{-1}; 100 mg L^{-1}	85% at HRT 325 min		[45]
		LAC from *T. versicolor*	Flask scale	5.8 mg L^{-1}	100% after 4 h without mediators, after 30 in with mediators		[50]
		LAC from *C. polyzona*	Flask scale	5 mg L^{-1}	65% after 4 h without mediator, 50% after 1 h with mediators	Oligomers (dimers, trimers and tetramers)	[48]

(continued)

Table 2 (continued)

Family drug	Drug	Fungal enzyme	Treatment	Initial concentration	Removal rate	Metabolites	References
		LAC from *G. lucidum*	Flask scale	0.2 mmol L^{-1}	56.5% after 24 h without mediators, 90% with mediators; reduction in toxicity demonstrated by bacterial inhibition methods	Oligomers in the absence of mediators (dimers and trimers of triclosan); 2,4-dichlorophenol (2,4-DCP) and dechlorinated forms of 2,4-DCP in the presence of mediators	[49]
		LAC from *T. versicolor*	Conjugates laccase-chitosan	5 mg L^{-1}	100% after 6 h (only 60% with free LAC)	Oligomers and dechlorinated oligomers	[46]
		LAC from *C. polyzona*	Continuous FBR with CLEAs	5 mg L^{-1}	100% at HRT 150 min		[42]
		VP from *B. adusta*	Flask scale with CLEAs	10 mg L^{-1}	26% after 10 min	VP was co-aggregated with glucose oxidase	[44]
Psychiatric drugs	Carbamazepine	LAC from *T. versicolor*	Flask scale with repeated additions of LAC or LAC + HBT	2×10^{-5} M	60% after 48 h, only with HBT	10,11-dihydro-10, 11-epoxycarba mazepine, 9(10 H) acridone	[11]
		MnP from *P. chrysosporium*	Flask scale	2×10^{-5} M	14% after 24 h		[11]
		VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	Negligible after 7 h		[9]
		LiP from *P. chrysosporium*	Flask scale	5 mg L^{-1}	<10% in 2 h		[10]
		LAC from *T. versicolor*/ MnP from *Bjerkandera* sp	Flask scale	10 mg L^{-1}	Negligible after 24 h, even with mediators		[3]
		LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	~35% in 3 h		[5]
	Citalopram	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	18% after 7 h		[9]
	Fluoxetine	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	< 10% after 7 h		[9]
Lipid regulators	Clofibric acid	LAC from *T. versicolor*/ MnP from *Bjerkandera* sp.	Flask scale	10 mg L^{-1}	Negligible after 24 h, even with mediators		[3]
		LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	~20% in 3 h		[5]
	Gemfibrozil	LAC from *T. versicolor*	Flask scale	10 μg L^{-1}	~80% in 3 h		[5]

Estrogens	17a-ethinylestradiol	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	100% after 15 min		[9]
		LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	100% after 5 h without mediators; 1–3 h depending on mediator		[8]
		MnP from *P. chrysosporium*	Flask scale	10^{-7} M	>95% after 1 h; estrogenic activity completely removed after 8 h		[55]
		LAC from *T. versicolor*	Flask scale	10^{-7} M	>95% after 1 h with mediators; estrogenic activity completely removed after 8 h		[55]
		LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h		[59]
		LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h; estrogenic activity completely removed after 8 h for a mixture of EDC		[60]
		LAC from *M. thermophila*	BSTR working in cycles/ continuous PBR with immobilized enzyme	5 mg L^{-1}	>94%/60%; reduction in toxicity detected		[58]
		VP from *B. adusta*	Flask scale with CLEAs	10 mg L^{-1}	93.1% after 10 min; 100% free VP		[44]
	17B-estradiol	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	100% after 15 min		[9]
		LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	100% after 3 h without mediators; 1–3 depending on mediator		[8]
		LAC from *T. pubescens*	Flask scale, biphasic system (buffer/ AcOEt)	5 g L^{-1}	n.d.	Two C–C and two C–O dimeric products	[57]

(continued)

Table 2 (continued)

Family drug	Drug	Fungal enzyme	Treatment	Initial concentration	Removal rate	Metabolites	References
		LAC from *Myceliophthora* sp.	Flask scale, adsorbed on glass beads in organic solvents (dioxane/water saturated toluene)	10 g L^{-1}	n.d.	Two C–C and two C–O dimeric products	[57]
		MnP from *P. chrysosporium*	Flask scale	10^{-7} M	>95% after 1 h; estrogenic activity completely removed after 8 h		[55]
		LAC from *T. versicolor*	Flask scale	10^{-7} M	>95% after 1 h with mediators;estrogenic activity completely removed after 8 h		[55]
		LAC from *Trametes* sp.	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h		[59]
		LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h; estrogenic activity completely removed after 8 h for a mixture of EDC		[60]
		LAC from *M. thermophila*	BSTR working in cycles/ continuous PBR with immobilized enzyme	5 mg L^{-1}	>95%/75%; reduction in toxicity detected		[58]
		VP from *B. adusta*	Flask scale with CLEAs	10 mg L^{-1}	90.1% after 10 min; 100% free VP		[44]
	Estriol	LAC from *Trametes* sp.	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h		[59]
		LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h; estrogenic activity completely removed after 8 h for a mixture of EDC		[60]

	Estrone	VP from *B. adusta*	Flask scale	2.5 mg L^{-1}	100% after 15 min		[9]
		LAC from *M. thermophila*	Flask scale	5 mg L^{-1}	37–100% after 24 h with mediators, 65% without mediators		[8]
		MnP from *P. sordida*	Flask scale	10^{-5} M	100% after 1 h		[56]
		LAC from *P. sordida*	Flask scale	10^{-5} M	100% after 1 h		[56]
		LAC from *Trametes* sp.	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h		[59]
		LAC from *T. versicolor*	Stirred batch reactor with the compound spiked in wastewater	100 ng L^{-1}	100% after 1 h; estrogenic activity completely removed after 8 h for a mixture of EDC		[60]
		LAC from *M. thermophila*	BSTR working in cycles/ continuous PBR with immobilized enzyme	5 mg L^{-1}	>87%/55%; reduction in toxicity detected		[58]
Iodinated contrast media	Diatrizoate	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	87%/29%/0%/0%	3,5-Di(acetamido)-2, 6-diiodobenzoate 3,5-Di(acetamido)-4, 6-diiodobenzoate 3,5-Di(acetamido)-2-monoiodobenzoate	[62]
	Iodipamide	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	90%/16%/0%/0%		[62]

(continued)

Table 2 (continued)

Family drug	Drug	Fungal enzyme	Treatment	Initial concentration	Removal rate	Metabolites	References
	Acetrizoate	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	93%/27%/0%/0%		[62]
	Aminotrizoate	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	68%/0%/60%/0%		[62]
	Aminotriiodoiso-phthalate	Extracellular extract from *T. versicolor*/ MnP from *P. chrysosporium*/ LiP from *P. chrysosporium*/ LAC from *T. versicolor*	Flask scale	1 mM	73%/18%/34%/0%		[63]
	Iopromide	Extracellular extract from *T. versicolor*	Flask scale	1 mM	98%	5-Methoxyacetyl amino-4-monoiodoi sophthalic acid (2,3-dihydroxy-propyl) diamide 5-Methoxyacetylamino-4(6)-monoiodoi sophthalic acid [(2,3-dihydroxy-propyl)-methyl)] diamide 5-Methoxyacetyl amino-2, 6-diiodoisophthalic acid [(2,3dihydroxy-*N*-methyl-propyl)-2, 3-dihydroxy-propyl)] diamide	[63]

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UV Filters Biodegradation by Fungi, Metabolites Identification and Biological Activity Assessment

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Abstract Organic UV filters used in cosmetics and sunlight protection of materials are considered emerging contaminants. These xenobiotic compounds occur in the environment in relevant concentrations and display critical properties such as environmental persistence and bioaccumulation. They enter the environment mainly through the liquid effluent of wastewater treatment plants (WWTPs), but, due to their high hydrophobicity, they are also adsorbed in WWTP sludge, that is, eventually spread on agricultural fields as fertilizer. The treatment of WWTP sludge with the white-rot fungi *Trametes versicolor* has emerged as a feasible alternative to current conventional treatment processes to degrade them in a range from 87% in the case of 3-(4′-methylbenzylidene) camphor (4-MBC) to 100% of benzophenone-3 (BP3) and its metabolite 4,4′-dihydroxybenzophenone (4DHB). When treating the sewage sludge to remove sunscreens content, it is crucial to establish the biological activity profile along the process. Oestrogenic activity was eliminated by the *T. versicolor* treatment, indicating that none of the resulting

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metabolites possessed significant oestrogenic activity at the produced concentrations. These results demonstrate the suitability of fungi to degrade sunscreen agents and eliminate their oestrogenic activity.

Keywords Degradation, Fungi, Biological activity, HPLC-MS/MS analysis, Metabolites, UV filters

Contents

Abbreviations

2,4,6-TCA	2,4,6-Trichloroanisole
3BC	3-Benzylidene camphor
4DHB	4,4′-Dihydroxybenzophenone
4HB	4-Hydroxybenzophenone
4-MBC	3-(4-Methylbenzylidene) camphor
ABTS	2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt
AhR	Aryl hydrocarbon receptor (also known as dioxin receptor)
AhR-RYA	AhR recombinant yeast assay
BC	Blank control
BMDM	4-*t*-Butyl-4′-methoxy-dibenzoylmethane
BP1	Benzophenone-1
BP3	Benzophenone-3
CID	Collision-induced fragmentation
Da	Daltons
DBEs	Double bond equivalents
DHMB	2-2′Dihydroxy-4-methoxybenzophenone
dw	Dry weight
E2	Estradiol
EC_{50}	Half maximal effective concentration

EHMC	Ethylhexyl-methoxycinnamate
ER	Oestrogen receptor
ER-RYA	Oestrogen receptor recombinant yeast assay
ESI	Electrospray ionisation
FP	Fragmentation pattern
FWHM	Full width at half maximum
hER	Human oestrogen receptor
HK	Heat killed
HMS	Homosalate
HPLC	High performance liquid chromatography
K_{OW}	Octanol-water partition coefficient
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NI	Negative
OC	Octocrylene
OD-PABA	2-Ethylhexyl-4-dimethylaminobenzoate
OT	Octyltriazone
PAHs	Polyaromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PI	Positive
PPCPs	Pharmaceuticals and personal care products
QqLIT	Quadrupole-linear ion trap
QqTOF	Quadrupole time of flight
rtER	Rainbow trout oestrogen receptor
RYA	Recombinant yeast assay
SRM	Selected reaction monitoring
TBBPA	Tetrabromobisphenol A
THB	2,3,4-Trihydroxybenzophenone
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
VTG	Vitellogenin
WWTP	Wastewater treatment plant

1 Introduction

Ultraviolet (UV) filters, also known as sunscreens, constitute a diverse group of chemical additives used as protective agents against solar radiation. Sunscreens can be classified into organic (chemical) absorbers and inorganic (physical) blockers on the basis of their mechanism of action. Organic UV filters absorb UV radiation with excitation to a higher energy state. Excess energy is dissipated by emission of longer wavelengths or by relaxation by photochemical processes such as

isomerization and heat release. They include camphors, benzophenones, cinnamates and triazines, among others. Inorganic sunscreens, i.e., titanium dioxide and zinc oxide, protect the skin by reflecting and scattering UV radiation.

The protection against UV sunlight is becoming an increasing need because of harmful effects of UV radiation on human skin, like skin drying and ageing, spots emergence, erythema and induction of skin cancer [1]. In particular, and due to the higher incidence of skin cancer in recent decades, several countries worldwide have promoted the use of sunscreen products. These chemicals can be found not only in cosmetics but also in other personal care products, food packaging, pharmaceuticals, plastics, textiles and vehicle maintenance products [2] to prevent photodegradation of polymers and pigments [3]. Therefore, as their use has risen also their presence in the environment has done.

1.1 Occurrence in the Environment

UV filters are currently considered as emerging environmental contaminants of increasing concern since most of the commonly used are known to cause endocrine disrupting effects in both aquatic and terrestrial organisms as well as in human skin cells [4]. These compounds bioaccumulate in fish at similar levels to polychlorinated biphenyls (PCBs) and DDT [5, 6].

Sunscreen agents enter the aquatic environment as a direct release or through sewage. Residues of more polar organic UV filters have been found in all kinds of water sources [5–8], including tap water [9], at the ng/L level. Due to the high lipophilicity and poor biodegradability of many UV filters, they end up in sewage sludge during wastewater treatment [10–13] and accumulate in river sediments [7, 14, 15] and biota [6, 7, 16, 17] at concentrations in the range of ng/g to mg/g.

1.2 Environmental Effects

Some reviews concerning the toxic effects of UV filters in the environment can be found in the literature [18–21]. In general, risk for acute toxic effects posed by sunscreens is unlikely to aquatic organisms at usual environmental detected levels [22], but chronic environmental toxic effects cannot be ruled out [6, 23]. Data of their effects in different aquatic organisms, such as the standard model *Daphnia magna* [6], other invertebrates [23, 24], fish [25–27], and tadpoles [28] among others, can be found. In those studies, *D. magna* was observed to be most sensitive over short-term exposures (48 h) to UV filters with higher log K_{ow}, such as ethylhexyl methoxycinnamate (EHMC) [6]. Long-term exposures (28–56 days) to 3-benzylidene camphor (3BC) and 3-(4′-methylbenzylidene) camphor (4-MBC) result in a significant reduction in reproduction and an increased mortality of benthic invertebrates [23]. However, no alterations in hormone levels in tadpoles

of frogs were detected at concentrations up to 50 μg/L [28]. Almost all the studies revealed that the observed effects occur at higher concentrations than those found in rivers and lakes. However, global environmental toxicity studies are needed in order to perform an appropriate risk assessment.

Besides these toxic effects, many studies showed the potential for oestrogenic activity of several sunscreen agents. These effects can be detected in vivo or at in vitro assays. The last are usually more sensitive than the former, concluding sometimes in an overestimation of the effects. Nevertheless, in vitro assays are faster and cheaper and allow a rapid screening of oestrogenic compounds. Therefore, a combination of different assays is recommended in order to have a wide spectrum of toxicity data that would allow performing a reliable risk assessment.

Many in vitro assays have been developed. They are mainly recombinant yeasts expressing human or rainbow trout oestrogen receptor (hER or rtER). But also human cell lines, e.g., MCF-7 cells, are used. Recombinant yeast assay (RYA) indicate that a wide range of UV filters have potential to cause oestrogenic effects. Benzophenone-1 (BP1) was found to be the most potent UV filter, with an EC_{50} of 0.8 μM (171 μg/L). Other benzophenones, including benzophenone-3 (BP3), present EC_{50} in a range from 2.96 to 298 μM, and reaching in many cases 100% of estradiol (E2) effect [26]. In the same study, no oestrogenic effects were detected for other UV filters such as 4-MBC, EHMC, octocrylene (OC), homosalate (HMS), 4-*t*-butyl-4′-methoxy-dibenzoylmethane (BMDM), and 2-ethylhexyl-4-dimethylaminobenzoate (OD-PABA). However, oestrogenic activity was detected in assays with MCF-7 cells in the following compounds, ordered from more to less oestrogenic: 3BC, HMS, BP1, EHMC, OD-PABA, 4-MBC and BP3 among others [29, 30]. EC_{50} in those studies ranged from 0.68 to 3.73 μM. It can be seen that, in general, in vitro assays using MCF-7 cell lines are more sensitive, resulting in lower EC_{50} than RYA assays.

In vivo assays using fish indicate that numerous UV filters display oestrogenic effects and adversely affect fecundity and reproduction as well [25]. 3BC appears to be the most oestrogenic compound inducing vitellogenin (VTG), after 14–21 days of exposure, depending on the fish species [26]. BP3 induces VTG production at similar concentrations as 3BC and also significantly decreases fertilized eggs hatchability [31]. Other UV filters, such as BP1, also induce VTG production but at concentrations tenfold higher than 3BC [26]. Studies using rats have indicated that 4-MBC affects the hypothalamus–pituitary–gonadal system in male rats thus altering gonad weight and steroid hormone production [32]. Therefore, other alterations in the endocrine system than simply binding to the oestrogen receptor can be generated by UV filters. In fact, alterations in androgenic, progestogenic and thyroid system have already been pointed [4, 33, 34]. Moreover, effects of mixtures are another point not deeply studied, although the first works pointed to a synergetic effect [35, 36].

In conclusion, a reliable assessment of the potential risk of UV filters to the ecosystems is, therefore, not yet possible, due to the scarcity of data on environmental concentrations and to the few species used to identify biological effects.

1.3 Degradation Processes

Monitoring data of wastewater treatment plants (WWTPs) indicate that current treatments based on conventional activated sludge are not effective at removing UV filters since several of them were found in untreated and treated wastewater in different countries [5, 7, 37], and in sewage sludge [10, 13]. Concentrations found in effluent waters were as high as 2.7 μg/L for 4-MBC and in sewage sludge from 0.04 to 9.17 mg/g dw. Therefore, advanced approaches need to be tested for a safer management of water and biosolids.

Due to the poor elimination observed for a number of contaminants under conventional treatment approaches, in recent years the development and implementation of advanced oxidation processes, membrane filtration and activated carbon adsorption have been used to improve removal efficiency. Among them, ozonation has the advantage of providing simultaneous oxidation of xenobiotics and disinfection of wastewater. This degradation technology is currently considered economically feasible, with cost below 0.2 euro/m^3 [38]. There is quite a high volume of information about removal of pharmaceuticals and pesticides via ozonation, but the data available for personal care products, and especially for UV filters, is rather limited and somewhat ambiguous. For instance, whereas Rosal et al. [39] did not observe any elimination of BP3 by ozone, other studies report removal efficiencies higher than 80% [40].

Because of UV filters are substances designed to absorb solar energy, photolysis and photocatalysis have been tested as a feasible treatment to degrade the recalcitrant compounds. To date, very few studies have examined UV filters response under UV radiation when exposed in aqueous samples [41–44]. Results indicate that the extent of degradation is quite variable, from no photodegradation of BP3 to complete mineralization of BP1 after 24 h of UV light irradiation.

One of the few studies on UV filters biodegradation [45] determined the microbial degradation of BP3 and 4-MBC in a vertical flow soil filter being only 12% of the total load in the first case and 75% in the latter. However, due to the high sorption affinity of the compounds, the elimination percentages reached almost 100% in a low load experiment and 82–86% for BP3 and 91–96% for 4-MBC in a high load experiment. All tests were done at environmental relevant concentrations (3 μg/L). Hernández Leal et al. [40] studied the removal of some UV filters (4-MBC, BP3, OC and avobenzone) in different biological systems under aerobic and anaerobic conditions. Based on their high log K_{ow} values, they assume that their high removal under aerobic conditions and also the variable yields of removal under anaerobic conditions are driven by adsorption and not by biodegradation. In a full-scale plant analysis, UV filters are also removed from the aqueous phase in a large extent, with percentages over 92% [46]. However, degradation percentages of 4-MBC, OMC and OC calculated from a mass balance analysis only achieved 45–90% of the initial load [46].

A novel biological technology for the efficient elimination of pharmaceuticals and personal care products (PPCPs) is based on the biodegradation by white-rot fungi.

The mechanism of action mainly relays on the activity of the unspecific oxidative enzymes extracellularly segregated such as lignin peroxidase, laccase and manganese peroxidase. This fungal degradation process has been proved to be successful at degrading a number of contaminants such as the pharmaceuticals carbamazepine, ibuprofen and clofibric acid [47]. There are two approaches to treat contaminated effluents by means of fungi: with purified fungal enzymes or with the entire fungus. Regarding UV filters, Garcia et al. [48] applied the enzymatic treatment with purified laccase to the degradation of BP3, also at low concentrations (10 μg/L). They achieved a complete removal of the compound in a primary effluent. However, the main inconvenient is that BP3, as many other xenobiotics, is not directly recognized by the enzyme laccase and the addition of some mediator such as 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) is necessary. Moreover, a higher mediator/contaminant molar ratio is needed as lower the contaminant concentration is. That raises the price of the treatment and adds extra compounds to be degraded in the effluent. Other drawbacks of the use of purified enzymes are the deactivation over time and a decrease in the range of treatable contaminants even with the addition of mediators in comparison with the use of the entire fungus. Thus, the use of the entire fungus is totally suitable in the cases where there is a complex mixture of compounds to oxidize. The use of those enzymes is still widely applied in industries such as olive, pulp and paper mill.

The potential ability of using the entire fungus, in particular *Trametes versicolor*, to degrade selected UV filters has been studied by Badia-Fabregat et al. [49] and Gago-Ferrero et al. [44] and it is described in next sections of this chapter. Nevertheless, degradation processes are only useful if they do not lead to the formation of new compounds with higher toxicity or bioaccumulation capacity. Therefore, it is necessary to identify and characterize the derivatives formed during the transformation processes and to assess the potential toxicity not only of parent compounds, but also of the degradation products formed, in order to draw a complete picture of the process.

2 Fungal Degradation by *T. versicolor*

To date, very few works deal with the degradation of UV filters both in liquid and in solid media. In particular, there is a lack of data on biodegradation of UV filters by fungi. Recently, an attempt to fill this gap has been carried out by assessing the potential of the white-rot fungus *T. versicolor* to degrade selected UV filters.

Degradation percentages of several UV filters and some of their metabolites in a solid-phase treatment of sewage sludge by *T. versicolor* are shown in [80]. As treatments were performed in sterile conditions, the near 100% removal efficiencies of those compounds can be attributed to fungal biotransformation. However, experiments in synthetic liquid media with the fungus in the form of pellets are preferable for the in-depth study of each contaminant.

2.1 Treatment in Liquid Medium

Unlike polyaromatic hydrocarbons (PAHs) and some pharmaceuticals, fungal degradation of UV filters and, by extension, of most of the personal care products compounds is not extensively studied.

Two of the most widely used and detected UV filters in the environment and WWTPs are BP3 and 4-MBC. Thus, they were the selected compounds to study individually their degradation by fungi [44, 49]. Studies with BP1, not only a BP3 metabolite but also an industrial UV filter (but its use in cosmetics is not allowed) itself have also been performed. Studies in liquid media allow a better analysis and monitoring of many parameters, both the contaminant concentration and the fungal metabolic state such as glucose consumption and enzyme production. In these studies, the degradation process was performed with the fungus in form of pellets.

If there is only interest in determining the elimination or degradation percentages, as in the studies mentioned above, experiments can be performed at low contaminant concentrations, similar to those found in the environment. However, if metabolites' identification analysis is wanted to be performed, higher concentrations are needed. That is, because degradation products are usually at much lower concentration than the initial parent compound.

Another important issue to take into account is the low solubility of many of the UV filters, which makes totally necessary to work with unit samples due to the indispensable solubilization step before the analytical determination by high performance liquid chromatography (HPLC).

Those studies show that the fungal elimination yields are quite high, reaching almost 100% at 24 h of culture (see Fig. 1). Contaminant was at the initial concentration of 10 mg/L, fungi was at 5 g/L dry weight (dw), and incubation conditions were 25°C and 130 rpm. In those experiments, where amber bottles were used to avoid a possible photodegradation of the compounds, it is necessary to include different types of controls as well. One of them would be the uninoculated control, without fungal addition. That control is useful to detect any kind of abiotic degradation such as spontaneous conjugation with glucose molecules present in the medium [50]. Then some controls with inactivated fungus are also necessary in order to evaluate the adsorption of the contaminant on the biomass. Fungus can be inactivated in different ways, the drawback is that all of them act different and thus the adsorption can become different. The most used inactivation procedures are the termicl one (autoclaving) and the addition of sodium azide. These differences come from the distinct action of high temperatures and sodium azide to the fungus. The first one causes physicochemical changes in the surface morphology of the fungus, resulting in variations in the biosorptive capacity of the heat-killed cells with respect to live cells [51], and the latter is a metabolic inhibitor with a yet unclear action mode. Some authors propose that it is a cytochrome c oxidation inhibitor [52], while others consider it as an ATPase inhibitor [53]. Anyhow, sodium azide stops the metabolism, thus inhibiting the active transport through the membrane but not avoiding the first step of binding to the surface, a rapid and energy independent phenomenon, or some

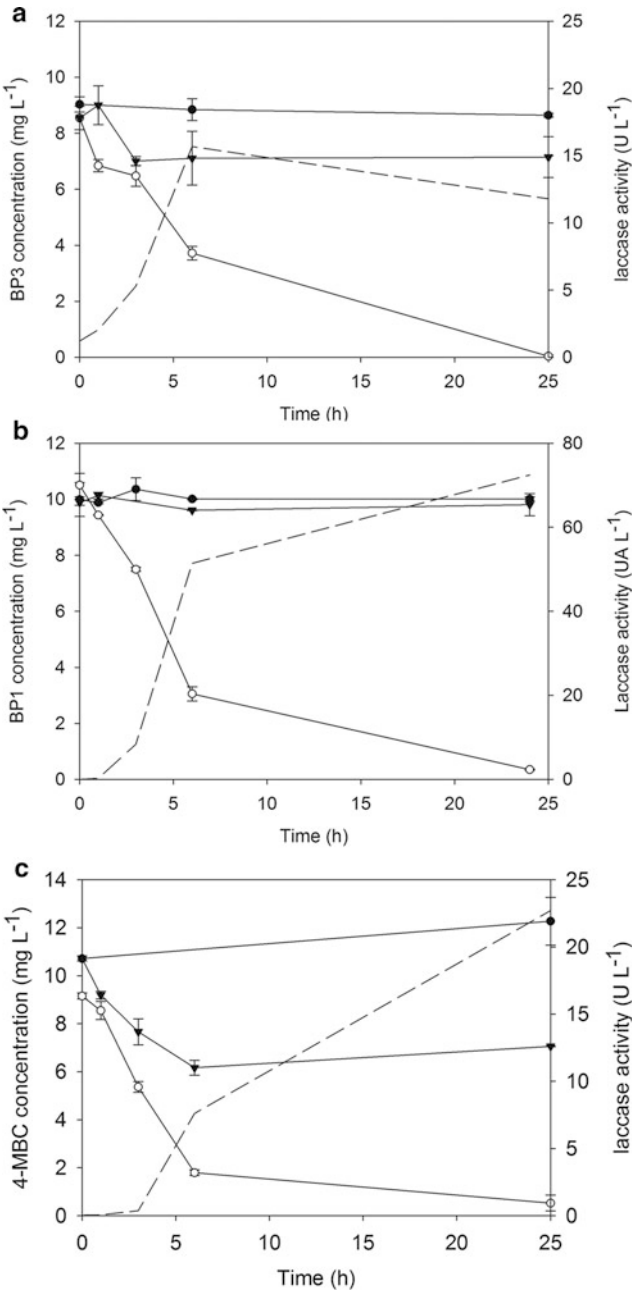

Fig. 1 Concentration profiles of (**a**) BP3, (**b**) BP1 and (**c**) 4-MBC concentration during 24 h degradation experiment by *T. versicolor* at Erlenmeyer scale and at 10 mg/L initial concentration. Treatments: (*filled circle*) uninoculated controls (UNI), (*open circle*) experimental bottles (EB) and (*filled inverted triangle*) heat-killed controls (HK). Laccase activity in EB is also plotted in *long dashes*. Values plotted are means ± standard error for triplicates. Modified from [44, 49]

passive membrane transport, energy independent as well [52, 54]. Finally, it is also recommendable to add a blank control, where no contaminant at all is added to monitor the normal metabolic activity of the fungus (growth, glucose consumption, enzymatic activity) and to report differences with the addition of the contaminant, i.e., to see whether the contaminant is toxic or not to the fungus or whether the observed enzymatic activity is endogenous or induced by the xenobiotic.

For the three UV filters studied (BP3, 4-MBC and BP1), the elimination in the experimental bottles can be completely assigned to fungal degradation. On the one hand, in BP1 and BP3 experiments because of including the solubilization step allows a complete recovery of the contaminant in the inactivated controls, and on the other hand, the case of 4-MBC, where the adsorption values reached almost 50%, all the initial contaminant is recovered after the fungal pellets' disruption. In this way, with the inclusion of a sonication step, it is possible to recover all the contaminant that the dead fungus had taken from the medium, probably by means of some passive mechanism. Elimination of contaminants is not only due to a single transformation but also due to a further degradation because quantification analysis of the main metabolites produced revealed that they also disappeared along the treatment period. More extensive information about the metabolites can be found in Sect. 2.2 of this chapter.

In a first scale-up to an air pulsed reactor of 1.5 L, it can be seen how at lower concentrations (250 μg/L) the removal of both studied benzophenones is also very high. In the case of 4-MBC, as well as other highly hydrophobic compounds, those kinds of studies in bioreactor are not suitable to perform because it is totally necessary a complete solubilization of the compound for a reliable quantification. Therefore, liquid samples cannot be taken at different time course because they would not be representative. That fact, at different scale, would be what happen with those compounds in the WWTP (and to extension in the environment): the more soluble compounds, like benzophenones, are those found in a higher proportion in the liquid effluent, while the UV filters with a higher log K_{ow} (such as 4-MBC and OC) are mainly found adsorbed onto the sludge.

2.2 Identification and Structural Elucidation of Biodegradation Products

New compounds formed during degradation must be identified and characterized in order to assess their potential biological activity and to get a complete picture of the process. To the authors' knowledge, there are no studies in the literature identifying fungal by-products from UV filter degradation. The studies on BP3, BP1 and 4-MBC carried out by Gago-Ferrero et al. [44] and Badia-Fabregat et al. [49] are the only providing data on fungal treatment by-products identification of UV filters.

2.2.1 Analytical Methodology

A detailed description of the sample preparation carried out for sunscreen analysis in sewage sludge is included in [81].

The instrumental analysis for the identification of UV filters degradation products formed during the fungal treatment process was performed by means of HPLC coupled to tandem mass spectrometry using a hybrid quadrupole-time-of-flight mass spectrometer (HPLC-QqTOF-MS/MS). Chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 5 μm, from Merck). In the optimized method, the mobile phase consisted of a mixture of HPLC grade water and acetonitrile, both with 0.15% formic acid. The injection volume was set to 10 μL and the mobile phase flow-rate to 0.3 mL/min.

MS full-scan analyses in the range *m*/*z* 50–700 were carried out on selected samples in both positive (PI) and negative (NI) electrospray (ESI) ionization modes at different cone voltages (15, 25 and 35 V) and with a capillary voltage of 3,000 V. Further MS/MS analyses were carried out on identified molecular ions for their structural characterization. Collision-induced fragmentation (CID) of selected *m*/*z* ions was evaluated at different collision energies between 10 and 40 eV, using argon as the collision gas at a pressure of 22 psi. Data were collected in the centroid mode, with a scan time of 0.3 s and an inter-scan delay time of 0.1 s, and with a full width at half maximum (FWHM) resolution of 5,000. Other MS/MS parameters were set as follows: 600 L/h for the desolvation gas at 350°C, 50 L/h for the cone gas and 120°C as source temperature. A valine-tyrosine-valine (Val-Tyr-Val) reference solution (*m*/*z* of $[M + H]^+ = 380.2185$) was used to tune the instrument and also as lock mass to ensure mass accuracy. The reference was analyzed by infusion in the MS analyzer by means of an independent reference probe (LockSpray™). Exact masses were calculated and the elemental composition of the molecular ions and their fragments were determined using the MassLynx V4.1 software.

2.2.2 Identification and Structural Elucidation of BP3 and BP1 Degradation Products

Figure 2a shows the full-scan chromatograms for samples at initial conditions and after 1, 5 and 9 days of BP3 degradation by the fungus *T. versicolor*. HK and BC chromatograms were recorded to discard the formation of other products than BP3 fungus degradation products. As depicted in Fig. 2a, solely BP3 ($t_R = 8.55$ min) was present before starting the biodegradation experiment ($t = 0$). BP3 levels decreased rapidly and were not detectable after 24 h of treatment ($t = 1$ day). At this time two new chromatographic peaks at t_R 4.69 and 5.20 min were observed in the chromatogram, corresponding to *m*/*z* ratios of 413 and 383 Daltons (Da), respectively. Maximum levels of these metabolites were determined at day 1, and they practically disappeared between 9 and 15 days of treatment, constituting the

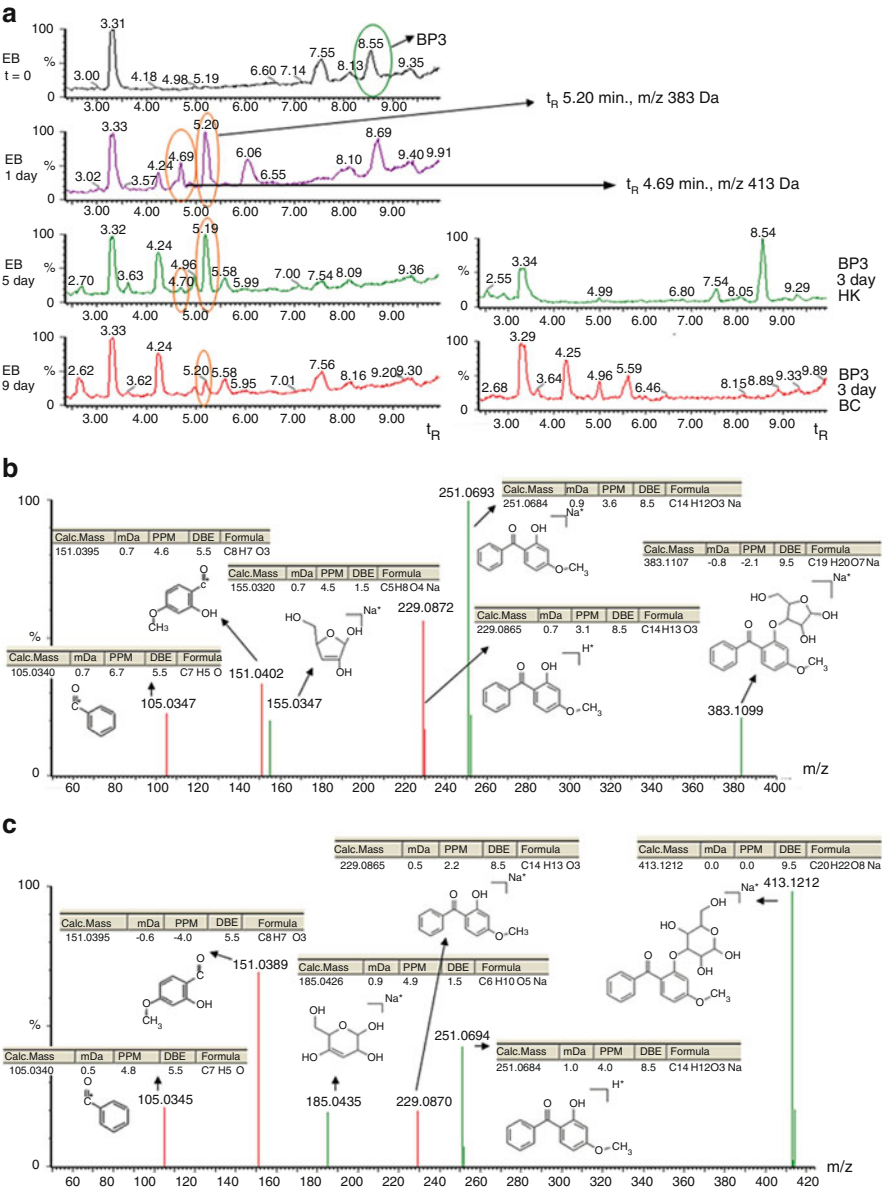

Fig. 2 (**a**) Total ion current chromatograms (TIC) of samples collected at $t = 0$, 1, 5 and 9 days. HK and BC TIC are also shown. (**b**) and (**c**) ESI(+)-MS/MS spectra of the molecular ions (in the form of sodium adduct) of the metabolites at (**b**) *m/z*: 383 and (**c**) $m/z = 413$ in $t = 1$ day samples [cone voltage =20 V, collision energies = 10 eV(*green*) and 35 eV (*red*)]. Accurate mass measurements of product ions are also provided. Figure taken from [44]

major degradation products of BP3 and may explain the decrease in the concentration of BP3 observed.

Figure 2b, c summarizes the MS/MS spectra obtained and the fragmentation patterns (FP) proposed for these degradation products, i.e. *m/z* 383 and *m/z* 413 at two collision energies. Additionally, the experimental and theoretical exact masses obtained for the molecular and fragment ions, expressed as *m/z*, the proposed elemental composition, together with recalculated mass errors (in mDa and mg/g) and double bond equivalents (DBEs) given by the software (mass measurements accuracy threshold of 5 mg/g) are provided. In both cases, higher sensitivity was obtained for the sodium adduct of the molecule ($[M + Na]^+$) as compared to that of the protonated molecule $[M + H]^+$, which was hardly detected. Thus, further MS/MS experiments were performed taking the sodium adduct as the precursor ion.

As it can be observed in Fig. 2b, CID fragmentation of *m/z* 383 at high collision energy completely fragmented the molecular ion and provided the $m/z = 229.0865$ as the major fragment ion. This accurate mass corresponded to the molecular formula $C_{14}H_{13}O_3$. Additionally, two other fragment ions were generated with $m/z = 151.0402$ and $m/z = 105.0347$, which gave the best-fit formula $C_8H_7O_3$ and C_7H_5O, respectively. The fragment with $m/z = 229.0865$ fits with the molecular formula of the BP3 molecular ion $[M + H]^+$, whereas the fragments with *m/z* 151 and 105 coincided with those product ions selected for selected reaction monitoring (SRM) acquisition of BP3. The fragment *m/z* 151 is produced by the loss of the phenyl group of BP3 $[M\text{-}C_6H_5]^+$, whereas the fragment *m/z* 105 corresponded to the benzoyl cation $[C_6H_5C = O]^+$. In the light of these results, it appears that BP3 is part of the molecular structure of the metabolite with *m/z* 383. In the spectrum obtained at low collision energy, the molecular ion $m/z = 383.1099$ is observed, which gave the best-fit formula $C_{19}H_{20}O_7Na$. At low collision energy, the most abundant fragment ion was $m/z = 251.0693$ ($C_{14}H_{12}O_3Na$), which probably corresponds to the adduct $[BP3 + Na]^+$. A fragment at $m/z = 155.0327$ with best-fit molecular formula $C_5H_8O_4Na$ was also detected, which would not correspond to any part of the BP3 molecule, unless aromatic ring cleavage occurred, which is not likely with CID experiments at low collision energy. Therefore, those data suggested that the metabolite with *m/z* 383 might be BP3 conjugated with a molecule of 132 Da. Several studies claim that the formation of conjugated metabolites with pentoses (mainly xylose and ribose) and hexoses (mainly glucose) is a predominant pathway in the degradation of chemicals by white-rot fungi, especially in phenolic hydroxyl groups [55, 56]. In this case, the addition of a pentose via glycosidic bond to the BP3 with the consequent loss of one molecule of water corresponds to an increase in the molecule mass of 132 Da. Thus, the metabolite at *m/z* 383 may be produced by conjugate addition of one pentose molecule to BP3.

CID spectra obtained for the metabolite with *m/z* 413 was similar to that of the previously discussed metabolite (*m/z* 383). At high collision energy the fragments with *m/z* 229.0870 ($C_{14}H_{13}O_3$), *m/z* 151 and *m/z* 105 that presumably correspond to BP3 and its main fragments were also detected. In the spectrum obtained at low collision energy, apart from the sodium adduct of BP3 ($m/z = 251.0694$,

$C_{14}H_{12}O_3Na$ $[BP3 + Na]^+$), two other fragment ions were observed. One at *m/z* 413.1212, which gave the best-fit formula $C_{20}H_{22}O_5Na$, corresponding to an increase of 162 Da of the BP3 molecular weight, and the other at *m/z* of 185.0435 ($C_6H_{10}O_5Na$). Following the rationale discussed above, it appears that the metabolite at *m/z* 413 might be generated by the conjugate addition of one hexose molecule, likely glucose, via glycosidic bond and consequent loss of one molecule of water.

BP1 degradation by the fungus *T. versicolor* was investigated following the same procedure. The results were very similar (data not shown) to those discussed above for BP3. After 24 h of treatment, BP1 disappeared and only one new chromatographic peak at $t_R = 5.09$ min, was observed. This compound reached maximum concentration levels after 24 h of treatment, and then, decreased until not detectable levels after 6 days of treatment. The *m/z* ratio of this compound was 347. MS/MS spectra obtained for *m/z* 347 were very similar to those obtained for BP3; however, in this case the most abundant molecular ion was the protonated form $[M + H]^+$. CID fragmentation of $m/z = 347$ allowed to observe the ion $[BP1 + H]^+$ and its two main fragments ($m/z = 137.0233$, $[BP1\text{-}C_6H_5]^+$ and $m/z = 105.03043$, $[C_6H_5C = O]^+$), also selected for SRM acquisition. Additionally the molecular ion $m/z = 347.1131$ ($C_{18}H_{19}O_7$) and the fragment $m/z = 133.0507$ ($C_5H_9O_4$) were also registered during CID experiments. As it was observed for the BP3 metabolite with *m/z* 383, this metabolite differed from the parent compound in 132 Da. Therefore, the conjugate addition of one pentose molecule to BP1 is proposed as the formation mechanism of *m/z* 347.

Target analysis was also carried out in order to detect small amounts of selected derivatives, namely BP1, 4HB, 4,4′-dihydroxybenzophenone (4DHB), 2-2′dihydroxy-4-methoxybenzophenone (DHMB) and 2,3,4-trihydroxybenzophenone (THB), which have been previously identified as metabolites in rats and humans [57–59]. These compounds were analyzed in the samples by HPLC-MS/MS using a hybrid quadrupole-linear ion trap-mass spectrometer (QqLIT-MS/MS) under the same chromatographic conditions. MS/MS detection was performed in positive ESI ionization in SRM mode. BP1, 4DHB and 4HB were determined as metabolites produced during the degradation experiments of BP3 with the fungus *T. versicolor*. BP1 was detected after 1 and 6 h of treatment at a concentration of 2.4 and 3.6 μg/L, respectively, and disappeared afterwards. These low values may be attributed to a high fungal degradation rate of BP1. 4DHB and 4HB were determined after 3 days of treatment. 4DHB was detected at 3, 5 and 9 days at a concentration of 11.5, 50.7 and 31.3 μg/L, respectively, while 4HB concentrations for 3, 5 and 9 days were below LOQ (0.5 μg/L). THB and DHMB were apparently not produced by the treatment with the fungus, since they were not detected in the analysed samples.

Fungal degradation of BP1 resulted in the formation of 4HB and 4DHB. The levels of these metabolites found after 3 and 6 days of treatment were similar to those achieved in BP3 degradation experiments. Likewise in BP3 degradation tests, THB and DHMB were not detected in any of the collected samples. The levels found of these metabolites were quite low; therefore, these products cannot be considered as major degradation products.

Nontarget identified metabolites constitute conjugated forms of BP3 and BP1, and thus, further enzymatic activity might revert them to the parent compound. Nevertheless, the fact that the two metabolites, together with BP3 or BP1, finally disappeared confirms further fungal degradation with a possible cleavage of aromatic rings.

2.2.3 Identification and Structural Elucidation of 4-MBC Degradation Products

For the identification and structural characterization of 4-MBC fungal degradation products, a procedure similar to that applied for BP3 and BP1 was carried out. In order to discard the formation of other products than 4-MBC fungus degradation products, HK and blank control (BC) chromatograms were recorded. Solely one peak of 4-MBC (*E*) (t_R = 10.27 min, [M + H] + =255) was present before starting the biodegradation experiment ($t = 0$). In accordance with that previously described, the intensity of this peak readily decreased to below the 5% of the initial area in 24 h. In the early hours of the experiment, a peak at t_R = 10.73 min. was present, with the same *m*/*z* ratio and identical MS/MS FP than that of 4-MBC. This fact suggested that a 4-MBC isomer, likely 4-MBC(*Z*), was also present. 4-MBC(*Z*) is a common photodegradation product [42] of the commercially available 4-MBC (*E*) and the isomerization of 4-MBC(*E*) to 4-MBC(*Z*) by the action of ten organisms has been described as well [60]. These two studies also mention the higher retention time of the *Z* isomer as occurs in this case.

Also from the early hours two peaks at t_R = 7.96 and 8.22 min were observed. These peaks reached their highest intensity upon 1 day of treatment, decreasing to be undetectable at $t = 3$ days. The two peaks had a base peak at *m*/*z* 271 and identical fragmentation pattern, fact that suggest that they were isomers of the same compound (denoted as Pr271). Figures 3 and 4a show the mass spectra and structures proposed for 4-MBC and Pr271, respectively. Considering that the mass of Pr271 was shifted 16 Da upwards relative to the parent compound and that the fragments obtained were also shifted 16 Da upwards relative to the parent compound fragments, hydroxylation by the •OH radical was assumed. Fragmentation of Pr271 yielded fragments with *m*/*z* 229 (detachment of the bridged moiety), *m*/*z* 187 (loss of C_5H_8O), *m*/*z* 173 (breaking up of the camphor moiety) and *m*/*z* 121. The fact that the mass of this last fragment ion, which corresponded to the aromatic moiety of the compound, was shifted 16 Da upwards relative to the parent compound fragment (*m*/*z* 105) suggested that the hydroxyl group, –OH, would be attached either to the aromatic ring or to the methyl group next to the aromatic ring. Nevertheless, on the basis of the MS/MS FP solely, an unequivocal assignment of the molecular structure was not possible, as the position of the hydroxyl group could not be elucidated for any of the fragments.

The less intense peaks, with *m*/*z* 287 (Pr287) found at t_R = 7.17 and t_R = 7.65 min (same MW and FP) showed an almost identical behaviour to that described above for the Pr271.

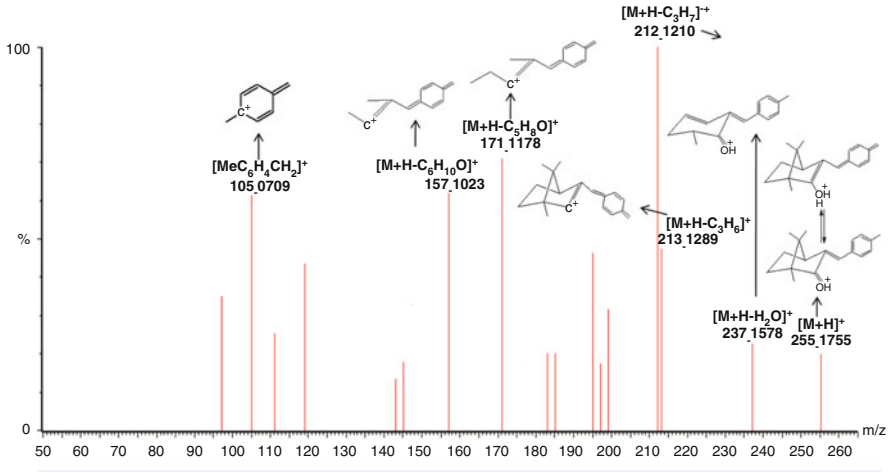

Fig. 3 4-MBC product ion spectrum obtained from MS/MS experiments performed by HPLC-QqTOF-MS/MS. Figure taken from [49]

In Fig. 4b fragment ions can be observed, which correspond to 16 Da upwards relative to Pr271 and 32 Da to the parent compound fragments. These data suggest that a double hydroxylation of 4-MBC occurred. The two hydroxyl groups were attached, as in the previous case, in the aromatic moiety of the compound.

The intensity of the peaks of Pr271 and Pr287 are very low compared with the intensity depletion for the signal of 4-MBC. This suggested that they were not the major degradation products. The main peaks in terms of intensity, which correspond to the major metabolites, were found at $t_R = 6.31$ and 6.74 min (same MW and FP) with *m*/*z* 425 (Pr425). The best fitting elemental composition for this mass was $C_{23}H_{30}O_6Na$. The protonated adduct of this compound could be detected but its rather low intensity made not possible to obtain a good MS/MS spectrum. Better results were achieved working with the sodium adduct.

Figure 4c shows the overlapping MS/MS spectra for *m*/*z* 425 and *m*/*z* 271 at $t_R = 6.31$ min (at $t_R = 6.74$ are exactly the same). Pr425 had the most intense ion fragment at *m*/*z* 293, which best fitted with the elemental composition $C_{18}H_{22}O_2Na$. This was consistent with the sodium adduct of the Pr271 metabolite. For the ion fragment at *m*/*z* 155, the proposed elemental composition was $C_5H_8O_4Na$. MS/MS spectrum for *m*/*z* 271, in addition to showing the peaks at $t_R = 7.96$ and 8.22 min previously described, showed just two higher intensity peaks at $t_R = 6.31$ and 6.74 min. This spectrum was identical to that obtained for *m*/*z* 271 at $t_R = 7.96$ and 8.22 min. Those pieces of data suggested that the metabolite Pr425 might be Pr271 conjugated with a molecule of 132 Da. Pr425 could correspond to one pentose molecule conjugated to Pr271 by glycosidic bond with losing one water molecule. This possibility is further discussed in more detail.

Two peaks of lower intensity were obtained at $t_R = 5.01$ and 5.36 min (same MW and FP) with *m*/*z* 441 (Pr441), whose elemental composition was elucidated as

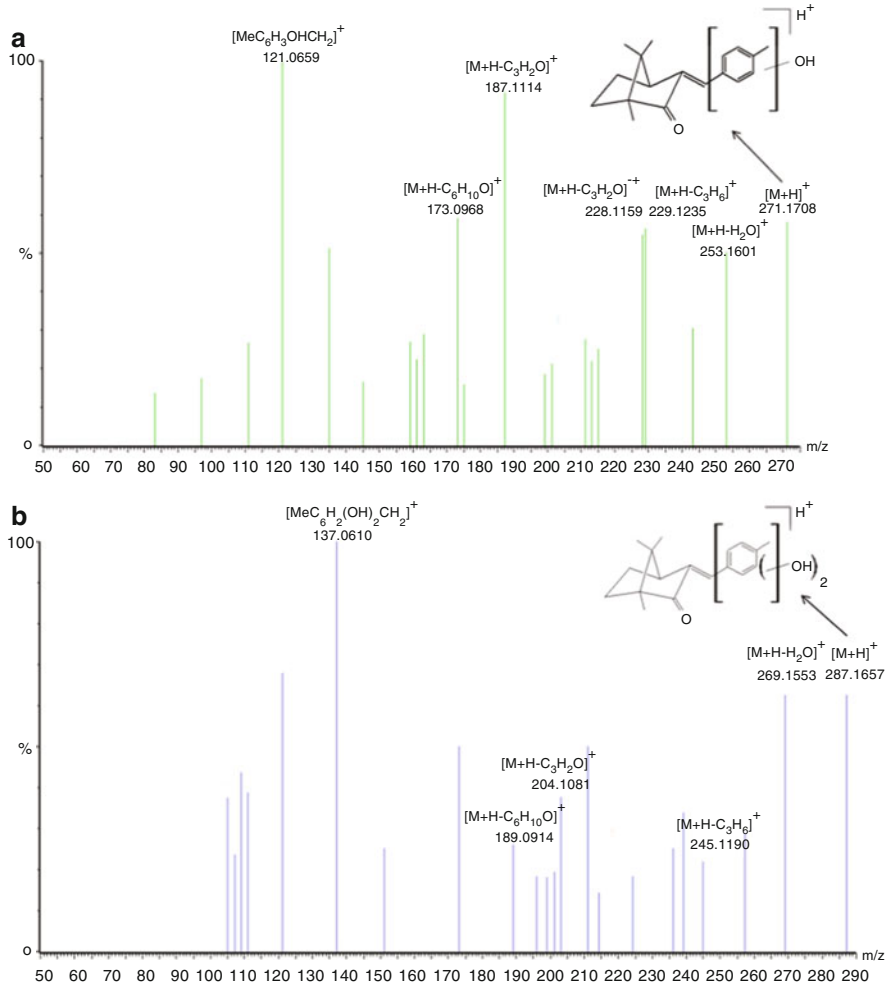

Fig. 4 (continued)

$C_{23}H_{30}O_7Na$. In this case, the protonated adduct could not be detected. Figure 4d shows the MS/MS spectra for *m/z* 441 and *m/z* 287 at $t_R = 5.01$ (spectra at $t_R = 5.36$ are exactly the same).

Such analogous behaviour was previously observed for Pr425 and Pr271. The MS/MS spectrum for *m/z* 287 at $t_R = 5.01$ min is exactly the same as *m/z* 287 at $t_R = 7.17$ min. On the other hand, the fragment ions obtained from the MS/MS analysis of *m/z* 441 are *m/z* 309 with molecular formula $C_{18}H_{22}O_3Na$ corresponding to Pr287 sodium adduct and *m/z* 155 with molecular formula $C_5H_8O_4Na$ (same as that obtained in the case of Pr425). These data suggested that Pr441 corresponded to one pentose molecule conjugated to Pr287 by glycosidic bond. Pr425 and Pr441 reached their maximum intensity after 3 days of treatment, and decreased until being undetectable, upon 12 days of fungal exposition.

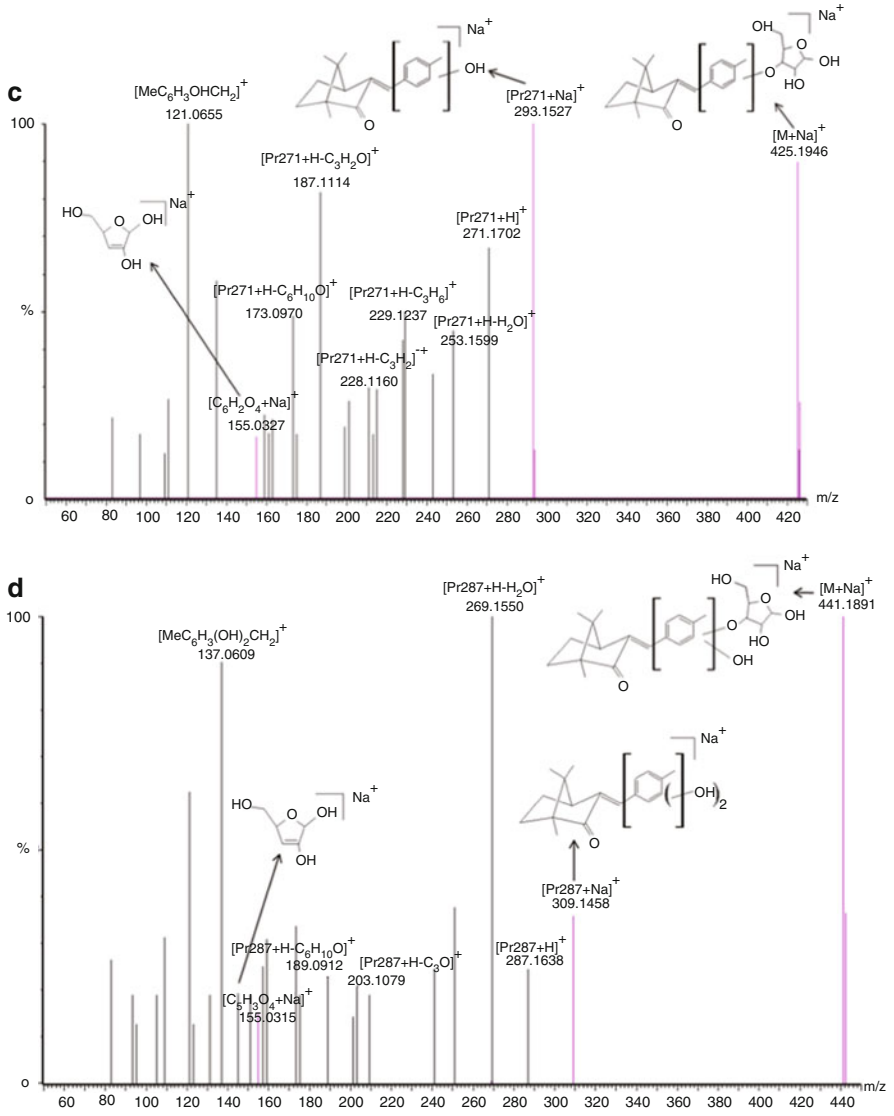

Fig. 4 4-MBC metabolites product ion spectra obtained from MS/MS experiments performed by HPLC-QqTOF-MS/MS. (**a**) Pr271, (**b**) Pr287, (**c**) Pr425 and (**d**) Pr441. Figure taken from [49]

2.3 Degradation Pathways

The study of the degradation pathways in fungi for PPCPs is relatively recent and not fully developed. In general, the most studied xenobiotic degradation pathways in fungi are those related to PAHs [61]. As PPCPs present aromatic structures it is

assumed that the enzymes implicated in their degradation might not differ considerably from the ones implicated in PAHs metabolism. Those are the extracellular enzymes laccase, manganese peroxidase and lignin peroxidase, and the intracellular cytochrome P450 system.

There are more studies related to PPCPs metabolism in rats or humans, as toxicokinetics and degradative metabolism have been analyzed to assess their associated risks. However, UV filters are an example where the degradation metabolism differs in mammals and in fungi [62], despite the enzymes involved are of the same family (cytochrome P450). Toxicokinetic studies in rats revealed that the main metabolic pathway of BP3 is the dealkylation of the methoxy side chain, which lead to the metabolite BP1. Secondary pathways would be aromatic hydroxylations carried out by the cytochrome P450 leading to the hydroxylated-metabolites THB and DHMB [59]. Nevertheless, the predominant metabolites in human urine and semen samples during the first 24 h after topic application are the glucuronide conjugates of BP3 and BP1 [63]. None of those compounds, except BP1, were found in the fungal culture. In 4-MBC assays, the metabolites identified in rats and humans were 3-(4-carboxybenzylidene)-6-hydroxycamphor (the major metabolite) and 3-(4-carboxybenzylidene) camphor and their respective glucuronide conjugates [64]. As in mammals, phase I and phase II enzymes appear to participate in the degradation of BP3, BP1 and 4-MBC by *T. versicolor* [44, 49].

In vitro laccase assays lead to the conclusion that this enzyme is not able to transform BP3 and 4-MBC by itself, although BP3 can be degraded if external mediators increasing the oxidizing range of the enzyme are added. Taking into account that fungi have their own mediators, the oxidation of BP3 (but not 4-MBC) by the endogenous laccase-mediator system is in principle feasible. These results are in accordance with those published by Garcia et al. [48]. They also observed that low concentrations of BP3 (1 ng/L) require higher mediator/BP3 molar ratios than high BP3 concentration (1 mg/L) to maintain the same degradation rate at a given enzyme concentration (1 U/mL). This laccase enzymatic treatment was found to generate oxidative coupling reactions, with dimmers, trimmers and similar products.

Fungi express other extracellular oxidative enzymes, like MnP and LiP. However, no studies about their involvement in the degradation of UV filters have been published so far.

Other important enzymes in the degradation of xenobiotics are the monooxigenases cytochrome P450. They are a broad family of intracellular enzymes present in all eukaryotic organisms and also in some prokaryotic microorganisms. In vivo studies, consisting in the addition of a P450 inhibitor in a fungal culture with a contaminant, allows to know whether that enzymatic system participates or not in the first steps of the contaminant's transformation. The presence of the inhibitor 1-aminobenzotriazole (ABT) affected the degradation rates of both, BP3 and 4-MBC, but in different ways. ABT inhibited 4-MBC degradation completely, as the first step of hydroxylation of the aromatic ring is apparently carried out by the cytochrome P450. On the other hand, ABT reduces, but not eliminates BP3 degradation. Unlike

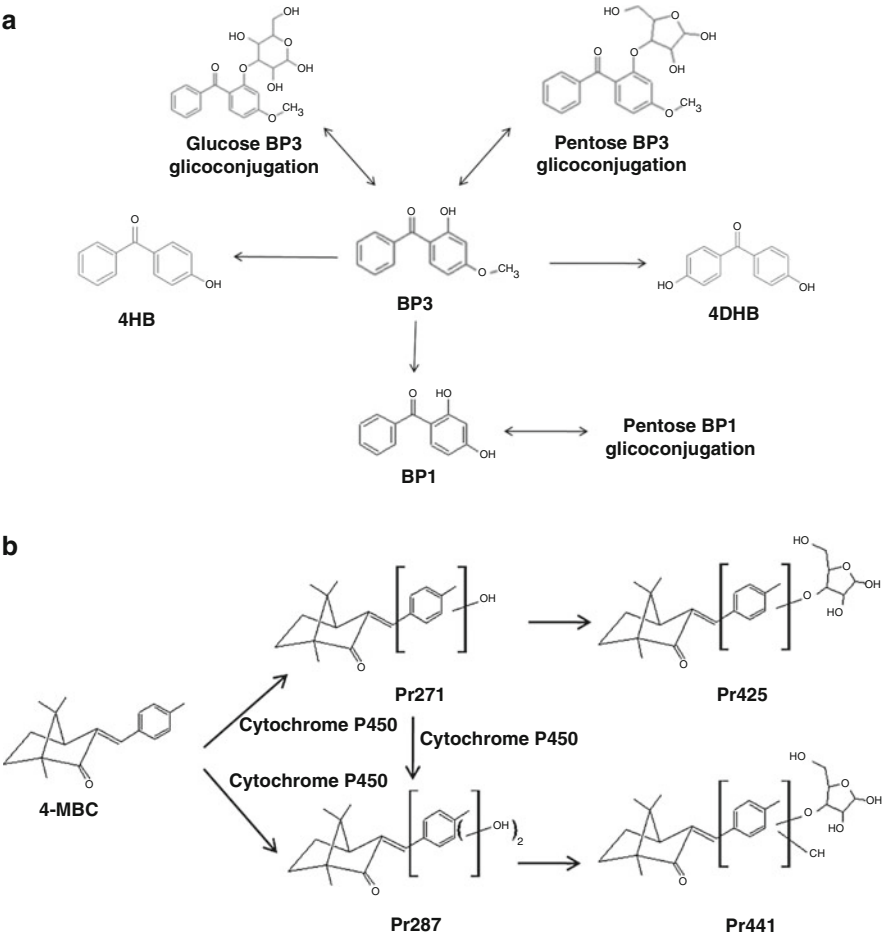

Fig. 5 Fungal degradation pathways of (**a**) BP3 and (**b**) 4-MBC. Modified from [44, 49]

4-MBC, BP3 presents hydroxylated groups that allow conjugation in the absence of cytochrome P450 hydroxylase activity.

Metabolites produced by laccases, P450 or other enzymes are more oxidized than the parental compounds, usually by addition of a hydroxyl group to an aromatic ring. In the case of BP3, cytochrome P450 might carry out also a demethylation as reported previously for 2,4,6-trichloroanisole (2,4,6-TCA) [65]. However, conjugated metabolites were also important in the transformation of the xenobiotics. Conjugation increases the polarity of the compounds, their bioavailability and their subsequent degradation. These processes generally result in a decrease of the toxicity of xenobiotics [66, 67], although in some cases the higher availability of the resulting metabolite may also increase its toxicity [47, 68]. Additionally, the problem of detecting conjugated metabolites is that they can be easily reverted to the parent

compound. Thus, it must be confirmed that at the end of the treatment both the parental compound and the conjugated forms have been degraded. In the case of BP3 it occurred after 9–15 days of culture, whereas conjugated 4-MBC metabolites disappear after a maximum peak at 3 days.

There are many fungal conjugated forms reported in the literature. The more common are glucuronide, glutathione, sulphate and glycoside conjugations [69, 70]. However, the conjugated metabolites of 4-MBC, BP3 and BP1 by *T. versicolor* are only formed by the addition of one pentose molecule to the parent compound or also of one glucose in the case of BP3. Ribose and xylose are the most common conjugated pentoses. As a consequence, the enzymes carrying out the transfer would be UDP-glucosyltransferase when the added molecule is glucose [71] and UDP-xylosiltransferase in the case that conjugation occurs with xylose, as it has been previously reported for other xenobiotics in *T. versicolor* [55]. Conjugation with ribose has been also described for other fungi [56], ant it can also occur in *T. versicolor* since the molecular weight of the resulting conjugate would be the same than for the xylose-conjugated metabolite. Sugar residues attach to the molecule through an O-glycosidic bond with the hydroxyl groups present in the aromatic ring [55, 56]. In BP3, the conjugation should occur through the unique free hydroxyl of the molecule, whereas BP1 presents two possible alternatives. These conjugated metabolites should contain the parental compound, since BP3 and BP1 structures were found without alterations in HPLC-MS/MS fragmentation analyses. In 4-MBC, conjugation would occur only after the mono- or di-hydroxylation of either the aromatic ring or the adjacent methyl group, but it was not possible to specify the exact position in our analyses.

For all the UV filters studied, the main metabolites identified were the conjugated forms. From them, the predominant product in BP3 degradation was the pentose conjugate, reaching at 24 h a peak of 40% of the initial amount of initial contaminant. The composition of the media could change the metabolites formed or, at least, the proportions as, in BP3, the absence of glucose in the media resulted in a decrease in the glucose conjugated form.

As stated before, any treatment does not make sense without a subsequent metabolization of both the conjugated and the free parental compound. Thus, as described above, metabolites gradually disappeared from the media, which means that O-glycosidic bond would gradually brake and other fungal enzymes would act. Then in the studied benzophenones, the monooxygenase cytochrome P450 would probably oxidize BP3 and BP1 by adding hydroxyl groups or by, the less extended, demethylation, only for BP3 in this case. It would lead to the formation of the detected BP1, 4HB and 4DHB metabolites. Nevertheless, the enzymatic system of fungi is broad [72] and other intracellular or extracellular enzymes could be involved. In 4-MBC, further degradation of glycoconjugated metabolites also occurred.

In conclusion, conjugation processes constitute one of the defensive mechanisms that fungi have against toxic hydroxylated compounds, decreasing their toxicity and increasing their bioavailability [71]. Thus, glycoconjugation would be the first step in the BP3 and BP1 (included in the degradation pathway of BP3) metabolism. For 4-MBC, any conjugation reaction requires a previous step of hydroxylation

(or dihydroxylation) by cytochrome P450. In all cases, conjugation would revert gradually and oxidation of compounds and subsequent ring cleavage would take place leading to smaller unidentified fragments. Figure 5 summarizes the proposed initial steps of the pathways for BP3 and 4-MBC degradation.

3 Biological Activity Evolution Profile Through the Biodegradation Process

Sunscreens and their degradation metabolites analyzed in this study are potential inducers of the oestrogen (ER) and aryl hydrocarbon receptors (AhR, also known as Dioxin Receptor). Ectopic activation of these pathways can cause severe damage to organisms and their ecosystem by altering reproduction, hormonal and/or circulatory systems [73–75] as well as they have been associated with carcinogenic and mutagenic effects [76–78].

Biological assays allow to complete sunscreens degradation analysis by measuring biological activity of the whole sample taking into account unknown metabolites and potential synergistic effects. Therefore, biological tests have been performed to assess oestrogenic and dioxin-like activity during the degradation process by *T. versicolor* of BP3, BP1 and 4-MBC, as well as sludge samples from the WWTP. Besides, parental and known metabolites (4-MBC, BP3, BP1, 4HB, 4DHB, THB and DHMB) have been tested individually by both assays.

3.1 Oestrogenic Activity

The oestrogen receptor recombinant yeast assay (ER-RYA) was performed as described in Noguerol et al. [79]. Oestrogenic activity of BP3 and BP1 determined with EC_{50} values of 12.5 and 0.058 mg/L, respectively, and an LOEC of 1.6 and 0.015 mg/L. These results indicate that BP1 is three orders of magnitude less oestrogenic than 17β-estradiol and 200-fold more oestrogenic than its parent compound (BP3) [44]. Metabolites 4HB and DHMB also show oestrogenic activity with EC_{50} values of 0.92 and 7.9 mg/L, respectively, whereas 4DHB and THB were considered nonoestrogenic in this assay. Similar values have been described by Kunz and Fent [33]. The oestrogenic potency of 4-MBC was half of BP3, with and EC_{50} value of 28.5 mg/L.

Analyses by ER-RYA during the BP3 and 4-MBC biodegradation process by *T. versicolor* showed no oestrogenic activity, indicating that any potential oestrogenic metabolite were not present at enough concentration to elicit biological response. In the case of BP3, this is in agreement with the low concentrations found for these metabolites during fungal degradation. Regarding BP1 biodegradation, the oestrogenic activity readily decreased; upon 4 h of treatment the activity was almost

completely eliminated. Finally, in the case of sludge samples, no oestrogenic activity was detected before or after the treatment.

3.2 Dioxin-Like Activity

Dioxin-like activity was assessed by the AhR recombinant yeast assay (AhR-RYA) performed as described in Noguerol et al. [79]. BP1, with an EC_{50} value of 0.61 mg/L showed tenfold more dioxin-like activity than BP3, with an EC_{50} value of 6.8 mg/L. However, two of their metabolites, 4HB and DHMB, presented 4 and 11 times higher activities, with EC_{50} values of 0.16 and 0.59 mg/L, respectively. No dioxin-like activity was observed for either 4DHB or THB.

Dioxin-like activity during biodegradation processes of BP3, BP1 and 4-MBC by *T. versicolor* was below detection limits for all three compounds. These data indicate that metabolites produced by *T. versicolor* were in small amounts or rapidly metabolized in non-dioxin-like compounds.

On the other hand, sludge samples showed a slight increase (two- to threefold) of dioxin-like activity after the fungal treatment, reaching values above the mg/L BNF equivalent mark. This data can be interpreted as an indicator for bio-activation of some compounds, other than UV filters, present in the sludge by the treatment with *T. versicolor*. These results emphasize the need of a broad screening of biological assays tests, as they differ in their capacity to detect specific hazardous effects.

4 Conclusions

Results of treating sewage sludge with the white-rot fungus *T. versicolor* can be found in [80], showing high degradation yields of all UV filters detected in the raw sludge, with percentages ranging from 87% to 100%.

Degradation products must be identified and characterized in any treatment in order to assess the suitability of the method with respect to biological hazard. These products are identified in liquid medium cultures, which facilitate the characterization procedure. During the degradation process of BP3, one of the most largely used sunscreens, BP1, another commercial UV filter, was formed. Its relative concentration, however, was almost insignificant because it also underwent fast biodegradation by *T. versicolor*. Using ESI(+)-QqTOF-MS/MS analysis (providing their exact mass and molecular formula), up to six derivatives were identified; BP1, 4DHB, 4HB, and the conjugates BP3-pentose (xylose or ribose) and BP3-hexose (glucose) for BP3, and the conjugate BP1-pentose for BP1. Regarding 4-MBC degradation in liquid medium, complete removal was achieved before 24 h as well. The main four metabolites identified were hydroxylated and pentose conjugated compounds. These results show the importance of phase II reactions besides oxidations in the degradation pathway of some toxic xenobiotics. Glycoconjugations decrease their

toxicity and increase bioavailability, allowing further transformations. However, this further transformation must occur during the fungal treatment to efficiently remove the original pollutants. Otherwise, conjugation reversion could occur.

As studied compounds present some biological activity, specific assays for monitoring endocrine disrupting activities, such as oestrogenic and dioxin-like (ER-RYA and AhR-RYA, respectively), during the degradation treatments are needed. Degradation did not result in a formation of oestrogenic metabolites at concentrations high enough to overcome the activity of the parental compounds. Same assays were performed in the treated sludge, in addition to *D. magna, Vibrio fisherii* and germination tests also reported in [80]. Those results showed a slightly increase in the dioxin-like activity of the treated sludge, highlighting the need of performing a broad spectrum of biological assays in order to assess the overall potential risk.

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Biodegradation of Technical Products of Brominated Flame Retardant by Fungi

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Abstract This chapter examines the degradation by the white-rot fungus *Trametes versicolor* of three commercial polybrominated diphenyl ether (PBDE) mixtures that have been used more in industry, and consequently, they are detected with great frequency in the environment. PBDEs are a group of compounds that are used in several products as flame retardants and they are highly recalcitrant to their degradation in the environment. The three products selected to study their degradation by the fungus in liquid media correspond to decabromodiphenyl ether (deca-BDE), octabromodiphenyl ether (octa-BDE) and pentabromodiphenyl ether (penta-BDE) mixtures. *T. versicolor* is able to degrade the three different PBDE commercial mixtures in aqueous media obtaining high final removal efficiencies. The degradation products obtained from the treatment by the fungus correspond to some hydroxylated PBDEs with different degrees of bromination, which suggests that the degradation consists of a hydroxylation enzymatic reaction. In addition, the intracellular enzyme cytochrome P450 is implicated in the first step

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of deca-BDE degradation by *T. versicolor*. However, the toxicity tests showed that toxicity in the aqueous media increases after the treatment by the fungus. On the other hand, this chapter reviews the biological treatments in aerobic and anaerobic conditions developed until present to remove PBDEs from aqueous media and soil or sewage sludge.

Keywords Biological degradation, Cytochrome P450, Hydroxylation reaction, Polybrominated diphenyl ether, *Trametes versicolor*

Contents

Abbreviations

ABT	1-Aminobenzotriazole
ABTS	2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
APCI	Atmospheric Pressure Chemical Ionization
BFRs	Brominated flame retardants
Cyp P450	Cytochrome P450
Deca-BDE	Decabromodiphenyl ether
DM	Defined medium
DMHAP	3,5-Dimethoxy-4-hydroxyacetophenol
EMRI	Mass spectrometer for isotope ratio
EPA	U.S. Environmental Protection Agency
GC–MS	Gas chromatography–mass spectrometry
HBCD	Hexabromocyclododecane
Hepta-BDEs	Heptabromodiphenyl ethers
Hexa-BDEs	Hexabromodiphenyl ethers
HOBT	1-Hydroxy-benzotriazole
hpf	Hours post-fertilization

LC	Liquid chromatography
LRAT	Long-distance atmospheric transport
MeO-PBDEs	Methoxy-polybromodiphenyl ethers
Me-PBDFs	Methoxy-polybrominated dibenzofurans
Mono-BDEs	Monobromodiphenyl ethers
Nona-BDEs	Nonabromodiphenyl ethers
Octa-BDE	Octabromodiphenyl ether
PBDEs	Polybrominated diphenyl ethers
PBDFs	Polybrominated dibenzofurans
PCBs	Polychlorinated biphenyls
PCE	Perchloroethylene
Penta-BDE	Pentabromodiphenyl ether
QqLIT-MS–MS	Quadrupole linear ion trap tandem mass spectrometer
TBBPA	Tetrabromobisphenol A
TCE	Trichloroethylene
Tetra-BDE	Tetrabromodiphenyl ether
Tri-BDEs	Tribromodiphenyl ethers
VA	Violuric acid
V-PDB	Vienna Pee Dee Belemnite

1 Introduction

1.1 Environment Pollution Due to the Presence of Polybrominated Diphenyl Ethers

The polybrominated diphenyl ethers (PBDEs) are a group of compounds included within the brominated flame retardants (BFRs), which are used in various applications such as electronic manufacturing, textiles and furniture, constituting between 5% and 30% of the product [1]. The main role of BFRs is to protect materials against ignition and prevent possible damages caused by fire, by interfering in the chemical reaction mechanism that takes place in the gas phase during combustion. Their production is estimated at 200,000 tonnes per year. Apart from the PBDEs, the most BFRs commonly used are tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD) [1].

There is a huge variety of PBDEs considering that there might be from one to ten bromine atoms bound to the diphenyl ether molecule. Among all the PBDEs, the commercial mixtures of decabromodiphenyl ether (deca-BDE), octabromodiphenyl ether (octa-BDE) and pentabromodiphenyl ether (penta-BDE) are the products that are most found in the environment. Related to octa-BDE and penta-BDE mixtures, their commercial use was banned in the European Union in 2004 and that year their leading manufacturers in North America stopped producing them on a voluntary basis [2]. Regarding the deca-BDE mixture, since July 2008, its use has been

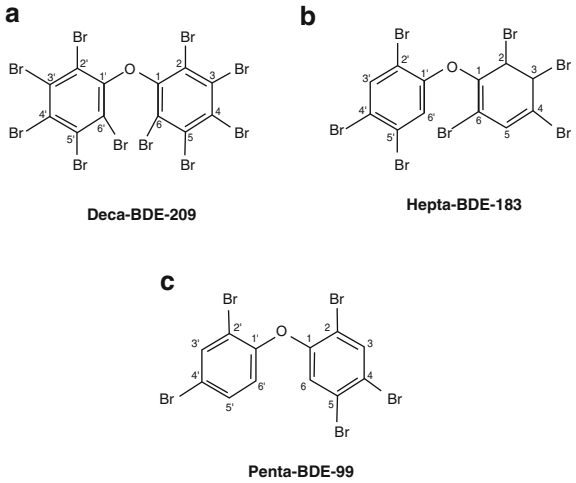

Fig. 1 PBDE structures examples: (**a**) deca-BDE-209; (**b**) hepta-BDE-183; (**c**) penta-BDE-99

banned in the EU in electrical and electronic applications. Two states of United States, Maine and Washington, banned its use in mattresses and furniture in 2008 [2]. In addition, the U.S. Environmental Protection Agency (EPA) reached an agreement with the major producers of this commercial mixture, Albermarle Corporation and Chemtura Corporation, and the leading importer, ICL Industrial Products Inc., for the final production and importation dismissal of this product for any commercial use by the end of 2013 [3].

The mixture of deca-BDE is composed almost exclusively of deca-BDE (BDE-209), with a rate of 97% or more and by 3% or less of nonabromodiphenyl ethers (nona-BDEs) and octa-BDEs. On the contrary, the commercial octa-BDE mixture usually contains between 10% and 12% hexabromodiphenyl ethers (hexa-BDEs), 44% of heptabromodiphenyl ethers (hepta-BDEs), between 31% and 35% of octa-BDEs, between 10% and 11% of nona-BDEs and $<1\%$ of deca-BDE [4]. The two compounds with a higher proportion in this mixture are, in first place, BDE-183 (2,2′,3,4,4′,5′,6-hepta-BDE) and then the BDE-153 (2,2′,4,4′,5,5′-hexa-BDE). In addition, the penta-BDE mixture, commercially known as DE-71, is primarily composed by tetrabromodiphenyl ethers (tetra-BDEs) (24–38%), penta-BDEs (50–60%) and hexa-BDEs (4–8%) being BDE-99 (2,2′,4,4′,5-penta-BDE) and BDE-47 (2,2′,4,4′-tetra-BDE) the main compounds of the mixture. Figure 1 shows different structures belonging to the three PBDE mixtures described.

PBDE compounds are highly insoluble in aqueous phase in all cases and very stable. Its insolubility increases as the higher the number of substituents in the molecule, ranging from a solubility of approximately 100 μg L^{-1} for monobromodiphenyl ethers (mono-BDEs) to a value of 25 ± 5 ng L^{-1} for deca-BDE [5].

The toxicity of these compounds is linked with its solubility and hence with its bioavailability, being mono-BDEs compounds the most toxic and deca-BDE the least. Because of its very low solubility, during long time deca-BDE was considered as a practically non-toxic compound due to its very low solubility and hence the great difficulty in moving up the food chain. But in recent years, there are several laboratory studies showing that deca-BDE is photodegradable and if exposed to ultraviolet light it can result in lower brominated PBDEs, which are more bioavailable and therefore more toxic. These studies were performed both with deca-BDE in liquid medium [6, 7] and adsorbed on solid matrices such as clay minerals or sediments [8]. These studies have shown a great variety of PBDE compounds formed from the deca-BDE photodegradation. In one case, different PBDEs from tribromodiphenyl ethers (tri-BDEs) to nona-BDEs were detected [6, 8] and in another case different species of polybrominated dibenzofurans (PBDFs) and methoxy-polybrominated dibenzofurans (Me-PBDFs), which are also regarded as toxic, were formed as well [7]. The loss of bromine atoms by the action of ultraviolet light is progressive and, therefore, deca-BDE is transformed into different nona-BDE isomers, which subsequently become different isomers of octa-BDE and so on until the formation of tri-BDE isomers, which are the lowest brominated PBDEs that have been detected in these studies. PBDEs decomposition rate by UV light decreases with the reduction of the number of bromine atoms bound to the diphenyl ether molecule, being deca-BDE the compound with the fastest rate and more susceptible to form lower brominated PBDEs [6]. On the other hand, it has been demonstrated that deca-BDE can be degraded under abiotic conditions by metal oxides present in soils and sediments [9]. In the case of compounds present in octa-BDEs and penta-BDEs mixtures, they are also susceptible to be debrominated by ultraviolet radiation, with the consequent increase in environmental toxicity [10, 11].

Regarding the toxicological properties of the three commercial mixtures studied in this chapter, it has been shown that these products have thyroid hormone disrupting properties [1, 12], which causes a competitive binding of PBDEs to thyroid hormone receptors due to the great similarity between the structures of these hormones and PBDEs. These hormones play an important role in cardiovascular, skeletal and muscular systems as well as in body responses related to heat production, oxygen consumption and regulation of other hormonal systems. In addition, PBDEs can alter liver function, causing changes in thyroid hormones and vitamin A homeostasis, resulting in over-elimination of the thyroid hormone T4 [13]. It has also been demonstrated that cell exposure to PBDE leads to cellular cytotoxic effects with a progressive decrease in their viability and an increase of apoptosis and necrosis [14], this being a last non-controlled process which leads to tissue damage. With regard to PBDEs carcinogenic effects, human exposure to these products is related to the formation of tumours and cancer. While concern over deca-BDEs carcinogenicity is low, congeners of lower bromination are expected to be relatively carcinogenic [12].

1.2 PBDEs Pollution Origin and Transport Process to Environment

PBDEs, and especially the mixtures studied in this chapter, have been detected in air, soil, sediment and aquifer samples [67]. Their concentration in environmental media depends largely on their distance from urban centres, with a progressive decrease in pollutants concentration as the distance increases, which indicates that the pollution sources are related to human activity [21, 68]. Specifically, in areas with high population density, the highest concentrations of PBDEs in air, water, soil and sediments are found in zones close to manufacturing and recycling products industries, and drop-off areas [15–17]. Moreover, the detection of PBDEs in remote areas, situated at a very large distance from pollution source, means that the long-distance atmospheric transport (LRAT) of PBDEs has a significant importance in movement and distribution of these pollutants in the environment at regional and global scales [18–20]. Initially, it was suggested that deca-BDE had a low potential in terms of being transported long distances by air, but recent studies that detected deca-BDE in remote lakes of Canada show that this compound can be transported long distances through the air [18]. Although the different studies carried out until now, further investigation is necessary with respect to PBDEs LRAT as it is demonstrated that there is a significant difference between the LRAT values calculated from different models, and also between the values predicted by models and values detected in situ [2].

Another source of environmental pollution by PBDEs corresponds to liquid effluents and sludge from urban wastewater treatment plants. There is a great variety of studies where PBDEs are detected, which indicates that both the housing and urban offices are a source of these pollutants contamination [21, 22]. In general, deca-BDE has been detected in higher concentration in sludge samples than in liquid effluents as it is the most insoluble of all PBDEs. In contrast, lower brominated diphenyl ethers are found in greater proportion in the liquid phase. However, the treatment plant effluent volumes returned to surface water are significant, and therefore it represents an important source of deca-BDE contamination, although its concentration is very low. On the other hand, brominated contaminants present in sewage sludge can accumulate in soil, once composted, due to its application in agricultural areas, this becoming the main source of PBDEs soil contamination [23]. Soil erosion also promotes the movement and distribution of these pollutants, because once they pass into surface water, they can be transported long distances and accumulate in sediments [21], marine fauna [24] and agricultural soils [23]. Finally, the fauna can also extend PBDEs distribution in the environment after these compounds will be accumulated in the successive levels of the food chains. This distribution can take place at regional or global level because of long-distance migrations, especially in case of birds and fish [25]. In both cases, the PBDEs amounts transported through animals are small compared to water and air distribution channels, but they are

very important for their potential to introduce these compounds directly into the upper levels of the food chains [25].

1.3 Biological Degradation of PBDEs

Different studies evidence the degradation of different PBDE compounds by biological processes in both aerobic and anaerobic conditions. However, it must be considered that these compounds are emerging contaminants that have been considered recently as toxic and carcinogenic agents and, as a consequence, the number of published studies is still scarce and most of them are very recent. In aerobic conditions, there are few studies of PBDEs degradation and in most cases correspond to low brominated congeners [26, 27], such as mono-BDEs or di-BDEs, which were the first one studied as they are the most bioavailable compounds among all the PBDEs. On the contrary, a more extend research has been carried out on anaerobic degradation of PBDEs mixtures, both in liquid phase and in soil or sludge.

1.3.1 Aerobic Degradation

The first study on PBDEs degradation in aerobic conditions, which was conducted by Schmidt et al. [26], showed that bacterial strain *Sphingomonas* SS3 is able to degrade diphenyl ether molecule and their halogenated derivatives (4-fluoro, 4-chloro and 4-bromodiphenyl ether) using them as the main carbon and energy source. In the case of 4-bromodiphenyl ether, this report proposes a double degradation pathway by a non-specific hydroxylation of the dioxygenase enzyme, forming phenol and 4-bromocatechol whether the enzyme attacks the halogenated ring or catechol and 4-bromophenol if the enzyme attacks the non-halogenated ring. This bacterial species has also been used in another study of low brominated PBDEs degradation, where *Sphingomonas* strain PH-07 degraded five different PBDE compounds (4-bromo-BDE, 2,4- and 4,4′-dibromo-BDE, and 2,4,6- and 2,4,4′-tribromo-BDE) [28]. In the case of 4-bromo-BDE, the degradation pathway is different from that described by Schmidt et al. [26], because hydroxylation occurs in a different phenyl ring position, forming 4-bromophenol, which in part is transformed into 4-bromocatechol and 2-hydroxymuconic acid as degradation products. For the other higher brominated PBDEs, this bacterial strain was able to degrade all of them, except for 2,4,6-tri-BDE, by means of an enzymatic hydroxylation, leading to the formation of different bromophenols and bromocatechols depending on the degraded compound.

Regarding the degradation of PBDEs by white-rot fungi, the first evidence of their ability to degrade a PBDE compound corresponds to a study published by Hundt et al. [27], which studied the degradation of 4-bromo-BDE by *Trametes versicolor*. The degradation occurs initially by hydroxylation reaction with the possible formation of three different isomers of hydroxy-diphenyl ether followed

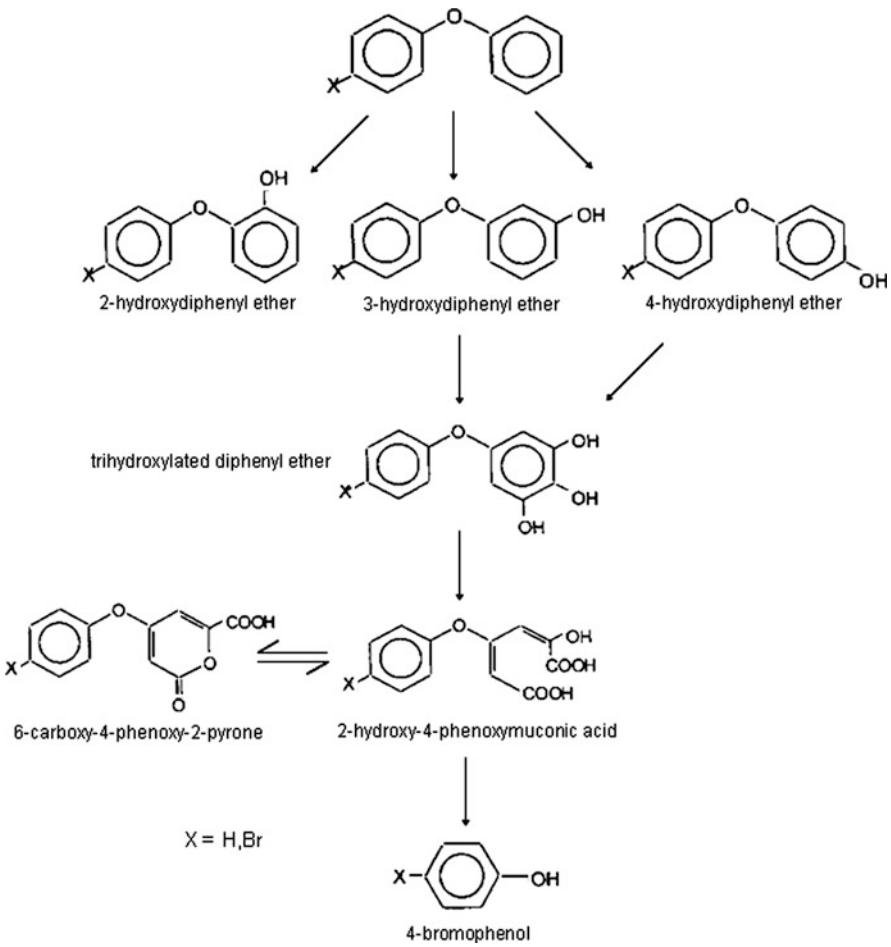

Fig. 2 Proposed pathway for 4-bromo-BDE degradation by *T. versicolor*. Adapted from Hundt et al. [27]

by a breakdown of the aromatic ring leading to the formation of 2-hydroxy-4-fenoxymuconic acid and the corresponding lactone. The hydroxylation always occurs in the non-halogenated phenyl ring, and this reinforces the idea that there is a trihydroxylated intermediate product of the non-halogenated ring, which was not detected in this study, between muconic acid and hydroxyphenyl ethers. Finally, 4-bromophenol is obtained as final degradation product, which cannot be confirmed to occur from muconic acid or from a direct enzymatic breakdown of the ether bond of the halogenated diphenyl ether. Figure 2 shows the degradation pathway proposed in this article.

Hundt et al. [27] studied the involvement of different enzymes produced by fungus in the degradation of the brominated compound. It was checked that there

was no compound degradation by the fungus in presence of 1-aminobenzotriazole (ABT), which is an inhibitor of intracellular enzyme cytochrome P450 (Cyp P450). On the other hand, there was no pollutant degradation in presence of supernatant with a high activity of laccase and manganese peroxidase. These results suggest that a non-ligninolytic intracellular enzyme system would be involved, which could be the Cyp P450, in the first step of 4-bromo-BDE degradation by *T. versicolor*, which corresponds to the hydroxylation reaction.

Apart from the work published by Hundt et al. [27], deca-BDE commercial mixture degradation by an unspecified white-rot fungus was studied by Zhou et al. [29]. This study corresponds to the first evidence of the ability of white-rot fungi to degrade deca-BDE mixture. In addition, this report examines the effect of surfactant Tween 80 and oligosaccharide β-cyclodextrin concentrations in liquid medium on deca-BDE degradation. Surfactant is used to increase pollutant solubility in aqueous phase and consequently its bioavailability to be degraded, and cyclodextrin is used in applications to promote environmental degradation in soil and in liquid medium. In all experiments, deca-BDE theoretical initial concentration was 1.6 mg L^{-1} and the fungus was able to degrade the pollutant with a maximum degradation yield of 96.5% for a period of 10 days and a Tween 80 concentration of 500 mg L^{-1}. For higher surfactant concentrations, the fungal growth was inhibited, and therefore it is important to choose the suitable surfactant initial concentration to maximize the degradation yield. Moreover, the presence of β-cyclodextrin promotes the degradation of deca-BDE in a lower range than the presence of Tween 80, obtaining a maximum degradation percentage of 78.4% after 10 days. This study shows that deca-BDE is degraded in presence of this ligninolytic fungus but no products were indentified from the degradation process neither the enzymatic system involved in this process.

Later, the studies on the degradation of 13 separate PBDEs, from mono-BDEs to hexa-BDEs, by aerobic bacteria were reported [30]. This was the first study that showed the degradation of the most relevant tetra-BDEs and penta-BDEs and one hexa-BDE. Four different bacterial strains were tested: *Rhodococcus* RR1, *Pseudonocardia dioxanivorans* CB1190, *Rhodococcus jostii* RHA1 and *Burkholderia xenovorans* LB400, considering that the last two had demonstrated previously its ability to degrade polychlorinated biphenyls (PCBs) [31, 32], contaminants with a similar structure to PBDEs. The strain RHA1 was chosen to study the degradation of penta-BDE commercial mixture with an initial concentration of 17 μg L^{-1} for each congener, which corresponds to significantly lower concentrations than deca-BDE degradation experiment by white-rot fungus [29]. The results show that the two PCBs degrader bacterial strains are more efficient both in percentage and in a variety of degraded PBDEs, being only BDE-149 (2,2′,3,4′,5′,6-hexa-BDE) and BDE-153 the compounds that RHA1 was unable to degrade and also BDE-138 (2,2′,3,4,4′,5′-hexa-BDE) in the case of LB400. The other two strains tested were only able to degrade the BDE-3 (4-mono-BDE) and one of the two di-BDEs tested, the BDE-47. Thus, they are not suitable to degrade PBDEs with a high number of bromine atoms. Moreover, this study showed that the degradation efficiency decreases as the number of bromine atoms in the molecule

increases. Experiments carried out with high concentration of BDE-3 and BDE-47 by the two more efficient degrading strains showed the formation of bromide ion in all cases but with different magnitudes. In the case of RHA1, debromination reached a very high percentage, between 80% and 100%, implying that the bacterium is able to break all the carbon–bromine bonds, and no accumulation of brominated products occurs because a practically total debromination is obtained. This is the first study that has demonstrated a complete PBDE debromination in stoichiometric conditions. However, only 10% of debromination was obtained in presence of strain B400, resulting in the accumulation of brominated products instead of pollutants debromination. In the latter case, a hydroxylated mono-BDE compound was detected from 4-mono-BDE degradation, suggesting that this strain caused the degradation through a hydroxylation reaction, as described in other cases detailed above. Subsequently, the implication of biphenyl and ethylbenzene dioxygenases of *R. jostii* RHA1 in PBDEs transformation was described and a dihydroxylated-BDE was detected as the primary metabolite of 4-bromodiphenyl ether [33].

1.3.2 Anaerobic Degradation

With respect to anaerobic degradation, there are a greater number of references related to commercial PBDEs mixtures degradation, both in liquid and in solid media.

The PBDEs degradation process by bacteria in anaerobic conditions always follows the debromination reductive pathway, which consists of the gradual replacement of bromine atoms bound to ether diphenyl rings by hydrogen atoms forming lower brominated PBDEs. This type of degradation corresponds to the same reaction that occurs in the anaerobic degradation of perchloroethylene (PCE) and trichloroethylene (TCE) by bacteria [34]. The main problem associated with this degradation route, as with the anaerobic degradation of TCE and PCE [35], is that the resulting products are more toxic than their precursors, because they have a higher solubility and bioavailability due to its lower number of bromine atoms. Therefore, a treatment based on anaerobic degradation can lead to an increased toxicity of the environment rather than its treatment.

Deca-BDE and octa-BDE mixtures degradation in anaerobic conditions in aqueous phase was first demonstrated by He et al. [36]. This study was performed with the bacterial strain *Sulfurospirillum multivorans* and different strains of *Dehalococcoides* species [*D. ethenogenes* 195 and EC195, *D.* BAV 1 and a culture with different species of *Dehalococcoides* (ANAS)]. Considering that PBDEs degradation is produced by co-metabolism in all cases, the presence of a compound that acts as an electron acceptor during the degradation process is required. In this case, TCE was used as an electron acceptor. The results showed that *S. multivorans* is able to degrade deca-BDE into different hepta-BDE and octa-BDE congeners, but it is unable to degrade the octa-BDE mixture into lower brominated congeners. These results agree with chlorinated ethylenes degradation results by this strain, as

it has been demonstrated that it can only degrade the higher chlorinated compounds (TCE and PCE), which agrees with the fact that only can debrominate deca-BDE. With regard to *Dehalococcoides* species, they were able to degrade the octa-BDE mixture, but none was able to degrade the deca-BDE mixture. The resulting products from PBDEs degradation are lower brominated congeners than those initially present in the mixture and they are different depending on the strain used, obtaining hepta-BDE to penta-BDE compounds (*D. ethenogenes* 195), and tetra-BDEs and di-BDEs congeners (mixture of *D.* BAV1 and *D. ethenogenes* EC195).

Later, Robrock et al. [37] studied the anaerobic degradation in aqueous phase of the five main components of the octa-BDE mixture. In addition, they studied the degradation of the two PBDEs with a greater presence in the environment: BDE-47 and BDE-99 [6, 38], which can be formed from higher brominated congeners degradation, and they are also included in penta-BDE commercial mixture composition. Using a mixture of *Dehalococcoides* strains (ANAS195), the PCE degrader strain *Dehalobacter restrictus* and pentachlorophenol degrader bacterium *Desulfitobacterium hafniense* PCP-1, it was found that all compounds tested were biodegraded by the three cultures, following a reductive debromination pathway very similar for all the cultures and exhibiting a preference for eliminating bromine atoms bound to phenyl ether rings in positions *meta* and *para*. In the case of *D. restrictus* and *D. hafniense*, it was suggested that degradation occurs by co-metabolism with PCE as electron acceptor or that degrading enzymes produced by bacteria are not only induced by the presence of PBDEs. However, in the case of ANAS195 culture, PBDEs were degraded without the presence of an electron acceptor.

Regarding the degradation of commercial PBDE mixtures in solid media, the fact that a relatively significant concentration has been detected in sediments [15, 39] and sewage sludge [23, 40, 41], especially in the case of deca-BDE, implies that the study of their degradation is also important. To date, deca-BDE mixture anaerobic degradation has been more studied than the degradation of the other two commercial mixtures.

The first study of deca-BDE mixture degradation in sewage sludge was published by Gerecke et al. [42]. The sludge where degradation took place and that was used as inoculum came from a mesophilic anaerobic digester of an urban wastewater treatment plant located in Dübendrof, Switzerland. The results showed that deca-BDE degradation in solid media occurs by reductive debromination, forming two different nona-BDEs and several octa-BDE compounds. This study was further complemented by a subsequently report published by Gerecke et al. [43], which compared the deca-BDE degradation in a sewage sludge from the same anaerobic digester with the degradation of other compounds that are also used as flame retardants, such as HBCD and TBBPA. The results of this study showed that the three compounds can be degraded in anaerobic conditions and that deca-BDE is the compound with the longest half-life (700 days). Other published reports also show significant deca-BDE degradation in both sludge from an urban wastewater treatment [44] and sediments [45]. In this second study, it was found that deca-BDE

is degraded via reductive debromination causing an amount increase of less brominated congeners (nona-, octa-, hepta- and hexa-BDEs).

Penta-BDE and octa-BDE mixtures degradation studies in solid media in anaerobic conditions are very scarce. In the case of penta-BDE mixture, Vonderheide et al. [2] inoculated a culture with different anaerobic microorganisms in a contaminated soil by this mixture. After a short time, the major compound in the mixture, BDE-99, was almost completely degraded, as well as other PBDEs present in lower proportion (hexa-BDEs and penta-BDEs). Moreover, for the same time, it was observed an increase of the amount of BDE-47, which corresponds to the second major compound in the mixture. The authors argue that BDE-47 amount increase could be caused by the fact that it is a degradation product of other higher brominated PBDEs such as BDE-99. In fact, it was demonstrated later that BDE-47 is formed from BDE-99 degradation in anaerobic conditions [37].

In relation to octa-BDE mixture degradation in soil and sediment, it has been recently studied by Lee and He [46]. Soils and sediments from three different locations: Guiyu Wuhan (China), Singapore and San Francisco (USA) were used to carry out a bioaugmentation process of native anaerobic microorganisms before the mixture addition. Results show that degradation occurs by reductive debromination leading to the formation of a broad range of products, from hexa- to mono-BDEs, with predominance of tetra-BDE isomers. In this work, a genetic analysis of microcosm was also carried out, and it was found that in most cultures *Dehalococcoides* species were present, which had been already proved their ability to degrade PBDEs [36, 37].

1.3.3 PBDE Degradation in Mammals

PBDEs degradation in mammals has also been studied in order to understand the behaviour of these compounds and the corresponding degradation products inside the body considering that they can be bioaccumulated in different organs [47, 48] with the consequent risk to the animals and humans health [12, 13, 49]. Among all the PBDEs, deca-BDE has been the most studied through experiments in rats. Most of these studies suggest that deca-BDE is metabolized to less brominated hydroxylated and methoxy-hydroxylated metabolites through dehalogenation oxidative reactions. Moreover, there are also degradation studies of other PBDEs in rats, such as BDE-154 (2,2′,4,4′,5,6′-hexabromodiphenyl ether) [50] and BDE-100 (2,2′,4,4′,6-pentabromodiphenyl ether) [51]. The degradation products detected for the latter were several mono- and di-OH-tetra-BDEs and a mono-OH-penta-BDE, which are presumably formed through an oxidative process. The enzymatic system involved is not reported in this paper.

In the case of BDE-154, the suggested degradation pathway results in different types of hydroxylated PBDEs: mono- and di-OH-hexa-BDEs, mono-, di- and tri-OH-penta-BDEs, and a mono-OH-tetra-BDE. It was checked that Cyp P450 is

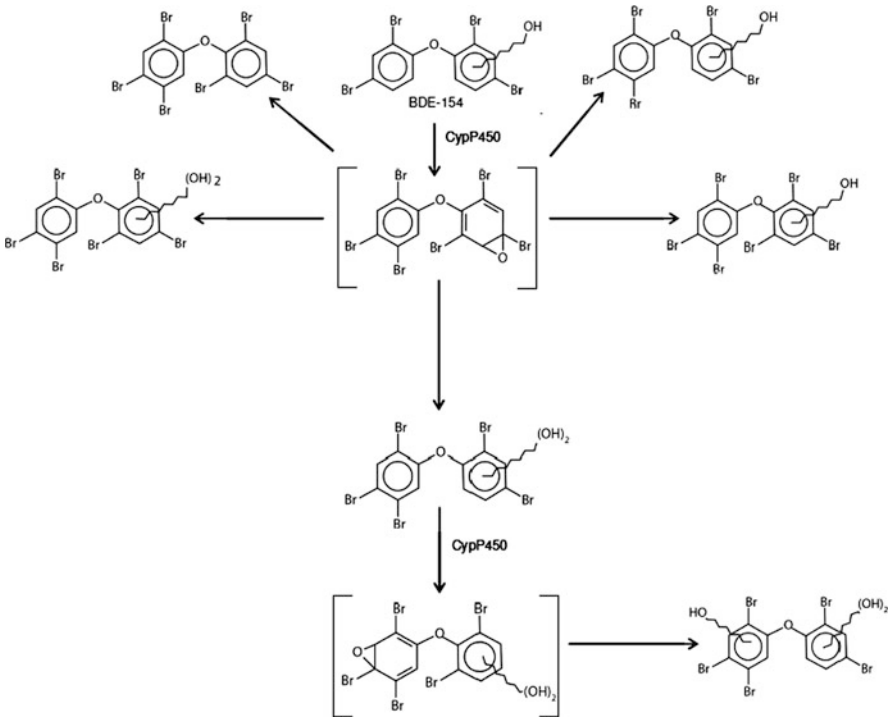

Fig. 3 Metabolic degradation pathway suggested for BDE-154 on rats. Adapted from Hakk et al. [50]

involved in the first degradation step of the compound, which corresponds to the formation of a reactive epoxy intermediate, which leads to different hydroxylated PBDEs. Figure 3 shows the suggested pathway by BDE-154 degradation in mammals.

In the case of deca-BDE, its suggested degradation process in mammals consists of a first reductive debromination where one, two or three bromine atoms can be replaced by hydrogen atoms followed by an oxidation to form hydroxylated metabolites, which are presumably formed from an intermediate epoxy [52]. This study detected traces of three nona-BDEs, which may be an indication of reductive debromination as a first step of degradation, and thirteen hydroxylated metabolites. Otherwise, the possibility of a deca-BDE oxidation as a first step to form the epoxy without an intermediate reductive debromination is also suggested [52]. The study conducted by Morck et al. [53] detected several hydroxylated products, from methoxy–hydroxy-pentabrominated to methoxy–hydroxy-heptabrominated compounds, which coincide with part of the metabolites obtained by Sandholm et al. [52]. In addition, both authors found that methoxy and hydroxy substituents are always on the same aromatic ring when both are present. Moreover,

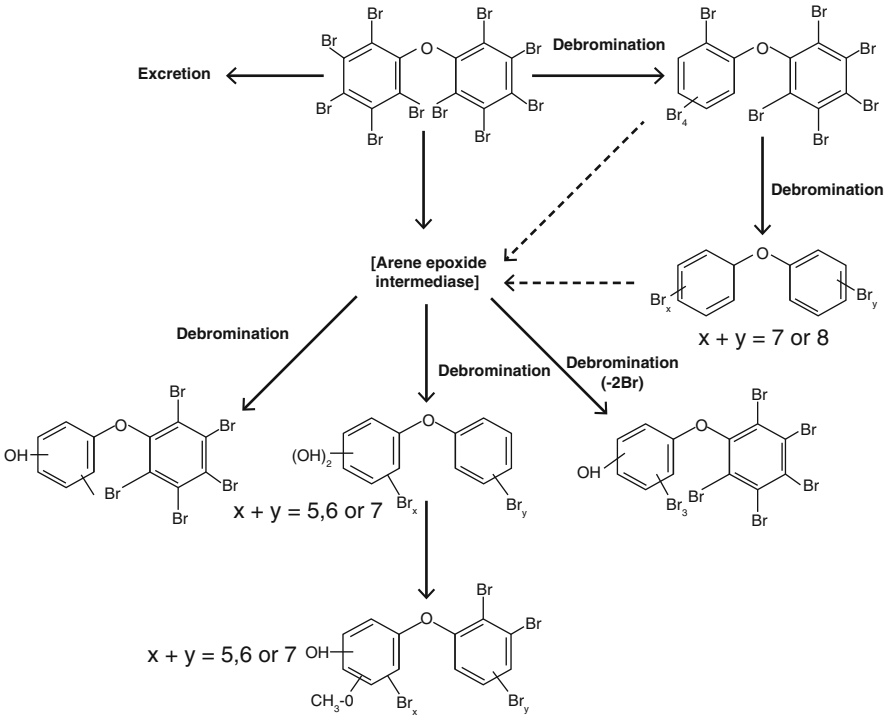

Fig. 4 Proposed deca-BDE degradation pathway on mammals. Adapted from Morck et al. [53] and Sandholm et al. [52]

Morck et al. [53] found traces of less brominated PBDEs than deca-BDE, indicating that the reductive debromination would be the first step in its degradation. In both articles, it is suggested the involvement of Cyp P450 in the oxidation reactions to form hydroxylated metabolites, but not in the reductive debromination proposed as the initial step.

Regarding the reductive debromination as the first step of deca-BDE degradation in mammals, Huwe and Smith [54] detected the formation of different PBDEs (three nona-BDEs, four octa-BDEs and one hepta-BDE) from deca-BDE degradation in rats, which also suggests the existence of a reductive debromination process as the first step in deca-BDE degradation in mammals. In this case, it was not identified whether the specific enzymatic system responsible for the reductive debromination and the corresponding analyses to detect the formation of hydroxylated metabolites were not carried out.

Figure 4 shows the proposed deca-BDE degradation pathway in mammals according to the research studies in rats.

2 Deca-BDE Degradation in Aqueous Phase by *T. versicolor*

2.1 Degradation Capacity

First, the capacity of the fungus *T. versicolor* to degrade deca-BDE was evaluated. To carry out the treatment, the fungus was used in the form of pellets; 100 mL amber serum bottles were filled with 10 mL of defined medium (DM) with Tween 80 (500 mg L^{-1}) as surfactant to increase the solubility of the contaminant and deca-BDE at a concentration of 10 mg L^{-1}. The bottles were inoculated with the necessary amount of pellets to reach an approximate biomass concentration of 3.5 g L^{-1} (dry weight).

Figure 5 shows the evolution of glucose concentration and deca-BDE degradation yield. The degradation yield is calculated by comparing the pollutant concentration value obtained in experimental bottles with fungus with that obtained in heat-killed controls, which consisted on autoclaved pellets. Moreover, abiotic controls were used to verify that there was no abiotic degradation or pollutant losses non-associated to fungal degradation. Deca-BDE degradation percentage increased along the time until it reached a value of 73 ± 10% at a time of 88 h. Subsequently, degradation yield did not improve although a glucose pulse was added. In this case, there is no clear reason to justify the degradation stop, because there were no significant variations in glucose consumption at any time of the experiment, which means that the metabolic system of the fungus was active and the fungus was presumably not inhibited by the presence of the pollutant in the broth or any of the degradation products.

Deca-BDE degradation at lower concentration (2 mg L^{-1}) was also assayed and Fig. 6 shows the results obtained. The degradation yield obtained after 182 h was 68 ± 5%, which is slightly lower than the degradation obtained in the previous experiment with a higher deca-BDE initial concentration. Results confirm that the fungus is also able to degrade deca-BDE at a lower concentration in a significant way. Regarding glucose consumption, at a time of 90 h, the initial amount added, corresponding to a concentration of 15 g L^{-1}, was nearly exhausted and a glucose pulse was added to avoid a possible limiting effect on pollutant degradation due to lack of primary substrate.

For both initial concentrations tested, pollutant mass adsorbed on the fungus was high, which means that it is bioavailable and can enter into fungal internal structure. This fact must be taken into account to quantify deca-BDE mass degraded. Figure 7 shows the deca-BDE amount decrease due to the adsorption on the fungus for both experiments, which was quantified from the bottles with heat-killed fungus. For an initial deca-BDE concentration of 2 mg L^{-1}, the adsorption percentages were 11 ± 6% and 24 ± 6% at 13 and 91 h, respectively, while the adsorption percentage was 13.5 ± 4.8% for a period of 161 h when the initial concentration was 10 mg L^{-1}. The high deca-BDE adsorption on the fungus implies that only a very low percentage of residual pollutant is detected in the aqueous phase at the end of the degradation process, because removal percentages are higher than 85% for both initial concentrations at the end of the experiment.

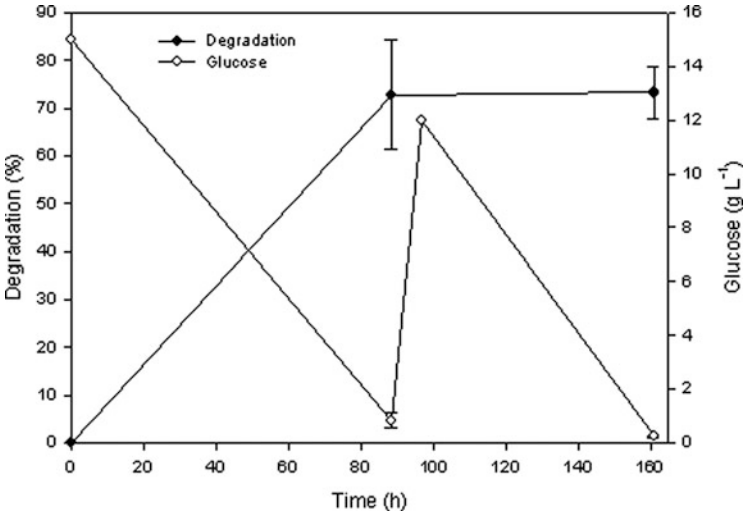

Fig. 5 Evolution of glucose concentration and deca-BDE degradation yield in aqueous phase by *T. versicolor* pellets. Deca-BDE initial concentration = 10 mg L^{-1}

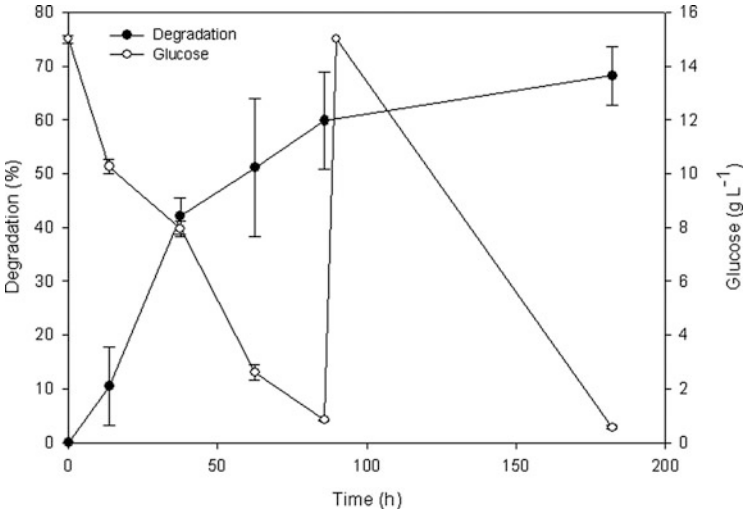

Fig. 6 Glucose concentration and deca-BDE degradation evolution in aqueous phase by *T. versicolor* in the form of pellets at an initial concentration of 2 mg L^{-1}

As mentioned previously, deca-BDE was considered for a long time as a practically non-toxic compound, due to its low availability. However, the results of both experiments have clearly demonstrated that this pollutant is bioavailable in aqueous phase in the presence of Tween 80. Considering that wastewater discharged into the environment often contains different types of surfactants,

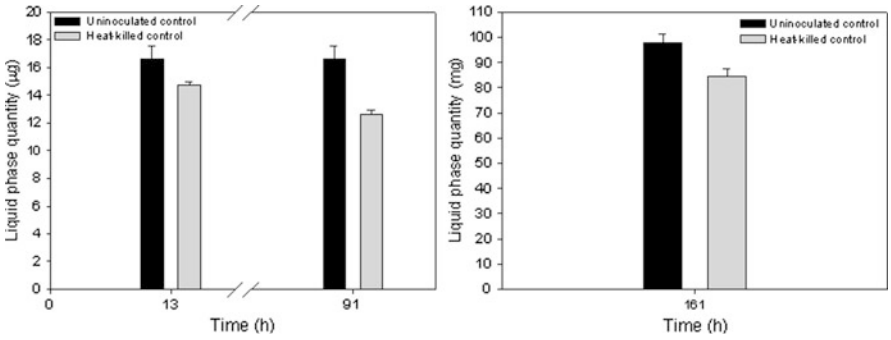

Fig. 7 Deca-BDE amount in the abiotic and adsorption control for an initial concentration of 2 mg L^{-1} (*left*) and 10 mg L^{-1} (*right*)

such as linear or composite polyethoxylated alkylbenzenes sulfonates [55, 56], deca-BDE present in these conditions may also be bioavailable and toxic for living organisms.

2.2 *Surfactant Effect on Deca-BDE Degradation by the Fungus*

Deca-BDE degradation studies by white-rot fungi have shown that surfactant Tween 80 is very effective to enhance pollutant degradation [29]. These authors show that an increase of 54.3% on deca-BDE degradation is obtained if Tween 80 is added to the medium, in a concentration of 500 mg L^{-1}, in relation to not adding surfactant. The main drawback of this surfactant, with a view to a possible application in situ bioremediation, is its high cost. Therefore, the effect of other surfactants on deca-BDE degradation was assayed but using more economical surfactants. The surfactants selected were two commercial surfactants that are used for in situ bioremediation processes, BS-400 (IEPSorbents) and Goldcrew (GoldCrew_Company), and Tween 20 (Sigma-Aldrich), which was used to evaluate the effect on deca-BDE degradation of peroxyl radicals produced by laccase in the presence of Tween 80 [57], because these radicals are not produced in the presence of Tween 20.

The experiment with different surfactants was carried out with a deca-BDE initial concentration of 10 mg L^{-1}, 10 mL of defined medium and a pellets concentration of approximately 3.5 g L^{-1} (dry weight). The surfactant's concentrations were 500 mg L^{-1} for Tween 20 and 5% and 14% (v/v) for BS-400 and Goldcrew, respectively. Figure 8 shows deca-BDE degradation using the different surfactants compared to pollutant degradation with Tween 80 from a previous experiment (Fig. 5). The maximum degradation rate was obtained with Tween 80, but the degradation obtained with Tween 20 was very similar. Therefore, in the case of deca-BDE degradation, although Tween 80 may become a substrate

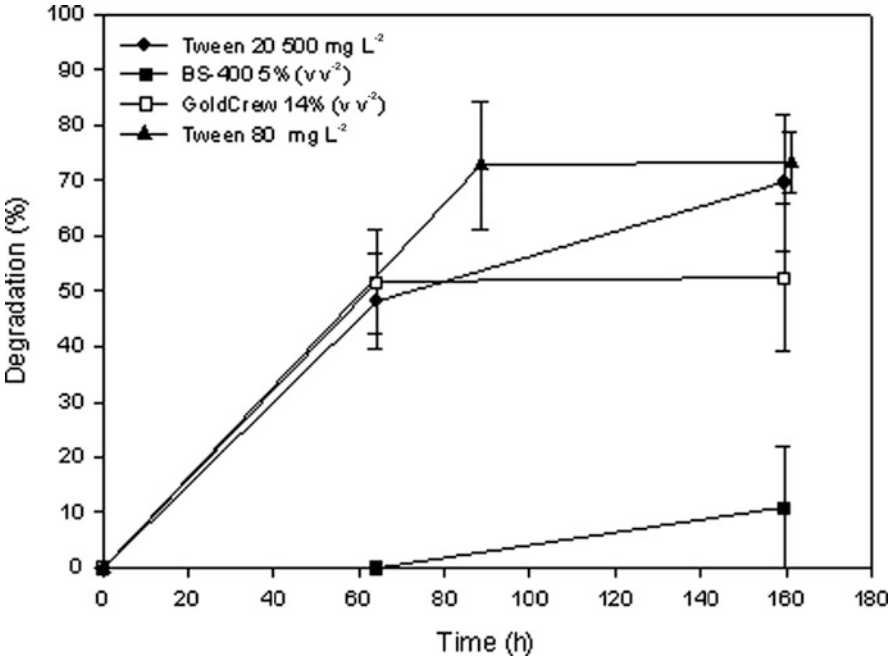

Fig. 8 Evolution of deca-BDE degradation by *T. versicolor* using different surfactants. Initial pollutant concentration of 10 mg L^{-1}

for the oxidizing species formation, it does not mean an increase in terms of degradation in relation to the use of Tween 20.

Regarding the commercial surfactants, on the one hand, a final pollutant degradation of 11 ± 11% was obtained if BS-400 is used. As a consequence, this compound is not suitable to promote deca-BDE degradation under the experimental conditions tested, probably because pollutant concentration does not increase in the presence of this surfactant. On the other hand, a degradation percentage of 52 ± 13% was obtained in the presence of Goldcrew, which was not significantly lower than degradation obtained in the presence of Tween 80 or Tween 20. For this reason, this product is an interesting candidate to study its effect on deca-BDE degradation by the fungus in a more extensive way with a view to on in situ bioremediation processes.

2.3 Deca-BDE Degradation Pathway

Biological degradation of different PBDE compounds in aerobic conditions reported the formation of hydroxylated PBDEs of lower bromination than the original pollutant [26–28, 30]. There is only one report about PBDEs degradation

by *T. versicolor* [27], where the involvement of the intracellular enzyme Cyp P450 and extracellular laccase and manganese peroxidase in the degradation process is studied. Authors suggest that Cyp P450 is involved in the first step of 4-bromo-BDE degradation and not the extracellular enzymes. Considering this reported study and the involvement of Cyp P450 in deca-BDE degradation in mammals [52, 53], the involvement of this enzymatic system in deca-BDE degradation by *T. versicolor* was studied. Moreover, the involvement of laccase in deca-BDE degradation was studied by an experiment using the laccase-mediator system.

The oxidation of pollutants by laccase is normally limited to aromatic compounds with one phenolic group, but in the presence of low molecular weight compounds, known as mediators, it can also oxidize other compounds, such as dyes [58] or alkenes and polycyclic aromatic hydrocarbons [59].

Although the use of mediator compounds is scarcely applicable in deca-BDE in situ bioremediation processes, it is important to consider whether their presence enhances the degradation of the pollutant by the enzyme laccase because it is known that the fungus is able to produce naturally certain compounds that can act as mediators in pollutant degradation by laccase and this study would show the extent of their effect on deca-BDE degradation in case that laccase was involved.

Although white-rot fungi degrading ability has been associated with peroxidases and laccase, it has been demonstrated that they are not involved in several degradation processes, such as TCE and PCE degradation by *T. versicolor* [60, 61] or TCE degradation by *Phanerochaete chrysosporium* [62]. Therefore, white-rot fungi use alternative enzymatic mechanisms to degrade pollutants, such as monooxygenases belonging to the system Cyp P450, whose genes for *T. versicolor* were identified and characterized recently [63].

2.4 In Vitro Study with Laccase-Mediator System

The study of deca-BDE in vitro degradation by *T. versicolor* commercial laccase was performed in the absence and presence of mediators, which correspond to 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1-hydroxy-benzotriazole (HOBT), violuric acid (VA) and 3,5-dimethoxy-4-hydroxyacetophenol (DMHAP), with a concentration of 1 mM in the case of HOBT and 0.8 mM for the other mediators. In all cases, the enzymatic activity in aqueous phase was about 180 U L^{-1}, corresponding to similar values measured in deca-BDE fungal degradation experiments. The results shown in Table 1 evidenced no significant pollutant degradation after 38 h of reaction because deca-BDE mass measured in degradation bottles with laccase and laccase-mediator was similar to the control. Only in the case of laccase-DMHAP system, a slight reduction in contaminant mass, equivalent to a rate of 14%, was obtained. This pollutant decrease could suggest that some degradation has occurred through the action of laccase-mediator system, but it was not comparable with that obtained by fungal degradation, corresponding to a value of

Table 1 Deca-BDE degradation obtained by laccase and laccase-mediator system with an initial pollutant concentration of 2 mg L^{-1} at 38 h

	Deca-BDE mass in serum bottles (μg)
Control	17 ± 2
Laccase without mediator	17.0 ± 0.7
Laccase + ABTS	16.3 ± 0.3
Laccase + HOBT	17.5 ± 0.9
Laccase + VA	18.6 ± 0.8
Laccase + DMHAP	15 ± 1

Table 2 Degradation of deca-BDE in presence and absence of ABT at a time of 38 h

	Deca-BDE mass in experimental bottles (μg)
Heat-killed controls	12.6 ± 0.3
Cultures without ABT	6.7 ± 0.3
Cultures with 5 mM ABT	14.4 ± 0.1

42 ± 3%. Therefore, the results obtained do not evidence that laccase is involved in the first step of deca-BDE degradation.

2.5 In Vivo Study with Cyp P450 Inhibitor

The experiment was conducted in the presence of the fungus and the compound ABT at sufficiently high concentration, 5 mM, to cause the inhibition of Cyp P450 enzyme system expression by the fungus.

Table 2 shows important differences between the final deca-BDE mass measured in culture without inhibitor with respect to inhibited culture. Moreover, the final pollutant mass detected in cultures with ABT was higher than the mass detected in heat-killed control. Consequently, the results suggest that Cyp P450 is involved in the first step of deca-BDE degradation by *T. versicolor*. The fact that pollutant degradation occurs by intracellular enzyme means that the compound must be bioavailable, because it needs to enter into the intracellular structure to be subsequently degraded by Cyp P450. Therefore, the results of deca-BDE fungal degradation and the implication of this intracellular enzyme on deca-BDE degradation confirm that this pollutant is clearly bioavailable by the fungus in presence of surfactant.

As it has been shown previously, the amount of deca-BDE adsorbed on the fungus is high and it is possible that part of it would access to fungal internal structure and it could be degraded by intracellular enzymatic system. Thus, the final degradation percentage could be higher than the values obtained, but it is not possible to quantify which proportion of pollutant adsorbed on the fungus is subsequently degraded.

Table 3 Isotopic ratio obtained from marked deca-BDE degradation by *T. versicolor* after 7 days

	$\delta^{13}C$ (‰VPDB)
Culture with [^{13}C]-deca-BDE	−26.3
Control with [^{12}C]-deca-BDE	−26.6 ± 0.1

The results obtained in this study are consistent with deca-BDE degradation pathway described for mammals [52, 53] and degradation pathway of 4-bromo-BDE by *T. versicolor* [27]. The fact that the same fungus enzymatic system is involved in deca-BDE and 4-bromo-BDE degradation could indicate that Cyp P450 enzyme is responsible for the degradation of any PBDE compound.

2.6 Deca-BDE Mineralization

Degradation experiments were performed with [^{13}C]-deca-BDE to measure the pollutant mineralization degree by the fungus.

The production of $^{13}CO_2$ from [^{13}C]-deca-BDE degradation was analyzed using the technique of gas chromatography coupled to mass spectrometer for isotope ratio (GC–EMRI). This technique measures the isotopic ratio $^{13}C/^{12}C$ of CO_2 in the gas phase with respect to an international standard V-PDB (Vienna Pee Dee Belemnite). This isotopic ratio is reported in δ notation relative to an international standard per mil (‰):

$$\delta^{13}C = \left(\frac{R_S}{R_r} - 1\right) \times 1{,}000,$$

where R_s and R_r are the sample $^{13}C/^{12}C$ ratio and the V-PDB standard ratio, respectively. The analysis error associated with the technique is ±0.5‰ V-PDB.

Table 3 shows the isotopic ratio values obtained at the end of [^{13}C]-deca-BDE degradation experiment where a degradation percentage of 70% was obtained after 7 days. Although the high degradation level reached, the difference in isotopic ratio between the culture with marked isotopic deca-BDE and the control was lower than the associated error. Consequently, no significant formation of $^{13}CO_2$ from pollutant degradation was detected, and therefore it is not possible to state that deca-BDE is mineralized by the fungus. Nevertheless, [^{13}C]-deca-BDE concentration in liquid phase was low (1.6 mg L^{-1}) and it is possible that the $^{13}CO_2$ moles produced from pollutant degradation were below the analysis detection limit.

3 Degradation of Penta-BDE and Octa-BDE Commercial Mixtures

Apart from deca-BDE mixture, the two PBDE mixtures most frequently used in the past were the penta-BDE and octa-BDE mixtures. Although its use was banned in the European Union from 2004 and their production was ceased in the United States

Table 4 Characterization of octa-BDE and penta-BDE mixtures compounds

Octa-BDE mixture components				BDE-153	BDE-183	
Content (%)				14 ± 2	86 ± 3	
Penta-BDE mixture components	BDE-47	BDE-100	BDE-99	BDE-153	BDE-183	BDE-154
Content (%)	34.2 ± 0.9	11.4 ± 0.4	42.7 ± 0.6	6.0 ± 0.6	1.9 ± 0.4	3.8 ± 0.2

in the same year, they are currently detected in significant concentrations in the environment due to their high recalcitrant nature. Moreover, it is important to study the degradation of the compounds present in these mixtures because they can also accumulate in the environment from deca-BDE photodegradation [6–8].

Before degradation experiments were performed, a characterization of the composition of both mixtures, which is summarized in Table 4, was carried out. Note that in the case of penta-BDE mixture, due to the lack of analytical standards, it was only possible to quantify 6 of the 27 compounds present in the mixture. These compounds correspond to BDE-47, BDE-100, BDE-99, BDE-154, BDE-153 and BDE-183. These compounds are the major components of penta-BDE mixture, representing the 92.5% of the total composition, and they are usually detected in the environment. Respect octa-BDE mixture, two compounds were detected, BDE-153 and BDE-183, which correspond to the major components of the mixture according to the reviewed literature [4], and probably the other compounds present in the mixture were not detected due to the lack of the corresponding analytical standards.

Fungal degradation of these mixtures in aqueous phase was carried out under the same conditions as deca-BDE degradation experiments performed previously, except for the initial mixture concentration, which was 5 mg L^{-1} for both mixtures, and the extension of the degradation period, which corresponded to 14.5 days in both cases.

Table 5 shows the degradation percentage obtained for the components detected in both mixtures. In the case of octa-BDE mixture, the concentration of both compounds decreased significantly. Furthermore, the adsorption of the two compounds on the fungus was very high, which caused an important difference between the degradation percentage, calculated from the difference between the mass of contaminant in the heat-killed fungal control and the active fungal culture, and the removal percentage, which was measured by the difference between the abiotic control and the active fungal culture. For the total sum of PBDEs included in octa-BDE mixture, the minimum degradation reached was 27 ± 7% and the removal was 67 ± 7%.

Comparing the adsorption results obtained from octa-BDE mixture components with deca-BDE results (Fig. 8), it is obtained a higher adsorption for the less brominated compounds. This is due to its greater solubility in aqueous phase, and hence it has higher bioavailability to interact with biomass.

Regarding the degradation of penta-BDE mixture components, Table 5 shows that there was a significant removal percentage for all components detected. BDE-183, which corresponds to the highest brominated component and the less abundant

Table 5 Degradation and removal percentages by *T. versicolor* for the detected components of octa-BDE and penta-BDE commercial mixtures

Octa-BDE mixture components				BDE-153	BDE-183		**Total PBDEs**
Degradation (%)				5 ± 12	31 ± 7		**28 ± 7**
Removal (%)				98 ± 22	62 ± 8		**67 ± 7**
Penta-BDE mixture components	BDE-47	BDE-100	BDE-99	BDE-153	BDE-183	BDE-154	**Total PBDEs**
Degradation (%)	33 ± 10	33 ± 11	38 ± 17	62 ± 18	46 ± 35	49 ± 9	**38 ± 13**
Removal (%)	90 ± 10	90 ± 11	79 ± 17	93 ± 18	46 ± 35	99 ± 9	**85 ± 13**

in the mixture, was the component removed in a lower percentage. The minimum degradation of the sum of all components was 38 ± 13% and the removal was 85 ± 13%. The high difference between removal and degradation percentage shows that adsorption should not be underestimated. In summary, it is important to mention that the results presented in Table 5 evidenced that the fungus *T. versicolor* is able to remove a high percentage of all the components present in penta-BDE and octa-BDE mixtures.

4 Identification of PBDE Degradation Products

In order to detect possible degradation products of deca-BDE as well as of penta-BDE and octa-BDE mixtures, the formation of less brominated compounds was studied through the analysis by gas chromatography–mass spectrometry (GC–MS) of samples at different times of degradation. The GC–MS methodology is optimized for the detection and quantification of different PBDE congeners with different degrees of bromination, including three mono-BDEs (BDE-1, -2 and -3), seven di-BDEs (BDE-7, -8, -10, -11, -12, -13 and -15), eight tri-BDEs (BDE-17, -25, -28, -30, -32, -33, -35 and -37), six tetra-BDEs (BDE-47, -49, -66, -71, -75 and -77), seven penta-BDEs (BDE-85, -99, -100, -105, -116, -119 and -126), six hexa-BDEs (BDE-138, -140, -153, -154, -155 and -166), three hepta-BDEs (BDE-181, -183 and -190), four octa-BDEs (BDE-194, -196, -197 and -203), three nona-BDEs (BDE-206, -207 and -208) and deca-BDE (BDE-209) [15].

In the case of deca-BDE mixture, the formation of less brominated compounds was not detected for any of the samples (time of degradation corresponding to 24, 36, 60, 84 and 182 h). Regarding octa-BDE and penta-BDE mixtures, the analysis of samples at time 168 h did not detect an increase in concentration for any of the components present in each of the mixtures or the formation of any PBDE not present in the original composition of both mixtures. Therefore, the results obtained for the three mixtures demonstrated that the PBDEs degradation by the fungus does not follow the reductive debromination pathway, which is logical because this

degradation route is characteristic of bacterial degradation processes in anaerobic conditions and in this case, degradation by the fungus occurs in aerobic conditions.

Moreover, considering the involvement of Cyp P450 in the first step of deca-BDE degradation by the fungus and previously reported for deca-BDE, BDE-154 and BDE-100 in mammals [50–53], it was expected that PBDE mixtures degradation products by the fungus would be very similar to those detected in degradation processes in mammals. These products correspond to hydroxylated and methoxy-hydroxylated metabolites.

The methoxy-polybromodiphenyl ethers (MeO-PBDEs) were analyzed together with PBDEs by GC–MS. The GC–MS methodology is optimized for the detection and quantification of eight different congeners including the 5-MeO-tetra-BDE-47, 6-MeO-tetra-BDE-47, 4-MeO-tetra-BDE-49, 2-MeO-tetra-BDE-68, 5-MeO-penta-BDE-99, 5′-MeO-penta-BDE-100, 4′-MeO-penta-BDE-101 and 4′-MeO-penta-BDE-103.

For the analysis of hydroxylated metabolites, a method by liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometer (LC–QqLIT-MS–MS) was developed. LC system Symbiosis Pico was used for chromatographic separation, using a C18 Betabasic column (100 mm × 2.1 mm, 3Î¼ m particle size). Water and acetonitrile were used as solvents A and B, respectively, with a 200 μL min^{-1} flow rate. Elution gradient started with an initial composition of 75% of solvent A and 25% of solvent B and was increased over the first 10 min to 40% A and 60% B, was hold for 15 min and then reached 100% of B in 10 min. Finally, system was returned to initial conditions in the final 5-min step. MS–MS conditions, using a hybrid triple quadrupole/linear ion trap Applied Biosystem MSD Sciex 4000QTRAP, and working in Atmospheric Pressure Chemical Ionization (APCI) mode, were optimized to reach the highest sensibility for each monitored transition. Optimized parameters were declustering potential (from −55 to −70 eV), collision energy (from −60 to −120 eV), and collision cell exit potential (from −1 to −13 eV). The LC–MS–MS methodology is optimized for the detection of the following different congeners: 4-OH-tri-BDE-17, 3-OH-tri-BDE28, 3-OH-tetra-BDE-47, 5-OH-tetra-BDE-47, 6-OH-tetra-BDE-47, 6-OH-penta-BDE-85, 4-OH-penta-BDE-90, 5-OH-penta-BDE-99, 6-OH-penta-BDE-100, 3-OH-hexa-BDE-154 and 6-OH-hexa-BDE-157.

Regarding penta-BDE and octa-BDE mixtures, samples corresponding to a degradation time of 12, 24, 72 and 168 h were analyzed to detect the formation of OH-PBDEs and MeO-PBDEs. In the samples corresponding at time 12 h, two different degradation metabolites were identified for each mixture. A peak corresponding to the transition of an OH-tetra-BDE ($501 > 79$, $501 > 81$) was detected in the penta-BDE mixture with a retention time of 38.44 min, while a peak corresponding to an OH-hexa-BDE ($654 > 79, 654 > 81$) was detected in the octa-BDE mixture with a retention time of 40.60 min (Fig. 9). The identification of OH-PBDEs was based on the following criteria: (1) simultaneous responses to the two selected transitions (SRM1 and SRM2) are needed, (2) the area of signal must be at least three times higher than the signal noise, and (3) the difference in the relative intensity of a peak with respect to theoretical values obtained with standard

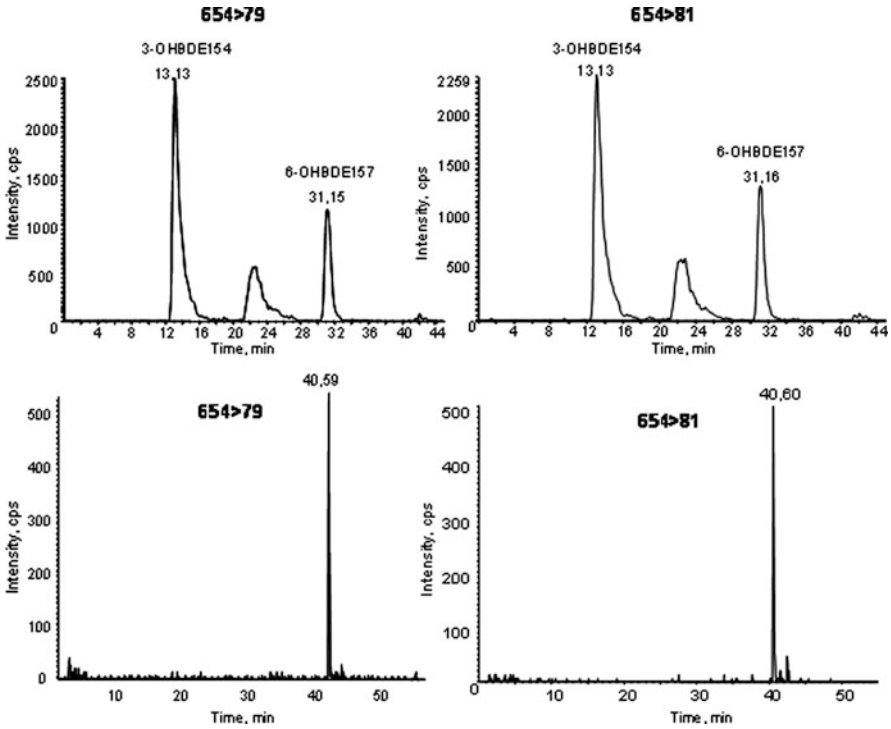

Fig. 9 Chromatograms obtained for the two monitored transitions for available OH-hexa-BDE standards are showed on the *top*. *Below* is showed the peak found for a degradation time of 12 h

solutions cannot exceed ± 15%. All these criteria are met for the two peaks detected with an SRM1/SRM2 ratio of 0.98 (1.09 for analytical standard) and 1.1 (1.17 for analytical standard) for OH-tetra-BDEs and OH-hexa-BDEs, respectively. These results confirm its identification as OH-PBDE congeners. However, the retention time for these two peaks detected did not match any of the available OH-PBDE standards. Therefore, we conclude that they correspond to a different OH-tetra-BDE and OH-hexa-BDE congeners than the available analytical standards.

For samples corresponding to higher degradation times (24, 72 and 169 h), any peak corresponding to an OH-BDE was detected. It is assumed that the OH-PBDE generated for a period of 12 h was degraded by the fungus during the following 12 h of experiment. This assumption could also explain the results obtained in deca-BDE degradation experiment by the fungus, where it was not possible to detect any hydroxyl product, but the first sample was taken at a time of 24 h. The possibility that methoxylated PBDEs were also generated by the degradation process was dismissed because they were not detected in any of the samples analyzed.

The two OH-PBDEs detected indicate that the degradation pathway of octa-BDE and penta-BDE mixtures by the fungus corresponds, in both cases, to a hydroxylation of the PBDE compounds. In the case of deca-BDE mixture, it was not possible to detect any hydroxylated product, but it cannot be excluded the formation of OH-PBDEs and their subsequent degradation for a time lower than 24 h. Therefore, it is possible that deca-BDE degradation by the fungus also occurs through the progressive hydroxylation of the compound, but it would be necessary to have results from treatment times of 12 h to confirm this possibility.

The degradation pathway of the different mixtures with *T. versicolor* proposed in this section agrees in part with PBDEs degradation pathways in mammals published before [50–53]. Regarding BDE-154 and BDE-100, which are components of penta-BDE mixture, it was not possible to identify the OH-tetra-BDE formed in the degradation process by the fungus, so it was not possible to check whether this product matches the compound formed in BDE-154 and BDE-100 degradation in mammals. However, the similarity of the products formed during the degradation process by the fungus with respect to degradation products in mammals leads to suppose that, in the case of penta-BDE and octa-BDE mixtures, Cyp P450 is also responsible for their degradation.

5 Toxicity Results in Aqueous Samples of PBDE Degradation

Toxicity analyses were performed by the individual exposure of zebrafish embryos to the different PBDEs commercial mixtures degradation samples from 4 to 48 hours post-fertilization (hpf), including negative controls and controls of solvent in the test. Additionally, two independent experiments were performed on undiluted samples and a last experiment was performed on a dilution series of 50%, 5% and 0.5% of the original samples. The embryos were observed after 24 and 48 hpf, and categorized as dead or alive.

No mortality was found in any embryo exposed to the controls. On the contrary, all the embryos exposed to the non-diluted samples of penta-, octa-, and deca-BDE commercial mixtures were dead after 24 h (Fig. 10). When the untreated PBDEs samples were diluted at 50%, a gradient of toxicity was observed: penta $>$ octa $>$ deca. After dilution at 5%, no embryos exposed to untreated samples were dead. In agreement with our results, it has been demonstrated that the toxicity of deca-BDE is commonly lower than for octa- and penta-BDE commercial products exposures with mammalian models [64]. The different toxicity found in mammalian models and also in zebrafish should be related to the higher accumulation of lower brominated congeners in the body, because of their greater partitioning and retention in lipid-rich tissues and lower rates of metabolism and elimination in relation to deca-BDE.

Treatment with the fungus strongly increased the toxicity of the samples. First, embryos exposed to the non-diluted treated samples, at a degradation time of 12 h and 7 days, exhibited almost a 100% of mortality and only 2 of the 24 embryos

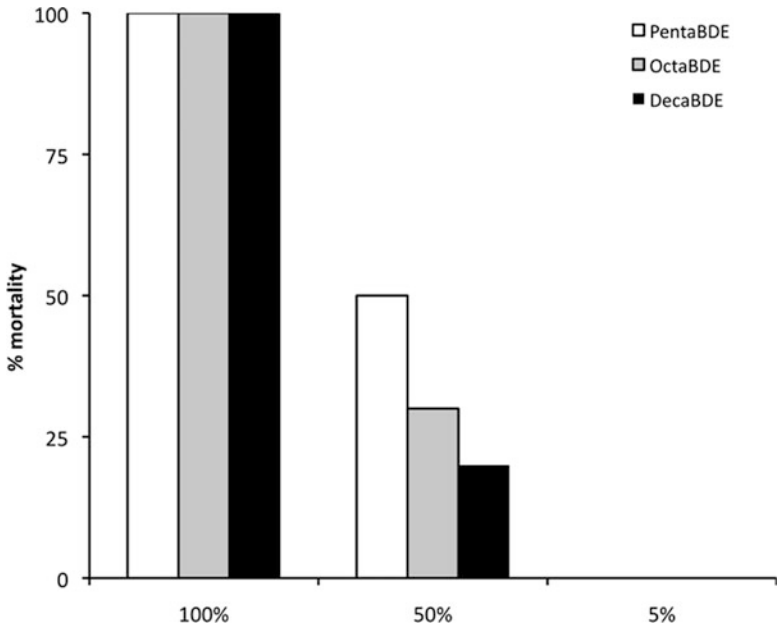

Fig. 10 Percentage of mortality of zebrafish embryos after 48 h of exposure to PBDE commercial mixtures samples at different dilutions (100%, 50%, and 5%)

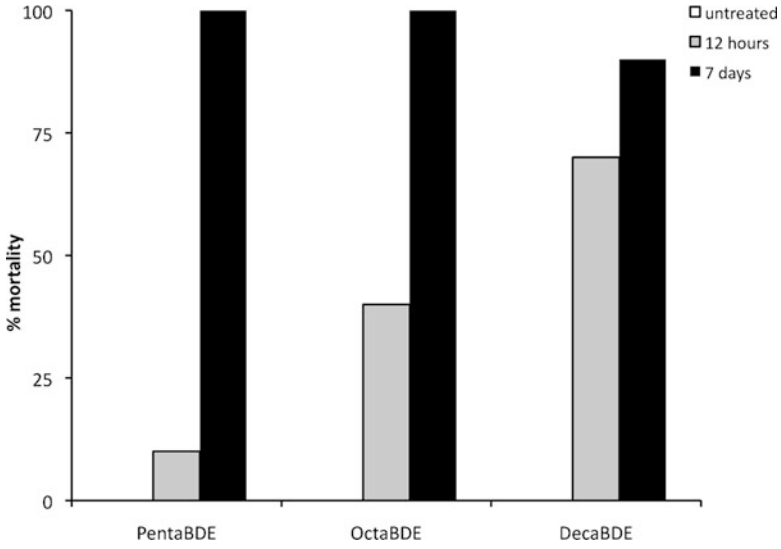

Fig. 11 Percentage of mortality of zebrafish embryos after 48 hpf to PBDE samples (before and after 12 h and 7 days of treatment with fungus). All the samples were diluted at 5%

survived to exposition to the sample of deca-BDE treated for 12 h. Even when samples were diluted to 50%, the 100% embryos exposed to the PBDEs treated were dead after 48 h. It has been previously reported that the presence of an OH group increases in a significant way the toxic potential of PBDEs [65, 66].

Embryos exposed to the 5% of the treated samples exhibited a slight mortality, although those exposed to a 5% of the three untreated PBDEs showed no mortality. The toxicity gradient after 12 h of treatment was the contrary of the observed for the untreated PBDEs, deca > octa > penta (Fig. 11). Furthermore, more than 90% of the embryos were dead after exposure to the samples treated with the fungus for 7 days.

Finally, when embryos were exposed to 0.5% of the untreated of treated samples, the survival was not different than for the controls (90–100%).

6 Conclusions and Perspectives

The degradation of higher brominated PBDEs by the white-rot fungus *T. versicolor* in aqueous phase has been shown to be high efficient in terms of pollutant removal. The products detected from penta-BDE and octa-BDE commercial mixtures degradation correspond to hydroxylated PBDEs and the deca-BDE degradation pathway studies performed reveal the involvement of the intracellular enzyme Cyp P450. These results agree with the ones obtained in previous PBDEs biological degradation studies in aerobic conditions. In relation to liquid media toxicity, it increases after the treatment process with the fungus, which should be related to the presence of an OH group in the degradation products obtained. The toxicity results obtained state the necessity to identify the products obtained after a degradation treatment, which can help to predict the final toxicity of the matrix.

The high potential of *T. versicolor* to degrade higher brominated PBDEs in liquid media suggests that the treatment with fungus would be a viable option for other PBDEs, such as lower brominated PBDEs, which are also present in the environment and they are more bioavailable than higher brominated congeners.

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Conclusions and Future Trends

D. Barceló, E. Eljarrat, G. Caminal, and T. Vicent

Abstract This chapter summarizes the main conclusions drawn from the eight different chapters of this book, as well as the future trends in the field of research on emerging organic pollutants in sludge. The chapter is divided into four sections. First of all, we discuss the wide range of emerging contaminants that continuously appear in the literature related to sludge. After that, we present the new developments in analytical methodologies and the future trends for their determination. Then, a review and evaluation of various sludge treatments was presented. And finally, the different results obtained in studies of biodegradation by fungi were summarized.

Keywords Analytical approaches, Biodegradation by fungi, Emerging contaminants, Sludge treatments

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Contents

Abbreviations and Symbols

4DHB	4,4′-Dihydroxybenzophenone
AD	Anaerobic digestion
ADBI	Celestolide
AHDI	Phantolide
AHTN	Tonalide
APE	Alkylphenolic polyetoxylated
ATII	Traseolide
BFR	Brominated flame retardant
BP3	Benzophenone-3
BTH	Benzothiazole
BTR	Benzotriazole
cVMS	Cyclic siloxanes
D3	Hexamethylcyclotrisiloxane
D4	Octamethylcyclotetrasiloxane
D5	Decamethylcyclopentasiloxane
D6	Dodecamethylcyclohexasiloxane
DP	Dechlorane Plus
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EDC	Endocrine disrupting compound
EHMC	Ethylhexyl-methoxycinnamate
GC	Gas chromatography
HHCB	Galaxolide
ICR	Ion cyclotron resonance
L2	Hexamethyldisiloxane
L3	Octamethyltrisiloxane
L4	Decamethyltetrasiloxane
L5	Dodecamethylpentasiloxane
LAC	Laccase
LC	Liquid chromatography
LiP	Lignin peroxidase
MnP	Manganese peroxidase
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry

NM	Nanomaterial
NP	Nonylphenol
NPEO	Nonylphenol polyetoxylated
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PCE	Perchloroethylene
PFC	Perfluorinated compound
PhC	Pharmaceutical
PLE	Pressurized liquid extraction
QqLIT	Quadrupole linear ion trap
QqQ	Triple quadrupole
Q-ToF	Quadrupole time of flight
SPE	Solid phase extraction
TCE	Trichloroethylene
TP	Transformation product
UV	Ultraviolet
VMS	Volatile methyl siloxanes
VP	Versatile peroxidase
WRF	White-rot fungi
WWTP	Wastewater treatment plant

1 Emerging Contaminants in Sludge

Continually, scientific studies are revealing the presence in the environment of substances that we use on a daily basis in our homes, industries, and agricultural fields. These substances are called emerging contaminants. This term could be divided into two subcategories: on the one hand, there are "new" emerging contaminants, which are chemicals that are recently manufactured and suddenly appear everywhere, such as decabromodiphenylethane which appears as a Deca-BDE replacement [1]. On the other hand, there are "old" emerging contaminants, which are those that were used and applied for a several decades, but they were not environmentally detected until recently. This is the case of Dechlorane Plus (DP) which was used since 1986 as a replacement of the banned Mirex, but it was not detected in the environment until 2006 [2]. Nor should we forget the emerging contaminants listed as transformation products (TPs) of legacy or emerging contaminants, or benign chemicals, as well as pernicious metabolization products. The overall purpose of metabolism is to make the chemical less toxic and more hydrophilic. However, metabolism does not always render a chemical less toxic.

The list of new chemicals whose presence in the environment represents an ascertained or potential risk is sizeable and broadly contains resins, plastics, and plastic additives (e.g., plasticizers and flame retardants); pharmaceuticals and personal care products (e.g., disinfectants, fragrances, sunscreens, antibiotics, drugs of abuse, and natural and synthetic hormones); detergents and other cleaning

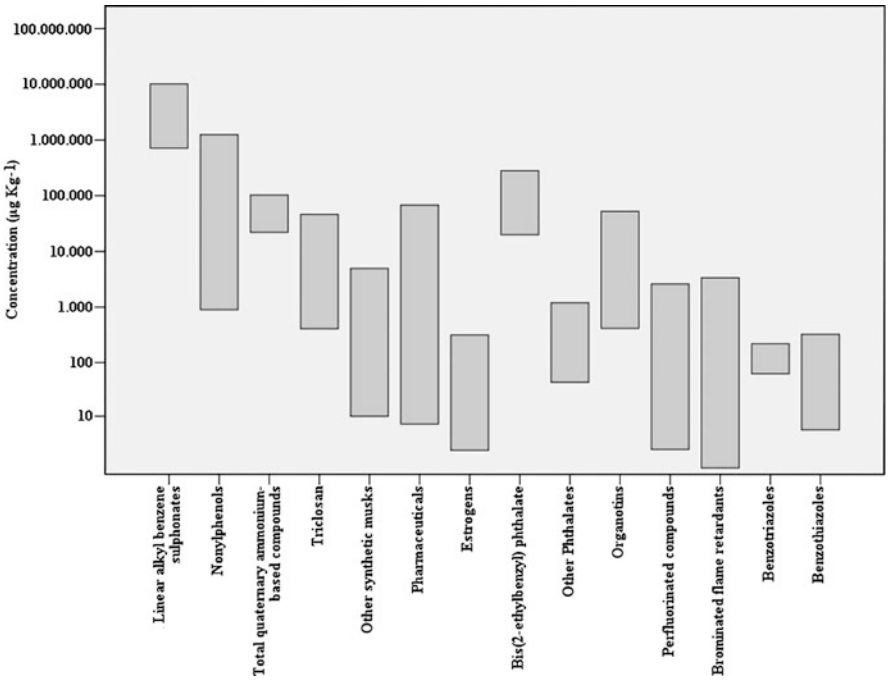

Fig. 1 Reported ranges of emerging contaminant concentrations in sludge samples. Reproduced from [4]

agents; pesticides and biocides (e.g., weed killers, fumigants, wood preservatives, and antifouling agents); petroleum-derived chemicals; and products of waste/drinking water treatment, of landfill, and incineration.

The adjective "emerging" implies that little is known about these contaminants, from their production volumes, to their physicochemical properties (water solubility, log Kow, etc.), to their effects on humans and the environment, to how best to regulate the unknown risks they pose [3]. In the context of environmental concerns of sludge, it is very important to know whether these new contaminants accumulate or not in sewage sludge (Fig. 1). These organic chemicals may ultimately enter urban wastewater. Degradation and attenuation during wastewater and sludge treatment remove significant amounts of organic contaminants. However, many compounds have lipophilic properties and hence transfer to sewage sludge and may be present in residual concentrations in the dry solids depending on the initial amounts present, their lipophilicity, and the extent of destruction during wastewater and sludge treatment.

Among the large variety of compounds, this book is focused on pharmaceutical compounds, estrogens, illicit drugs, UV filters (sunscreens), brominated flame retardants (BFRs), and perfluorinated compounds (PFCs) due to their endocrine-disrupting purposes, being as well present in sewage sludge samples. However, other emerging contaminants are also receiving special attention by the environmental

scientific community in last years [4, 5]. Thus, future research should be focused to study the presence of these new chemical groups of emerging pollutants in sludge. Among these new emerging contaminants we can mention synthetic musk compounds, benzothiazoles and benzotriazoles, nanomaterials, and volatile methyl siloxanes. Because these new families of compounds have not been treated throughout the different chapters of this book, a brief description of them will follow.

Synthetic musk compounds are part of synthetic fragrances which became cheaply available during the 1950s. They were widespread used as a substitute for natural musks in fragrances and can be found in a number of consumer products such as laundry detergents, fabric softeners, cleaning agents, and cosmetic and hygiene products (soaps, shampoos, body lotions, perfumes, deodorants, cosmetics, etc.). However, it took until the 1980s that these compounds were detected in the environment as a consequence to vast usage and incomplete removal in wastewater treatment plants. The main classes of synthetic musks include nitroaromatic musks, such as musk xylene and musk ketone, as well as polycyclic musks, such as HHCB (galaxolide), AHTN (tonalide), ADBI (celestolide), ATII (traseolide), and AHDI (phantolide). It is well known that these compounds are emitted into the wastewater, reach freshwaters as well as the marine environments. As most of these compounds are very lipophilic, they tend to accumulate in sediments, sludges, and biota [6].

Benzothiazoles (BTHs) and benzotriazoles (BTRs) belong to high production volume chemicals that find broad application in various industrial processes and household products [7]. BTHs are used as biocides in paper and leather manufacturing, as vulcanization accelerator in rubber production and as corrosion inhibitors. BTRs are commonly used as a corrosion inhibitor in dishwasher detergents and de-icing/anti-icing fluids, an ultraviolet light stabilizer in plastics, and an antifogging agent in photography. BTHs and BTRs are water soluble, resistant to biodegradation and only partially removed in wastewater treatment. BTRs have been classified as emergent pollutants because even at low concentrations, they elicit negative effects to aquatic organisms. The German Government has proposed limit values in biosolids for two rubber vulcanizing agents: 2-mercaptobenzothiazole and 2-hydroxybenzothiazole.

Nanomaterials (NMs) have one dimension <100 nm and possess physicochemical properties dictated by their unusually small size, large surface area, shape, and chemical composition. New properties of nanomaterials have boosted their production and industrial applications in many fields (e.g., microelectronics, catalysis, fuel cells, materials science, textiles, biotechnology, and medicine). The fast development of nanotechnology and its expected growing will increase the products containing NMs in next coming years. However, NMs may also have negative effects on biological systems. Nanotechnology is a major, innovative, scientific, and economic growth area, but the increasing production and use of nanomaterials have led to calls for more information regarding the potential impacts that their release may have on human health and the environment. NMs include materials with one dimension on the nanoscale (nanolayers) and two dimensions on the nanoscale (nanowires and nanotubes), and particles (e.g., quantum dots, metal

nanoparticles, and fullerenes). To date, little is known about the potential risks associated with engineered NMs [8].

Volatile methyl siloxanes (VMS) are chemicals composed by R_2SiO units where R is a methyl group. VMS include cyclic siloxanes (cVMS), the most important being hexamethylcyclotrisiloxane (D3), octamethylcyclotetrasiloxane (D4), decamethylcyclopentasiloxane (D5), and dodecamethylcyclohexasiloxane (D6), and linear siloxanes, e.g., hexamethyldisiloxane (L2), octamethyltrisiloxane (L3), decamethyltetrasiloxane (L4), and dodecamethylpentasiloxane (L5). Special attention is paid to D4 and D5 siloxanes that are persistent, bioaccumulative, and toxic substances, according to tests performed on animals [9, 10]. Due to their volatility, low surface tension, transparency, hydrophobic nature, and lack of odor, siloxanes are used in a number of products such as cosmetics, deodorants, soaps, hair conditioners, hair dyes, car waxes, cookware, cleaners, furniture polishes, water-repellent windshield coatings, and in industrial applications such as solvents, lubricants, and sealants. Both cyclic and linear VMS are emitted into the environment as industrial refuses, e.g., in manufacturing high molecular weight silicone polymers, or owing to use of silicon products. Due to their widespread use, siloxanes are found in wastewaters, in landfill solid wastes, and also in biogases generated by the anaerobic degradation of organic materials [11].

2 Analytical Approaches

The continuous progress in analytical techniques has improved the capability of detecting chemicals and recognizing new substances and extended the list of detectable contaminants widespread in all environmental compartments by human activities. Over recent years, different methodologies for the analysis of emerging compounds have been developed. Some recent reviews have been published and some of them focused on emerging contaminants [12–14]. However, for some of these emerging pollutants, very few studies are referred to the analysis of sewage sludge. The key issue in the analysis of emerging pollutants in complex matrices continues being to assess the quality of analytical data. These quality parameters must be tested through the performance of interlaboratory tests and combined, if it is possible and available, with the use of reference materials.

Sample preparation procedure plays a fundamental role in developing analytical methodology for complex matrixes, such as sludge. Extraction and cleanup steps have presented a high improved during recent years, especially in terms of automation and the development on online solid phase extraction (SPE) technologies, reducing the sample manipulation, the time of analysis and minimizing the quantities of sample required for the analysis. Techniques such as pressurized liquid extraction (PLE) providing fast, safe, and easy preparation procedures, and using smaller amounts of solvents, have received significant attention.

Regarding the instrumental determination, developed methodologies are based on the use of mass spectrometry (MS), either coupled to gas chromatography (GC) or

Table 1 Parameters of mass spectrometers used in LC-MS. Adapted from [14]

Mass analyzer	Resolving power ($\times 10^3$)	Mass accuracy (ppm)	m/z range (upper limit) ($\times 10^3$)	Acquisition speed (Hz)	Linear dynamic range	Price
Q	3–5	Low	2–3	2–10	10^5–10^6	Low
IT	4–20	Low	4–6	2–10	10^4–10^5	Moderate
ToF	10–60	1–5	10–20	10–50	10^4–10^5	Moderate
Orbitrap	100–240	1–3	4	1–5	5×10^3	High
ICR	730–2,500	0.3–1	4–10	0.5–2	10^4	High

Q Quadrupole, *IT* Ion trap, *ToF* Time of flight, *ICR* Ion cyclotron resonance

liquid chromatography (LC). The market of MS and LC-MS is extremely dynamic and manufacturers invest into the development of new technologies (Table 1). In the analysis of emerging polar organic compounds, LC-MS techniques are the best established ones. However, the required sensitivity needs the use of tandem mass spectrometry (MS-MS). Different configurations have been applied, being the most common the use of the triple quadrupole (QqQ). However, in recent years other hybrid MS configurations have also appeared, such as quadrupole linear ion trap (QqLIT), quadrupole time of flight (Q-ToF), or Orbitrap. QqQ instruments allowed the confirmation of detected contaminants even at very low concentrations. On the other hand, ToF analyzers allow to finding additional non-target organic contaminants. And Orbitrap, an ultra-high resolving power analyzer (>100,000), is increasing their use to identify unknown contaminants, typically environmental TPs.

The analysis of environmental TPs has become a major trend in environmental chemistry, and increasingly, researchers are taking this a step further in proposing complex transformation pathways. It is expected to see a gradual shift from parent compound analysis to the analysis of metabolites and TPs. It is evident that more research is needed to determine the breakdown pathways and to evaluate the fate of TPs. Therefore, development of future generic analytical protocols should permit the simultaneous determination of parent compounds and their metabolites.

3 Sludge Treatments

Nowadays there is already lots of information about detection and emerging contaminant concentrations in liquid matrices, such as industrial and agricultural effluents and influent/effluent of the wastewater treatment plants (WWTPs). On the contrary, there is lack of knowledge about these pollutants in solid matrices, despite the hydrophobicity of a wide range of them and therefore it is expected that a large part of them are adsorbed in sludge, manure, sediments, etc. One of the reasons for this lack of information, as has been revealed in Chap. 2, is the analytical difficulty in solid matrices that is much greater than in liquid matrices, not only for the

analytical method itself but also for the difficulty in obtaining representative samples in solid wastes.

Recently, the studies have been focused on emerging pollutants adsorbed in sludge since, due to its high content of organic matter and nutrients, they are used as fertilizers or soil conditioning. The common practice is to stabilize them by anaerobic digestion (AD) (approx. 70% of the sewage sludge generated) or composting, before their application to the soil. In some WWTPs sludge is first treated by AD and then is subjected to a composting process to be able to be used as fertilizer.

Some scientists have focused their research on knowing the aerobic and anaerobic biodegradability of emerging contaminants detected in sludge (Chap. 3). Among the different families of pollutants, those whose estrogenic effects have been detected in the sludge have received greater attention, such as alkylphenolic polyetoxylated (APE) detergents. The main derivative of APE, nonylphenol (NP) has already been included in the list of priority pollutants by the European Union.

It can be concluded, because there are already many published results, that it is important to know both aerobic and anaerobic biodegradation of pollutants. However, this is not enough; it is also necessary to determine its TPs, since many of the emerging pollutants are aerobically biodegradable in the WWTPs but not their TPs. This is the case of the long chain nonylphenol polyetoxylated (NPEO), which are aerobically degradable. These compounds are broken and they convert into less hydrophilic compounds and with more tendency to adsorb in sludge. The detection of short-chain NPEOs in digested sludge shows that they are recalcitrant to methanogenic degradation and that they accumulate in the sludge.

In the case of hormones entering the WWTPs, almost 60% are conjugates. Most studies conclude that its biodegradation is an aerobic process although the levels of which are detected in treated effluents show that they are not completely biologically degraded and that part of them are adsorbed into the sludge. Although there are very few studies on degradation of hormones in sludge, the published results confirm that the hormones are recalcitrant to the anaerobic treatment. This is corroborated by the results on composting of manure which points out that percentages of elimination of up to 80% can be achieved.

Pharmaceuticals (PhC) are the most studied family of emerging pollutants in the WWTPs, but only most recent studies have considered PhC adsorbed in sludge. Most of these works try to establish which is the main factor that determines its presence in sludge, and they concluded that are multiple. On the one hand, it is demonstrated that a large number of different PhC (antibiotics, analgesics, beta-blockers, etc.) are very biodegradable aerobically although their degradation is not complete and it is influenced by the properties of a particular drug, its concentration, as well as by the characteristics of the process (hydraulic retention time, solid retention time, structure and activity of biomass, etc.). On the other hand, there are different studies that could not demonstrate that the organic matter content of the sludge is the most determinant factor to promote its adsorption.

The elimination of PhC in the sludge treatment, both anaerobic and aerobic, seems to be the function of the molecule and also of the process conditions

(mesophilic or thermophilic temperatures). The results suggest that aerobic conditions allow a more efficient elimination of a wide spectrum of molecules, although it is noticeable in the case of carbamazepine. Numerous studies show that carbamazepine is detected in similar concentrations in influent and treated effluents in WWTPs, showing its persistence to biological degradation. But in addition it is also detected in sludge, although at low levels, and even in composted sludge. Therefore it is recalcitrant to the bacteria in the sludge treatment processes, both aerobic in the composting process and anaerobic in anaerobic digestion process.

Further researches are still need to better know:

- Microorganisms involved in the degradation of pollutants in sludge under anaerobic and aerobic conditions
- Parameters that affect the elimination of pollutants in sludge treatment processes (AD and composting)
- Novel microorganisms and processes for pollutant biodegradation

In Chap. 5, and later, the results on degradation of emerging contaminants by ligninolytic fungi are presented. The ability of the white-rot fungi (WRF) to degrade xenobiotics compounds, with complex molecules similar to the structure of lignin, has been well known since many years ago. It has been widely published the capacity of the WRF and its extracellular enzymes to degrade organochlorine compounds (PCE, TCE, PCB, ...), polycyclic aromatic hydrocarbons, munition wastes, etc., as well as decoloration and detoxification of effluents as paper-mill, textile, olive-oil mill, etc. Therefore, WRF cannot be considered as the "new microorganisms" but the development of treatment processes of effluents and sludge by fungi can be considered one of those "novel processes" on which it would be necessary to devote efforts [15]. Up to now, there are very few published papers on treatment of sludge with fungi, mostly deal with studies of degradation in liquid media or bioremediation of contaminated soils. And still the results on degradation of adsorbed emerging contaminants in sludge are much rarer.

Before achieving the development of an industrial scale process, the research must follow different stages. First of all, the researchers have as an objective to demonstrate that the WRF are capable of degrading different families of emerging pollutants. Most of the studies have been carried out in liquid media, and a review is presented in Chaps. 6, 7, and 8.

The endocrine disrupting compounds (EDC) comprise the most studied group. Approaches for the removal of PhCs are more recent and belong to analgesics, antibiotics, psychiatric drugs, lipid regulators families, but researches on the biodegradation of some classes of drugs that are also consumed regularly as the cytostatic, contrast media and others that are also detected in WWTPs are missed. The panorama is similar for BFRs and UV Filters, whose degradation by WRF has been barely studied. Moreover there are other families of emerging pollutants as the PFOS, some derivates from NPEOs, pesticides, on which there are not still studies on degradation by fungi.

The research carried out shows good results on pollutant biodegradation and there is an increasingly knowledge about the characteristics of the WRF which make it possible, and can be summarized as follows:

- WRF presents an extracellular oxidative system nonspecific and nonselective.
- The pollutant degradation by WRF is a co-metabolic process in which additional C and N sources are required. This capacity represents an advantage respect bacteria as it prevents the need to internalize the pollutant, thus avoiding toxicity problems and permitting to attack low-soluble compounds.
- WRF presents an intracellular enzymatic system, cytochrome P450 monooxigenases, similar to those mammalian cells, that catalyzes a broad range of intracellular degradation reactions of released metabolites after the pollutants breaking by extracellular enzymes.
- The hyphae growth facilitate colonization in solid matrix.

This last characteristic is especially interesting in sludge treatment. Several reports deal with the application of fungal bioaugmentation for bioremediation of contaminated soils with persistent pollutants, in slurry phase and solid phase (biopiles), but the research about their application in sludge treatment is limited. Chapter 5 presented the results on the elimination of different types of emerging pollutants in sewage sludge by fungal treatments in biopile and slurry reactors at laboratory scale. Biopile treatment (like composting) is interesting for treating dry sewage sludge obtained after dewatering in WWTPs with thermal dewatering step or filter press. In slurry phase treatment the sludge is a suspension with a solid concentration equal to that found in the outlet flow of the anaerobic digester.

The degradation of many hazardous compounds has been first demonstrated at laboratory scale and under sterile conditions, with the objective of avoiding the interaction with bacteria and other microorganisms contained usually in digested sludge. In this way it is possible to demonstrate that on the one hand, it is possible the colonization of sludge by the fungus, and on the other hand, the degradation of pollutants is only due to the action of the fungi.

The fungal treatment showed a high efficiency, by completely removing the estrogens (E1, E2, and E3), either in biopile or slurry reactors. On the contrary the removal of BFRs in both systems shows differences. Removal after the fungal slurry treatment was rather low compared to the removal obtained in solid-phase system. These results need more research because they are in contradiction with those stated by some researchers that point to the low solubility of these compounds as the limiting factor in its biodegradation.

The results about the removal of UV Filters were contrary to the expected. Higher removals were expected in slurry system for the least hydrophobic UV Filters and lower in solid-phase. 4DHB and BP3, least hydrophobic than OC and EHMC, were completely removed in solid phase and only BP3 was poorly removed in slurry treatment while 4DHB showed recalcitrance. In general, for all UV Filters detected in raw sludge, better removal values in solid phase treatment were obtained.

PhCs are the most abundant pollutants in raw sludge and as it is expected, a greater number of them are detected in digested sludge than in thermally dehydrated sludge. Removal of PhCs by fungi varied from 40% to complete elimination, showing differences between the compounds and between the two types of fungal treatment. The degradation results of the antiepileptic carbamazepine are highly remarkable, as its almost negligible removal has widely reported in bacterial processes. The removal values for carbamazepine are lower in slurry treatment (approx. 30%) as well as in biopila (approx. 43%), but they show the high power of the fungus to degrade molecules that cannot be degraded by the bacteria.

Comparison of the efficiency removal in both fungal treatments indicates that a solid phase treatment is more efficient than the bioslurry process. Therefore, once demonstrated the role of fungi, the following research objective must be to carry out the process with non-sterile sludge. The results published on bioremediation of contaminated soils by fungi are not in agreement about the interaction of bacteria and fungi. In some cases of highly contaminated soils by hydrocarbons, the survival of the fungus is very low and therefore the biodegradation is only due to bacteria. Recently, published results on sludge treatment under non-sterile conditions with the WRF *Trametes versicolor* show the highest percentages of PhC degradation to occur during the first days of fungal treatment in solid phase [16]. Molecular biology studies of the population present in the sludge reveal that the inoculated fungus survives and is predominant during this first period against bacteria, then bacteria are predominant, and no more significative pollutants removal is detected.

4 Biodegradation by Fungi

Chapters 6, 7, and 8 present a literature revision about fungal degradation in liquid medium of three groups of emerging contaminants detected in sludge: PhCs, UV Filters and BFRs. The former group of contaminants, due to their high consumption worldwide, is present in different compartments of the environment and also, as Chap. 5 describes, they are detected in sludge. Similarly, due to their high hydrophobicity and low degradability, UV Filters and BFRs are specially retained in sludge.

Degradability studies have generally been conducted in liquid media using synthetic samples spiked with a contaminant at higher concentration than that typically found in the environment. At high concentrations the degradation and the detection of TPs are facilitated. Although it is demonstrated that under certain conditions, fungi may not completely degrade (i.e., mineralize) and/or assimilate these products.

While information about degradation and product identification of UV Filters and BFRs is limited, drugs have raised higher interest among researchers as it is shown in the number of papers published in recent years (see Table 1, Chap. 6).

In previous studies of fungal degradation of pollutants, one of the main questions has been whether low concentrations may be sufficient to activate the fungal oxidative–reductive enzyme system to degrade these compounds. Many studies have demonstrated that at concentrations as low as ppm, ppb, or even at concentrations found in real effluents, fungi are in fact able to degrade pollutants. Despite this evidence, a minimal concentration requirement is yet not clear. For example, the studies by Subramanian and Yadav [17] show that the transcription of the cytochrome P-450 system of *Phanerochaete chrysosporium* is activated in the presence of pollutants. Regarding the extracellular system of fungi (i.e., laccase), studies have demonstrated an increase in activity when contaminants are in the medium, even when the enzymes were not directly involved in the early stages of degradation [18, 19]. Can we then state that the activation of fungal enzymes requires a minimal concentration? In the light of the latest findings, we conclude that this question remains unknown and further investigations are needed.

Among WRF, *P. chrysosporium* has been traditionally applied in biodegradation assays, mainly for being the best characterized fungus. Over time, other fungi have gained attention. Perhaps the second most studied fungus in biodegradation is *T. versicolor,* mainly due to its great performance [20]. Among its benefits is that laccase (LAC) production is associated with growth without nutrient-starvation conditions (i.e., low levels of carbon and/or nitrogen). Moreover, the presence of contaminants may stimulate laccase production of *T. versicolor*. There are also other works that demonstrate the capacity of other WRF to degrade pollutants, i.e., *Irpex lacteus*, *Pleurotus ostreatus*, *Bjerkandera* spp., etc. [21].

WRF are, by all means, the most studied group of *Basidiomycetes*. In addition, several fungi belonging to the phyla *Ascomycota* (*Tricchoderma, Geotrichium, Mucor, Candida*. . .spp.) have also demonstrated the ability to degrade contaminants (as can be see in Table 1 Chap. 6), although the number of publications is much lower compared to that related to WRF. Lately, researchers are paying attention to the capabilities of other group of wood-degrading fungi, the litter-decomposing fungi, which mainly oxidize contaminants using manganese peroxidase (MnP) [22–24].

The group of PhCs contains the largest list of contaminants. As the chapter of analytical methods (Chap. 2) describes, many compounds may be identified just in a single sample of sewage sludge. These compounds frequently belong to groups with different therapeutic activities (e.g., anti-inflammatory, psychiatric and depression drugs, lipid regulators, antibiotics, β-blockers, estrogens, iodinated contrast media . . .) and different chemical structure. Thus, their degradation mechanism is difficult to predict. Table 1 of Chap. 6 summarizes removal efficiencies of PhCs at different conditions and times. One can find compounds with 100% removal yield in 24 h, but also others whose removal rate is $<10\%$ in 2 weeks as fluoxetine per example. In general, inflammatory and antibiotic drugs are degraded fast, while for example, contrast media compounds degrade to a lesser extent and at lower rate. Even, some drugs, such as carbamazepine or clofibric acid, which bacteria cannot easily degrade, are better degraded by WRF, although even fungi have difficulties to degrade them compared with other drugs. The same table also includes possible degradation products of PhCs which may be obtained after fungal treatment in liquid medium.

In the case of the UV Filters, degradation yield may range from 87% to 100%. The parental compound usually is degraded very fast, but elimination of the transformation product occurs at a slower pace. The degradation mechanisms of UV Filters starts with a rapid conjugation step with a sugar (e.g., pentose or hexose), in order to make the compound more soluble. Thereafter, this conjugate is slowly transformed by the fungus.

Regarding BFRs, only three mixtures of polybrominated diphenyl ethers (PBDE), deca-BDE, octa-BDE and penta-BDE which differentiate only in the number of bromide atoms (ten, eight or five, respectively) have been studied. For deca-BDE total elimination was obtained after 4 days. For octa- and penta-PBDE the removal was between 67 and 85%, respectively. The degradation products are not clear. Most likely, demobrination of the parental compound does not occur as not less brominated transformation products are found. Similarly than the mechanism to degrade UV Filters, the first step might be a solubilization of the parental compound via sugar adduction, although further research is needed to confirm it. Some compound may undergo hydroxylation because hydroxylated BDEs are detected as degradation products. In these studies a surfactant is used to aid BDE solubilization, but even so, the dissolved concentration is very low.

Although the exact mechanism of degradation at metabolic level for each compound or group of compounds is not well known, the involvement of extracellular oxidative enzymes such as LAC, MnP, LiP, and versatile peroxidase (VP) (see Tables 1 and 2 of Chap. 6) and intracellular monooxygenases as cytochrome P-450 is well documented for pollutants such as hydrocarbons, dyes, and halogenated solvents [25]. To determine the actual role of the extracellular enzymes, many studies are performed in vitro experiments with purified enzymes. In the case of cytochrome P-450, usually inhibitors are used.

In the case of degradation of emerging contaminants (PhCs, UV Filters, and PBDEs), generally an oxidation of the compound occurs. The main reactions observed are hydroxylations, formylations, desulphonations, dehalogenations, demethylations, ring cleavage. Often, fungi act similarly in the first degradation step than the degradation mechanisms of the liver of mammals [18, 19]. In some cases, transformation products may not always be mineralized and, consequently, they accumulate. But in the cases when transformation products disappear, mineralization and assimilation may be expected.

The fact that mineralization does not always occur justifies the large number of studies published as an effort to characterize the degradation products. In some cases these products are detected in human or animal urine, which may reach WWTPs and/or different environmental compartments. Probably, in the coming years more sophisticated analytical techniques to detect and quantify these metabolites will be developed.

From the point of view of risk analysis assessment, it might be needed both analytical methods as well as thorough toxicity assays and biological methods as it is not possible to detect all the compounds present in real samples. This is especially interesting in the case when, for example, a single sludge sample is toxic just due to a compound present at very low concentration. Combination of techniques is

necessary in order to anticipate the potential harmful effects of the application of sludge in agricultural land, especially when the contaminants are at low concentration. Moreover, techniques related to massive omics (e.g., metabolomics, proteomics, genomics) will be also very beneficial in this field. Further efforts are also needed to standardize, develop, and implement different toxicity tests that are complementary and cover the wide spectrum of possible adverse effects.

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