Environmental Chemistry for a Sustainable World

Eric Lichtfouse
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Pollutants in Buildings, Water and Living Organisms

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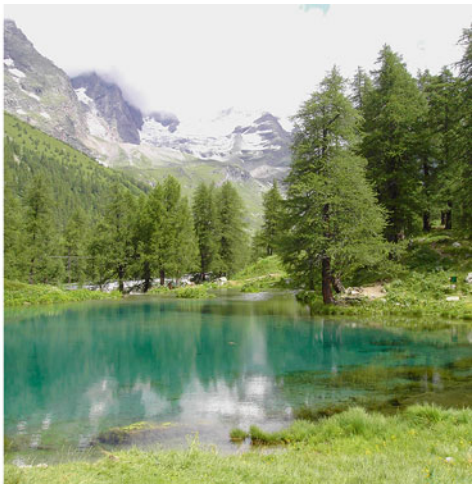
Preface

Chemosphere

If we take a look at the history of environmental chemistry from about 1970, the early days were mainly busy with trying to identify pollutants in various media. At that time analytical tools were very slow and far less precise than today. Most pollutants such as polar compounds, compounds occurring as traces, and compounds occurring in complex natural media, e.g. soils, wastes and sediments, were simply not analysable because there was no method available. As a consequence only few pollutants were identified, resulting in low to moderate public concern. With the further development of high-resolution techniques such as gas chromatography coupled with mass spectrometry, scientists realised that we are living in a 'chemosphere' filled with pollutants flowing from one media to the other, which is nicely depicted by the quote 'pollutants have no borders'. Public concern thus dramatically increased because ecotoxicologists and doctors found more and more causal links between pollutants and illnesses such as cancer. Now research is mainly focussed on designing non-polluting processes and methods to clean pollutants.

Left: erosion effect on a limestone statuary, from Chap. 1 of this book. *Right*: Blue Lake, Aosta Valley, Italy, from Chap. 4 of this book

This book presents advanced methods to monitor and remediate pollutants. Alves and Sanjurjo-Sánchez review the effects of pollution on building materials and methods to clean materials in the first chapter. Water treatment methods are described in Chap. 2 by Baruah et al., with emphasis on nanotechnology. Da Silva et al. explain the use of bacteria and fungi to degrade pesticides in Chap. 3. The most advanced analytical tools of chromatography and mass spectrometry, and their application to water analysis, are reviewed by Gosetti et al. in Chap. 4. Kasiotis and Emmanouil review methods to analyse polycyclic aromatic hydrocarbons (PAH) in Chap. 5; they also present the toxicological effects of PAH on bivalves. The cycle of selenium in plants is described by El Ramady in Chap. 6. Pereira et al. present methods to analyse and remediate aromatic amines in Chap. 7.

Thanks for reading.

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Contents

Chapter 1
Maintenance and Conservation of Materials in the Built Environment

Carlos Alves and Jorge Sanjurjo-Sánchez

Contents

Abstract Materials on the built environment are exposed to several agents that promote alteration processes resulting in features that might be considered detrimental of its value. Here we review the main issues related to the struggle against these alteration processes, from the consideration of the intervention criteria, including the non-intervention option, to strategic considerations on the organisation of the intervention that must consider the temporal and spatial features of the alteration processes, as well as possible interventions on the surroundings of the materials, e.g. atmosphere, terrain and structure, and in relation to treatments of materials,

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including its replacement. It is highlighted the problem of testing using small clean specimens aggravated by comparative studies based on one specimen by case. Assessment of short-term an effects is discussed in relation to intrinsic aspects of the interventions. The long-term effectiveness is linked with the global strategy namely in relation to the conditions that promote the alteration processes. Some sustainability questions related to the intervention operations are also considered such the use of toxic substances and the consumption of resources.

Keywords Stony materials • Alteration processes • Decay assessment • Intervention strategy • Dating • Materials treatment and replacement • Sustainability

1.1 Introduction

Materials applied in the built environment are subjected to diverse agents that promote alteration processes (Siegesmund and Snethlage 2011; Sanjurjo-Sánchez and Alves 2011, 2012). The resulting alteration features can be described in very general and summary terms in coatings/stains and erosive features (for detailed classifications see Fitzner and Heinrichs 2002; ICOMOS-ISCS 2008). There could be diverse types of coatings or stains relate to fixation of exogenous matter, reactions between pollutants and the substrate and the development of organism (biological colonisation). In the case of erosive features there is loss of material that have been attributed to several causes such as freeze-thaw, wetting-drying, chemical dissolution and specially the crystallisation of soluble salts that can have diverse sources and permeate the porous media, crystallising in the pore walls and provoking the physical disruption (Goudie and Viles 1997).

These alteration features can be considered of the object aesthetics or hazardous to its physical integrity and that might be considered as requiring interventions aimed at eliminating or mitigating the alteration Reviews of the conceptual framework and rationale for the intervention operations as well as the different terminology applied to the general scheme of interventions (conservation, preservation, care, maintenance, repair, rehabilitation, restoration, prevention, etc.) can be found among others in Caple (2000), Wood (2003) and Muñoz-Viñas (2005). Conservation is used here in the widest possible sense (see Muñoz-Viñas 2005) to encompass all these operations aimed at avoiding the alteration of materials.

The present review will focus on strategic and tactical aspects of the interventions in relation to the characteristics of the alteration processes. In a perspective of Environmental Chemistry for a Sustainable World it will be focused on the relevance of the understanding of the problem (diagnosis) including the characterisation of the materials and alteration agents, the substances used in the interventions and sustainability aspects related to these interventions. This review will be mostly focused on alteration processes that affect stony materials and the interventions in these materials and its surroundings (including atmosphere, terrain and structures) with a view towards the conservation of the stony materials. Interventions for stabil-

ity or usability of the structure or the treatment of other materials (metallic, plastics, glass, paper, wood, etc.) will not be considered here. However, some weathering processes of stony materials could favour the alteration of other materials as occur to iron reinforcement in concrete structures as consequence of carbonation of the cementitious paste and, in this sense, the questions considered here will could be also relevant for the conservation of other materials.

1.2 Intervention Criteria (Including Non-Intervention Option)

The classical medical expression *Primum non nocere* can be used to initiate this section since the first concern that one must keep in mind is whether the intervention will be beneficial for the object. Besides the aesthetical reasons, there are factors that might imply that the intervention will be worst for the object than a non-intervention option, depending on the decay agent. One should consider whether the system to be treated is presently at either a steady state or a dynamic equilibrium and stabilized and or whether it is foreseen that decay will progress with time. In the first case, any significative intervention will affect the present state and equilibrium and this could go the wrong way. Sometimes the best policy is a non-intervention policy, referred as the "do nothing" option by Warke et al. (2003), where one lets the system keeps its equilibrium (a monitoring scheme and contingency plans could be included) or when the risk of possible interventions is considered higher than the possible benefits and that could be considered the real example of passive conservation since all the conservation strategies implies some sort of purposeful interaction with the object (e.g. when you change its surrounding environment). The do-nothing option is, however, not a popular choice; it is not easy to convince authorities that keep quiet is the best choice but the monitoring program is the way to keep an eye on things. One can argued that the now popular choice among archaeologist of reburial is conceptually similar to this one, being a case of letting things as they stand (or stood before excavation) as long as this could be considered a less damaging option which will depend on the objects and the burying environment (Canti and Davis 1999; Wilson and Pollard 2002; Caple 2004; Crow 2008). Doehne and Price (2010) refer in relation to the effects of intervention procedures "a degree of scepticism would perhaps be justified over 'damage' that is observable only through a scanning electron microscope". The same could be applied to the effects of some alteration processes.

Besides, there is the question of what does constitute decay and how to assess decay, especially in terms of visual alterations without significative erosive impact. Some authors might consider, for example, that a patina can show relation to the passage of time and the use of the object (Mostafavi and Leatherbarrow 1993; Kirkwood 2004; Caple 2000), that consider it the kind of change that increases value (Muñoz-Viñas 2005) and also that it can act as protective layer (Caple 2000). Studies on patinas and coatings show radical differences depending on the substrate and the

environment (e.g. concentration of pollutants): coatings can grow at very variable rates in variable time-intervals from centuries to thousands of years (Sanjurjo-Sánchez et al. 2012) as a result of "natural ageing" or interaction with pollutants (Dorn 1998; Sanjurjo Sánchez et al. 2008, 2009). For example, Concha-Lozano et al. (2012b) refer to the protective effect of biological colonization and propose that endolithic organic matter resulting from lichens can act as a barrier ageing contaminants (e.g. sulphation). Curiously, there has been some polemics on whether oxalate films are product of alteration or represent a protective layer and presently there are protection treatments based on the promotion of the formation of oxalates (see below). Caple (2000) refers that cleaning decisions must balance the loss of information that could be contained in the substances to be removed by cleaning against the benefits (improvement of stability of the object and revealing more of the original visual form). The result of the interventions might be displeasing to some people (Brimblecombe and Grossi 2005; Muñoz-Viñas 2005). There are aesthetic issues that are more or less polemical including the assessment of the whole result in the surrounding context as could perhaps be discussed with the example shown in Fig. 1.1. An interesting approach (one could say "citizen-oriented") was undertaken by Brimblecombe and Grossi (2005) that researched the possibility of using questionnaires to define potentials levels of blackening that could be considered as aesthetic thresholds for a surface to be considered dirty by the public. It could be

Fig. 1.1 Example of façade cleaning where the evaluation of the whole result might be a polemic subject

considered that all the alterations are part of the historical evidence and any direct intervention will be excluded (Muñoz-Viñas 2005). It is necessary to assess the current risk situation and the risks related to the interventions, i.e., How would evolve things without (path 1) and with (path 2) intervention? Path 2 should diverge enough (and in the right direction) to justify the intervention.

In the case when it is necessary to do some intervention it is necessary to ponder the possible side effects. One needs to assess the global impact on the object of the intervention (there could be also other impacts on the surrounding environment). This applies to all possible interventions even preventive ones. In Fig. 1.2 it is possible to observe a situation where the use of a metallic net to protect the statuary of a church has contribute to the development of brownish stains in the statues. This must be kept on mind to be alert to some proposals such as the application of brass strips on object sites exposed to run-off rainwater having in mind the control of microbial contamination that, as discussed in Warscheid, and Braams (2000) could lead to greenish stains. Of course sometimes is a question of choosing the lesser of two evils (however in the previous case one could be critical about which would be

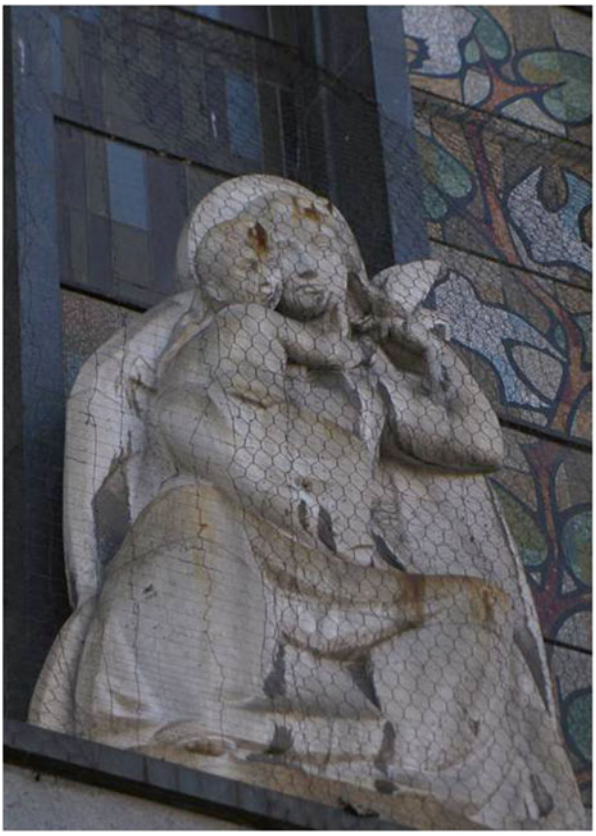

Fig. 1.2 Stains in statuary that seem related to alteration of metallic structures

the lesser evil). Further questions will be discussed in the presentation of details on the different procedures. A general principle could be the "minimum intervention" (Muñoz-Viñas 2005).

One of the main problems for what has been proposed above is the difficulty of making the risk assessment. As reviewed in Sanjurjo-Sánchez and Alves (2012) while there are some decay functions proposed for laboratory studies there is still much uncertainty in the specific prediction of decay rates for a given pollutants load. Of course one can establish that things are "dirty" (and define aesthetics thresholds as is done by Brimblecombe and Grossi 2005). If it is visible that a given object is experienced erosive processes the problem for the moment of intervention is how worst it will get and how soon? While Smith (1996) discusses some possible generic models, the evolution of decay in time has also been little researched and it is in fact almost impossible to predict for a given object in real field conditions its evolution. As referred above, risk assessment should also be done for the proposed intervention addressing questions such as what can go wrong? What could be the side effects? The assessment of these features should be included in the general strategic planning.

How can we assess the opportunity of one intervention if decay is difficult to assess? One of the most controversial questions is the assessment of alterations or decay. Different destructive and non-destructive methods have been proposed and tested on building materials to generally assess decay. A table with the most common methods is provided (Table 1.1). One problem of this part of the diagnostic work is to compare decay phases or states among materials and buildings. Also, it is not easy to compare the results obtained with different methods, above all if we compare physical and chemical analysis. If we assess the weathering state of some stone blocks in a building, this could result in different conclusions depending on the chosen method. As an example, the use of porosimetric techniques has provided different results than geochemical methods (Ng et al. 2001; Arel and Tugrul 2001; Gupta and Rao 2001).

Other kind of preoccupations presented in the previous paragraphs had lead to the notion of "reversibility" of interventions in the sense that it could be possible to go back to pre-intervention stage. Perfect reversibility is hard or even unattainable (some interventions such as cleaning being intrinsically irreversibly) and other terms such as "Removability" and "Retreatability" have been proposed (Appelbaum 1987; Muñoz-Viñas 2005). Muñoz-Viñas (2005) links the difficulties of reversing treatments to the notion of "minimum intervention".

1.3 General Strategy

Admitting that after the consideration of the issues presented above it is decided that there is need and advantage in intervening in a given objected it will be necessary to consider the action to implement. One will be begin by the discussion of the general strategy that will select the specific procedures to implement. The conservation

Table 1.1 Most frequent diagnostic methods for assessing damage in building materials

Method	Information	Effect of test/ sampling	Use
Schmidt hammer	Mechanical strength of surface	In situ non-damaging	Quick strength decay tests
Water porosity	Total porosity, hydric properties	Little sampling damage	Physical decay, structural damage
Mercury porosity	Porous system	Little sampling damage	Physical decay, structural damage
Gas absorption porosity	Porous system	Little sampling damage	Physical decay, structural damage
Mechanical strength tests	Strength and structural decay	Damaging (tests on equivalent or substituted materials)	Strength decay, structural damage
Ultrasonic pulse Velocity	Porosity, mineralogy, strength	In situ non-damaging	Physical decay
Optical Microscopy	Structure, porosity, mineral composition	Little sampling damage	Decay (weathering minerals and porosity), salts, stains
Scanning Electron Microscopy	Structure, porosity, mineral composition, geochemistry	Little sampling damage	Decay (weathering minerals and porosity), salts, stains
X-ray Diffraction	Mineral composition	Little sampling damage	Decay (weathering minerals), salts, stains
Thermogravimetric – Differential Thermal Analysis	Mineral composition	Little sampling damage	Decay (weathering minerals), salts, stains
Fourier Transform Infrared Spectroscopy	Mineral composition	In situ non-damaging	Decay (weathering minerals), salts, stains
Raman Spectroscopy	Mineral composition	In situ non-damaging	Decay (weathering minerals), salts, stains
X-Ray Fluorescence	Geochemical composition	In situ non-damaging	Decay (geochemical composition), salts, stains
ICP-MS	Geochemical composition	Little sampling damage	Decay (geochemical composition), salts, stains

strategies chosen for building materials should consider the possible historical and scientific value of some of such materials. Taking decisions should consider that written documents on the history of construction, use, restorations and reconstructions of buildings have been missed or never existed. Thus, the study of the history of some buildings including partial reconstructions and building phases can only be performed towards certain analytical methods. The strategic considerations should attempt to assess the interdependencies of the procedures considering its direct and indirect consequences as is done e.g. in environmental impact assessments (Carter 1996) and in relation to the existing problem and its possible evolution after

intervention (for an example see Moreno et al. 2006). A systematics of conservation strategies and tactics specially for concrete structures (but that can be generalised for other stony materials) can be found in Matthews and Bigaj-van Vliet (2013).

The selection of procedures to be performed could be described as an analysis in a multivariate space based on materials characteristics, pollutants, pollution sources, environmental conditions, financial considerations, object characteristics (including cultural value). The impact of the financial considerations and the object cultural value will be skimpily discussed in this review but it is easy to see that in some cases the object perceived value might imply that some options will not be financially feasible while in the other end of this component some intervention options will be ruled out from the very beginning giving the associated risk and limiting conditions could be defined as usually in risk assessment considering the hazard situation (the probability of occurrence of the negative process) and the vulnerability related to the value of the object that will be affected by the hazard should it occur (for an easily accessible general reference on urban risks see Dickson et al. 2012). Risk assessment could be ideally (but perhaps rarely in reality) be undertaken for the design of preventive measures in relation to new objects.

The materials characteristics might limit or even exclude the use of certain intervention procedures as, for example, the water-based cleaning of water-soluble objects (e.g. gypsum alabaster) or the acid cleaning of calcium carbonate rocks and there are also other aspects in the definition of the intervention procedure that would be considered in the tactical options.

The kind of decay processes that affect the cultural objects will determine firstly, in a trivial way, the general kind of procedures, to wit coatings imply cleaning of surfaces, salt contamination imply salt extraction from the porous media, erosive processes imply consolidation, etc. The characteristics of the pollutants might affect the type of intervention, e.g., the solubility of the substances on a given coating to be cleaned might exclude some cleaning agents (further on will also be considered the question of the concentration of the cleaning agents). Perhaps less trivially the higroscopicity of a given soluble salt causing decay actions affect the suitability of interventions regarding the environmental control, to wit, the creation of conditions that prevent crystallisation as in a case presented in Arnold and Zehnder (1991) where it is recommended the keeping of high relative humidity atmosphere to avoid crystallization cycles. The extension of the area affected can also have a significant relevance in terms of financial and time requirements (being that in the importance of the required time it is necessary to include the possible implications to other activities).

It is also necessary to consider the distribution of the alteration agents in the affected materials. If the alteration features are limited to the surface then it is only necessary to do a surface cleaning. However, in some cases surface features are evidence of a deeper contamination as in the case of salt efflorescences that marks salt contamination of the porous substrate. In this case it is necessary to implement processes that remove the pollutants from insider the substrate. The usefulness of a protective treatment in an already affected material must be questioned as is illustrated by the study of Polder et al. (2001) who concluded that under wet conditions,

hydrophobic treatment of concrete did not stop the corrosion by chlorides initiated before the treatment.

The characteristics of the pollution sources might be arguably seen as one of the major variables in the definition of the strategy of intervention, considering namely time and space components. In relation to time, the main question to assess is whether the observed decay is a "heritage" from the past (it corresponds to an inherited decay) or whether the sources of decay still exist in the present day and there is the hazard of recurrent occurrences. A first, perhaps evident, implication is that in the former case the conservation strategy must ponder how to treat the effects of past events while in the latter case it needs also to consider procedures to eliminate or mitigate the present day and possible future menaces. For example, in a case presented by Alves and Sequeira Braga (2000) there were observations of new salt crystallization spots and paint erosion along time after raining events. Other regular examples (see below for details) include the use of barriers for acting infiltrating or capillary rising sources of pollutants. Another example is illustrated in Fig. 1.3, where it is possible to see the presence of alkaline solutions (as indicated by a simple pH paper test) that are associated with the formation of carbonate-rich coatings. The study of cross-sections of samples of these carbonate crusts (Alves 2010) showed several layers marking successive episodes of solutions circulation and crystal formation in a kind of "urban travertine". In this case (as seen in practice) the cleaning of the carbonate crust was a temporary measure since after a while (around a couple of years) new crusts were formed again In this case (as seen in practice) the cleaning of the carbonate crust was a temporary measure since after a while (a few years) new crusts were formed again. For this example it would be necessary to eliminate the circulation of the solutions that transport the pollutants. Biological colonisation is an example of an alteration process that always looms over materials when conditions are favourable and that might be recurrent as long as those conditions exist as is illustrated in Fig. 1.4.

As part of a responsible strategic approach it is advisable to use tested procedures and to test the procedures to be used previously in similar conditions to those of the object to be treated. Ideally this will recur to the observation of previous interventions on similar materials or to do spot testing in the objects to be treated. One frequent source of preoccupation are possible colour changes but other issues

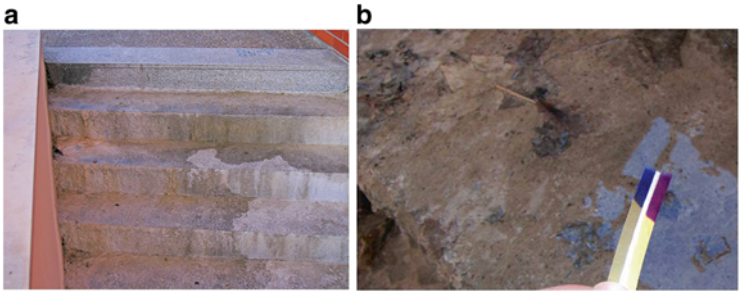

Fig. 1.3 Stair steps of *greyish* granite, as can be seen at the top of the stair, covered by carbonate rich-crusts (**a**) related to the circulation of alkaline waters (**b**)

Fig. 1.4 Comparison of stones cleaned in the first semester of 2010 showing the recurrence of biological colonisation (*greenish-greyish* stains)

can arise namely in relation to water migration. An advice from the experience of the authors is the need for a careful, detailed and very well documented description of the situation pre-intervention since afterwards there is always the risk that some aspects of the treated work are ascribed to the interventions, being a relative common case allegations of relation between cleaning and erosion, that might indeed occur. However, the information on the results of previous applications of treatments might be rarely available and the development of spot tests is generally incompatible with the time-frame of the interventions, with the exception of cleaning procedures where it is possible to see the immediate results, but the long term consequences might be a different tale, see below.

Another possibility is the realization of laboratory tests and there seems to be a significative trend for the performing of tests on small clean specimens under accelerated aging conditions, which will be perhaps understandable given the already referred difficulties and the research grant environment. Some research seems to

follow a "production-line" approach where there are some changes in the formula of the products that are applied to clean specimens and then there are performed some tests on the materials properties. However, as is discussed in the section regarding the general strategy, it is necessary to consider the circumstances of the decay processes that might affect the application of the products and the conditions that affect the treatment afterwards and care must be taken in the comparison of laboratory and field conditions (Torraca 1999). Another worrisome trend in diverse studies is the use of one specimen per treatment. This could lead to wide of the mark comparative results given the variability of stony materials (natural and manufactured), specially in the case of destructive techniques (e.g. mechanical tests, mercury intrusion porosimetry), as, for example, the curious result that the mechanical strength is higher after freeze-thaw cycles. An interesting approach in relation to stone materials is proposed in Cámara et al. (2011) consisting of testing in abandoned quarries. When treating objects composed of several components such as built structures it will be advisable to try to identify the different materials and the variations in those materials (e.g. petrographic variations in rocks) that might affect relevant properties of the materials (e.g. porous media, chemical reactivity). Another way is the testing of similar materials on structures of low historical value in the neighbourhood (Ludovico-Marques et al. 2012).

Additionally the effects of the interdependences between procedures should be discussed (for an example in relation to salt contamination see Moreno et al. 2006) as well as other issues such as the implications of the interventions on the other materials and on the users should. A complete strategic planning should include the methods for the assessment of the results and the follow up monitoring plan. This should include the evaluation of the changes in relation to the previous situation in terms of the problem and in relation to the properties of the materials.

In the following sections will be discussed details on the types of interventions that can be undertaken in relation to the alterations processes affecting the materials, considering initially interventions on the surroundings (atmosphere, terrain, structure) and afterwards treatments of the affected materials.

1.4 Dating of Materials

Usually, architects and archaeologists have used different methods to approach the age of building phase: identifying building techniques, continuousness and gaps on architectural elements, demolition tracks, etc. Such methods are stratigraphic techniques, chronotypology, mensiochronology or chemical characterization of some building materials. Stratigraphic techniques provide information on construction sequences considering the stratigraphy of façades (including horizontal and vertical stratigraphy), surface and subsurface elements (Bortolotto et al. 2005; Blanco Rotea et al. 2003). Performing building archaeological analysis requires an interdisciplinary study. Geometric surveys and topographic approaches are necessary for processing of stratigraphic data. The stratigraphic techniques combined with analysis

of materials, building techniques, observation of demolition tracks and even available (despite fragmentary) historical data can allow reconstructing the whole chronological sequence of a building. Analysis of materials usually includes physicochemical characterisation of the building materials, typically bricks, mortars and stones. Destructive techniques are avoided, although sometimes necessary. Therefore any modification introduced in a building for conservation purposes should be pondered, including substitution of materials.

Among dating methods, some of them are not destructive. Chronotypology consists in observing the different design features used in different historical periods, but requires local chronotypological catalogues or reference database (Boato and Pittaluga 2000). Mensiochronlogy is a kind of chronotypology applicable when the main dating features taken into account are the dimensional characters of the elements. The method is also based on databases and the precision of the data is strongly dependent on the geographical area and the quality of the database (Boato and Pittaluga 2000).

Most of the methods used in the chemical or physical characterization of building materials are destructive. Such study is usually performed by geochemical analysis of stones, bricks or mortars (Vendrell-Saz et al. 1996; Barba et al. 2009; Sanjurjo-Sánchez et al. 2010). It allows differentiating several historical phases in controversial cases as sometimes building materials were reused or some building parts are mimetic structures.

Absolute dating methods provide absolute ages for different geological and archaeological objects. They can also be a reliable and even definitive tool to get building chronologies. Different dating techniques have been tested and used for some building materials. However, some problems can been found with these methods: one of them is the reuse of materials in past restorations or reconstructions. When reused materials are dated, they usually provide the age of first use (or manufacture), and thus overestimated ages for some building elements. Also, deliberated or accidental man-made modifications on some materials can distort the properties of the dated materials, resulting in underestimated or overestimated ages. Frequently such modifications are due to restoration interventions in buildings.

The main absolute dating techniques used for building materials are dendrochronology for wood, radiocarbon for wood and lime mortars and luminescence dating for bricks and mortars. Dendrochronology is based on tree-ring dating of wood used as building material. The pattern of tree-rings provides the time at which rings were formed. It can be used to date timbers, commonly used as roofing materials. However, dendrochronological sequences provide ages of trees used as building materials, that is, ages probably older than the building moment. Moreover, the substitution or reuse of timbers commonly causes underestimation or overestimation of ages for building structures.

Radiocarbon dating of wood or organic materials provides absolute ages of mortars containing organic matter (e.g. charcoal, bones, vegetal fragments, organic matter). Radiocarbon has been successfully applied to date the $CaCO_3$ of the lime binder of lime mortars in some cases (Heinemeier et al. 1997), due to the slaking with water of the quicklime (calcium oxide, obtained after crushing and burning

limestone) and further reaction of atmospheric CO_2 as the mortar hardens (Heinemeier et al. 1997). Because of this, the 14C activity of the binder can be measured, the binder dated and converted to calendar years using normal calibration procedures. However, there are some well-known problems due to the mortar content in old limestone, either as lumps from incomplete conversion to calcium oxide (due to incomplete crushing and burning), leading to ages that are too old (Heinemeier et al. 1997, 2010; Nawrocka et al. 2005). In addition, the slow hardening of mortars in historic buildings and different chemical processes related with decay (e.g. dissolution, weathering) can cause changes on the lime ^{14}C content (younger carbon can be incorporated) due to dissolution and re-crystallization of $CaCO_3$, yielding dates too young (Elert et al. 2002; Hale et al. 2003). Also, any change in the composition or the decay pattern with conservative purposes can induce modifications in the CaCO3 content, hindering the possibility of dating. In such case valuable historical-scientific data is missed.

Luminescence dating has extensively been applied to date ancient bricks. Thermoluminescence dating (TL) provides the age of the last heating of a brick, commonly due to the firing of the brick in the manufacture process (Aitken 1985). Brick dating is routinely used to date construction phases but it implies some frequent problems such as reuse on later building phases (Bailiff 2008; Martini and Sibilia 2006; Blain 2010; Blain et al. 2010). The main condition for a brick to be reliable for dating is that it must remain in the place were it was firstly placed (TL dating is not possible on reused bricks). Optically Stimulated Luminescence (OSL) has been developed in the 1990s and it has been proven on bricks with success (Bailiff 2008). Even, altered bricks can be useful for dating but TL or OSL dating requires that the brick stay on the place. Removing of substitution of materials prevent the use of these techniques.

OSL has also been tested to date lime mortars with promising results, using the mortar quartz of the aggregate sand, although careful must be taken. As mortars cannot be reused they are an ideal material to date historical building phases. The use of the quartz sand requires the exposure to daylight of quartz grains during the mortar manufacture (before the mortar settling), enough time (or enough light intensity) to bleach the residual absorbed dose of ionizing radiation. This occurs during the extraction and transport of the sand, and the mortar manufacture. Also, the shielding of grains from daylight within the mortar is required. Such requirements have been shortly studied for dating purposes (Bøtter-Jensen et al. 2000; Zacharias et al. 2002; Goedicke 2003, 2011; Jain et al. 2002; Sanjurjo-Sánchez et al. 2013; Stella et al. 2013). An advantage of the OSL for dating mortars is that it can be used on any mortar containing quartz, including gypsum plasters or mud mortars (Feathers et al. 2008).

The substitution of mortars on some parts and façades of historical buildings is common in ancient and historical buildings, as well as the addition of mortars on eroded joints. Such praxis hinders the use of the mortars for dating (for any dating method) although sometimes remains of the original can remain inside the walls of interest.

1.5 Interventions in the Surroundings

In this section will be considered interventions that are done in the atmosphere, terrain or structure surrounding the material, either to alter the conditions of the alteration agents or to avoid further supply of moisture or other pollutants. The interventions on the surrounding environment or terrains or the addition of structures such as shelters has the advantage of not implying an intervention on the affected material and hence should be easier to modify or adapt according to the observed results.

One could begin by considering interventions aiming to modify the environment. This is usually done to avoid or at least mitigate the action of the alteration agents. A classic example is the study presented in Arnold and Zehnder (1991) where the study of the environmental conditions that controlled the cycles of crystallization-dissolution of soluble salts responsible for paintings erosion lead to recommendations regarding the temperature and relative humidity of the local. There has been some research attention to the behaviour of soluble salt mixtures in relation to environmental parameters in order to define the climatic conditions that promote the deteriorating effects of the soluble salts (Price 2000) that has even lead to the proposal of a software for prediction the behaviour of salt mixtures (RUNSALT (c) – http://science.sdf-eu.org/runsalt/).In that respect an example of comparison of actual observations of salt behaviour with model predictions can be found in Zehnder and Schoch (2009). Klenz Larsen (2006) defended the adoption of climatic measures based on the results of the RUNSALT software. Another approach relate to the surrounding environment will be illustrated by the proposals of Brimblecombe and Grossi (2005) for the limitation of the carbon emissions depending on the blackening thresholds of building surfaces.

In the case of objects affected by salt contamination, besides avoiding cycles of salt crystallization, and given that higher drying rates will promote salt crystallization inside the porous material and hence erosive decay (Hammecker 1995), interventions pursuing climatic control could be used to reduce drying conditions (Albero et al. 2004), protecting from the sun or the wind or promoting high moisture conditions. Laboratory experiments by Selwitz and Doehne (2002) showed that draft-free, high humidity environment promoted that most of the salt emerged as efflorescences without apparent damage to the substrate. Climate control measures could include the control of the opening of doors to mitigate drying (Albero et al. 2004). If the degrading processes are attributed to wet-drying cycles or to thermal cycles due to sun exposition then a shelter that protects the object from the rain, wind or sun can be considered (Agnew et al. 1996; Doehne et al. 2005; Hussein and El-Shishiny 2009). In the case of burying the cultural object, care must be taken in relation to material used and the environment that is created (Canti and Davis 1999; Wilson and Pollard 2002; Caple 2004; Crow 2008). Climatic control can also be used to avoid conditions that favour biological colonization (Warscheid 2000).

Of course the main question besides the climatic conditions to be pursued is how to obtain those conditions. The choice could be just to turn off the heating system in

order to keep a moist and cold atmosphere (as in Arnold and Zehnder 1991) or to promote actively favourable environmental conditions through the addition of shelters (Doehne and Price 2010). This might include the use of forestation such as in the example referred by Doehne and Price (2010) where a plantation of trees was suggested in 1996 by Thorn and Piper to protect salt-laden structures. A similar situation can be seen in the study by André et al. (2012) it was concluded that the woodland areas promote a less aggressive climatic environment (in that sense forestation can be seen as a less intrusive conservation measure).

Climatic measures will have some effect if the alteration process is affected by the climate modification. However, if the alteration processes is, e.g., related to successive infiltration cases of a salt with equilibrium relative humidity as related by Alves and Sequeira Braga (2000), environmental control could be ineffective (unless the option is a very high humidity environment near 100 %). In these cases it will necessary to fight the pollutants sources.

Interventions in the surrounding could also attempt to separate the alteration agent from the affected material. In the example presented in Fig. 1.5 it has been proposed (Alves 2009) that the profound erosive marks observed in the carbonate stones resulted from the circulation of the water (based in the field observations comparing zone with different exposition to circulating waters) and a possible intervention will be to alter the water flow in order to avoid contact with the stones. In the case of alteration procedures promote by moisture, such as biological colonization or the effects of salt laden solutions one path will to avoid moisture increase, using drainage systems or correcting architectural details that allow the infiltrating solutions. Warscheid and Braams (2000) propose that the control of biodeterioration processes should start with the adoption of measures that will prevent favourable conditions which might be achieved by the reduction of moisture, e.g., by optimizing drainage systems or correcting architectural details. A relevant example

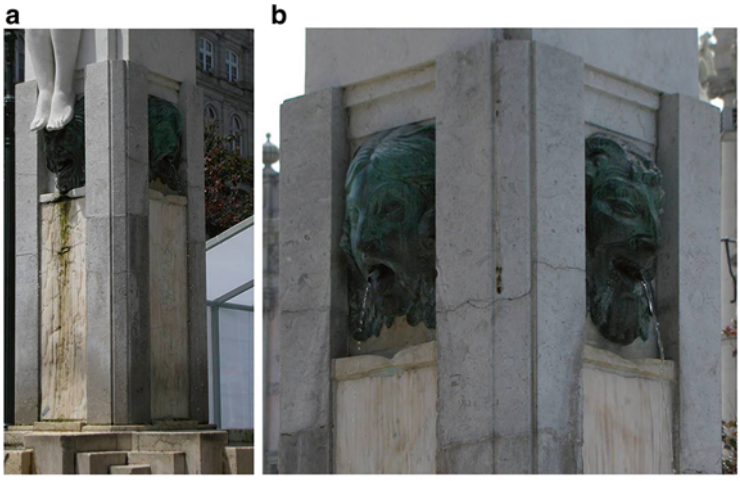

Fig. 1.5 Erosive marks on carbonate stones on a fountain related to the circulation of water (**a**, **b**)

presented by Caple (2000) refers to waterproofing works to stop water infiltration in the vault of the Sistine chapel that had lead to the development of salt efflorescences, cracking and delamination of plaster. A procedure against capillary rising pollution is the use of damp-proof courses (DPC) a several physical and chemical procedures are available (Young 2008) including the injection of water-proofing substances (chemical DPCs). In a long term test with walls built for that effect comparing silanes, silicones, silicates and plates in galvanized steel, Alfano et al. (2006) concluded that silanes presented the best result. However, the efficiency of application in existing building is troublesome (see survey by Lopez-Arce et al. 2008). Ahmad and Rahman (2010) present examples where a silicone-based chemical is injected in situ either by using gravity flow or pumps to form a barrier against dampness from moving upward in the masonry walls. Mortars properties can have an important influence on rising damp (Rirsch and Zhang 2010) and this could be useful in the case of new constructions or when rebuilding an old structure. For example, Cultrone and Sebastián (2008) concluded that the addition of an air-entraining agent to mortars joints partially hindered the capillary rise of salt solutions in masonry. One can include also here interventions for creation of structures that attempt to protect materials from aviary (pigeon) agents.

1.6 Interventions on the Materials

There are diverse possible procedures and substances that can be used according to the problem to be treated and that will be subsequently presented. The following sections consider, subsequently, the cleaning of superficial alteration products that is separated from the issue of salt remediation that can involve a different approach given that the salts can be located inside the pore space. This is followed by considerations on the consolidation of materials experiencing disruption processes that lead to erosion and the application of protective treatments against superficial-sourced agents. All these procedures involve some kind of treatment of the materials and its permanence in the object but it is also included in this section a kind of extreme intervention that consist of the replacement of the existing materials by new materials.

1.6.1 Cleaning of Surfaces

In the case of coatings and stains such as the reddish stains in Fig. 1.2, the carbonate crusts shown in Fig. 1.3, the biological colonisation of Fig. 1.4 and the very frequent darkish colorations related soiling such as shown in Fig. 1.1 or to the very frequent gypsum-rich black crusts (Fig. 1.6), the main goal is to remove exogenous material that is not part of the original surface, something that might be hard to define. As can be illustrated by the polemics in relation to oxalate coatings (Del Monte et al.

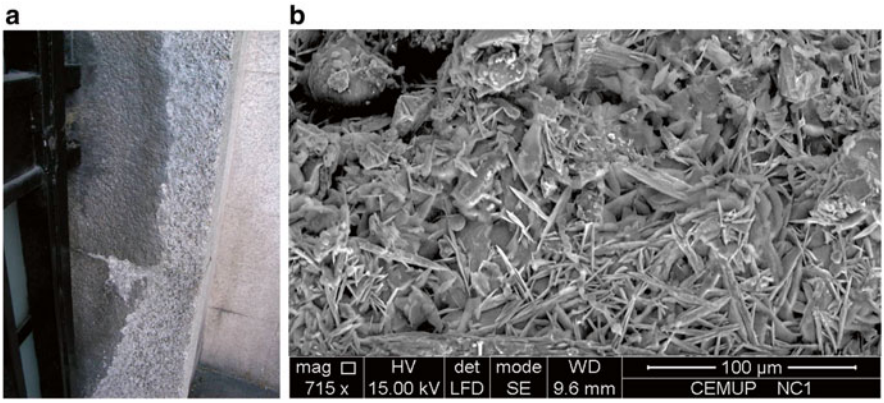

Fig. 1.6 Darkish colouration related to the presence of *black* crusts (**a**) with gypsum aggregates that contribute to fixate atmospheric particles (**b**) Scanning electron. Studies performed at CEMUP laboratory (University of Oporto, Portugal)

1987; Blázquez et al. 1997; Pavía and Caro 2006; Lazzarini et al. 2007 and see below a new proposal for protection using oxalates) there are situations where there is polemics whether a certain coating is a product of decay or addition from exogenous factors or substances intentionally added with the purpose of conservation and this must be carefully considered in the diagnostics. But even in the latter case it could be justified the removal of the coating if the diagnostics established that its presence contributed to the decay. An added problem is the question that coatings can grow on artificial man-made coatings (e.g. plasters, renders). This case is usually a result of the interaction of external pollutants (due to the high concentration of gaseous pollutants in the air near the surface). In such case, the man-made non-altered material can remain unaltered in the inner part and altered in the external part, causing aesthetical damage, but also an increasing risk of dissolution of salts from the external coating with penetration in the building exists (Sanjurjo Sánchez et al. 2008, 2009).

Besides influencing the choice of cleaning procedure, the constituents and thickness of the coating might imply different time or different intensity for the selected procedures. The general principles, procedures and questions related to cleaning of materials in the built environment can be found in reviews by Young et al. (2003), Doehne and Price (2010). Techniques involve the use of chemical products, physical removal processes and the use of biological agents. This section will be dedicated to the cleaning of surface products while the remediation of salt pollution inside porous media will be considered below.

A process that involves both chemical and physical aspects is the use of water washing, and easy and cheap technique that promotes the Softening, dissolution and transport (of loosely bound particles) and facilitates cleaning by brushing. This technique of course cannot be used with water-soluble substrates and it is necessary to be careful to adjust the used pressure in the case of poorly consolidated materials.

In a field study Fort et al. (2000) concluded by recommending the use of water cleaning highlighting that in the case of gypsum black crusts a previous treatment was required. In comparative terms, Marczak et al. (2008) found in a laboratory study that the best surface quality, corresponding to the lowest roughness, was is attained with the use of water blasting and abrasive cleaning with grinded walnut shells but, on the other hand, these methods were not so effective in cleaning as the laser cleaning (especially of irregular surfaces). De los Ríos et al. (2012) refer the greater effectiveness of water in mechanical cleaning at removing remnants of dead lichen. Moropoulou et al. (2002) with an in situ assessment of cleaning operations done by fibre optics microscopy concluded that the water spray method did not led to homogeneously cleaned surfaces and refer also that cleaning by water spray under high pressure caused detachment of grains and fissuring. In the case of whitish stains related to very soluble salts the required cleaning could be performed with simple water, but the minimal amounts should be used since the presence of the salt efflorescences indicates salt contamination and the presence of salts in the materials pore space, hence the use of water might promote further migration of salt that form new efflorescences or, even worst, crystallise inside the pore space and promote erosive decay. After cleaning of the efflorescences they might form again indicating the active migration of salts towards the surface, a situation that would require salt extraction (considered below).

A related more impacting technique (both in the coatings and in the substrates) is the use of steam jet where water is heated (see Young et al. 2003). According to Gaspar et al. (2003), in a laboratory comparison of different cleaning techniques, the use of steam cleaning for very long contact times (or when an incorrect choice of nozzle distance was used) could lead to topographical modifications and that the cleaning action could be considered as mild in the case of encrustations (which were likely to remain attached to surfaces).

A frequent physical removal treatment consists of cleaning with abrasives and diverse materials can be used in micro-particulate form such as carborundum, frozen carbon dioxide (Young et al. 2003), alumina (Moropoulou et al. 2002; Marczak et al. 2008), sand (microsand; sandblasting or siliceous grit blasting has a high time and cost efficiency but is now-a-days considered generally banned for historical works due to its strong impact on the substrates – Young et al. 2003), glass beads and grinded walnut shells (Marczak et al. 2008), that has even been featured in a NY CSI episode. There is a clear apprehension on the physical impact of abrasive procedures and there are also references to contamination of the substrate by the abrasives (Gaspar et al. 2003). Moropoulou et al. (2002) refer that microblasting did not lead to homogeneously cleaned surfaces and that it needed experience operators. But Marczak et al. (2008) considered abrasive cleaning with grinded walnut shells one of the methods that attained lowest roughness of the surfaces (but not the most effective). Perez-Monserrat et al. (2011) compared chemical methods, pressurized hot water, glass bead blasting and latex peeling, concluding that the most effective and which caused least alteration to the surface was abrasive cleaning with glass bead.

Laser cleaning is one of the cleaning techniques in fashion given its precision both in area and in depth, allowing cleaning of the surface layers without major

physical disturbances of the substrate even in the case of poorly consolidate materials and that the technological advances allow a more extensive use. There is even a regular conference on the use of Lasers in the Conservation of Artworks (LACONA) that is currently in its 9th edition. Recent reviews of its utilization can be found in Doehne and Price (2010), Siano and Salimbeni (2010), Pouli et al. (2011), Siano et al. (2011).

The conditions of laser cleaning need to be adjusted to the type of coating that is being cleaned with several examples for black crusts (Lanterna and Matteini 2000; Bromblet et al. 2003; Zafiropulos et al. 2003; Vergès-Belmin and Dignard 2003; Vergès-Belmin and Labouré 2007; Siano et al. 2008; Gioventù et al. 2011; Pouli et al. 2011) and also some examples for biological colonization (Klein et al. 2001; López et al. 2010; Siano et al. 2011). Laser cleaning has also been used for staining related to human interventions such as beeswax (Pan et al. 2011) and water repellents (Gómez-Heras et al. 2003). It can also be tailored so to preserve certain aspects of the coatings that might be considered as having cultural significance (Pouli et al. 2011).

The impact of the laser radiation can also cause damage to the substrate and usually a damage threshold (Siano et al. 2000) has to be established. The characteristics of the substrate can influence the damaging effect of the laser application such as higher decay in fine-grained than coarse-grained marble (Rodriguez-Navarro 2004) and colour alterations in granites, marbles and limestones that has been attributed to the elimination of mineral nanoparticles (Urones-Garrote et al. 2011), to alterations of iron minerals (Esbert et al. 2003; Grossi et al. 2007) and to calcite spalling depending on crystal orientation (Esbert et al. 2003).

In some cases there seems to be observed a discolouration effect resulting in a yellowish tonality after laser cleaning that has been known as "yellowing" that have raised concerns in relation to the use of laser cleaning. Yellowing has been attributed to the penetration of substances during the intervention (Siano and Salimbeni 2010; Siano et al. 2011) or to incomplete removal of coatings (Bromblet et al. 2003; Siano et al. 2008; Vergès-Belmin and Labouré 2007; Siano and Salimbeni 2010) and by the similar results obtained by other cleaning techniques as seems to be indicated by the easy removal of the yellowish tone by further cleaning and also to light scattering (Zafiropulos et al. 2003). There seems to be indications that controlling the conditions of laser application and the use of water can reduce the effects of yellowing (Vergès-Belmin and Dignard 2003; Bromblet et al. 2003; Pouli et al. 2011; Siano and Salimbeni 2010).

There are some other physical methods such as latex peeling (Perez-Monserrat et al. 2011; Young et al. 2003), nylon brushes (Moropoulou and Kefalonitou 2002), abrasive sponges, grinding disks, needle guns and other mechanical techniques (Young et al. 2003).

Several chemical products has been used for cleaning that can applied as solutions, gels or through poultices and that are reviewed by Young et al. (2003), including acids and alkaline products, chelanting agents (being the commonest Ethylenediaminetetraacetic acid-EDTA) that can be applied as solutions or through gels and poultices, non-ionic detergents, sodium citrate, sodium-hydrosulphite and ammonia salts. Other examples include hydrogen peroxide and water with glycerine

and urea (Moropoulou and Kefalonitou 2002). There are several issues (as reviewed in Young et al. 2003) to deserve attention and some examples will be referred. Perez-Monserrat et al. (2011) report the possible formation of by-products associated with chemical cleaning. According to Gaspar et al. (2003), using hydrofluoric acid caused severe staining in limestone surfaces, due to the long contact time that were necessary to treat the crusts. Moropoulou and Kefalonitou (2002) concluded that EDTA-based paste application caused a significant weight loss as well as a superficial alteration to such an extent that the method could not be proposed. Lauffenburger et al. (1992) report changes in the surface of marble tiles resulting from the application of poultices even using deionized water alone.

In the case of ammonium bicarbonate (Moropoulou et al. 2002) used to deal with gypsum-rich black crusts, transforming calcium sulphate in calcium carbonate there is production of ammonium sulphate as by product and it is hoped that this by product, being highly soluble, will be easily washed away. Gaspar et al. (2003) refer that the ammonium carbonate treatment was very effective in removing pollutants and presented a low impact on surface topography. A similar approach for gypsum coatings concerns the use of chemical additives to modify crystallization conditions by formation of insoluble salts, like the use barium hydroxide (Berlucchi et al. 2000; Lanterna and Matteini 2000) or barium aluminates (Messori et al. 2000) for treatment of calcium sulphate contamination.

The study of Moropoulou et al. (2002) found that different methods (chemical) were suitable to different situations according to the roughness of the surfaces. Ion exchange resins have also been used to remove superficial precipitations of salts, namely gypsum (Berlucchi et al. 2000; Guidetti and Uminski 2000; Casadio et al. 2000).

Another technique of delivering chemical substances and that combines chemical and physical processes is reviewed in Doehne and Price (2010) consist in the use of EDTA delivery through a latex poultice that can be peeled off afterwards that can be used for cleaning of soiling indoors but that apparently does not work in the presence of gypsum crusts and might have the risks of leaving residues.

Nanotechnology, that will figure further on in relation to other procedures, has also arrived to cleaning and an example of using carbon nanomaterials is presented in Valentini et al. (2012). Another possible set of cleaning procedures is related to the use of biological agents. At least as early as 1992 it was proposed by Gauri et al. (1992) that bacteria could convert calcium sulphate in calcium carbonate. Other examples of the use of microorganism for cleaning are those of Ranalli et al. (1997) and Polo et al. (2010) relative to the removal of gypsum-rich crusts, and there has been indication that biocleaning could be more efficient in the removal of black crusts than other treatments (Cappitelli et al. 2007; Gioventù et al. 2011). Microorganisms have also been applied for removal of nitrates (Ranalli et al. 1996; Alfano et al. 2011), human applied products (Lustrato et al. 2012) and even biological colonisation (Graef et al. 2005). In these biocleaning procedures could be included the use of enzymatic products that has been used for removal of biofilms (Valentini et al. 2010, 2012; Geweely and Afifi 2011), black crusts (Valentini et al. 2012) and human products (Ranalli et al. 2005). In a comparative study, Ranalli

et al. (2005) concluded in favour of the use of bacteria in relation to enzymes due to the wider versatility of the former.

Besides the immediate consequences that were being referred above it is also necessary to consider long term consequences. Young and Urquhart (1998) refer the possibility of some cleaning chemicals (phosphate-rich) act as nutrients sources and promote algal growth. Young et al. (2003) presents data from a report of Young et al. to Historic Scotland in 2002 reporting that surface covered by decay after 10 years was generally higher for cleaned sandstone than for uncleaned sandstone being that abrasive cleaning was worst for some sandstone types and chemical cleaning was worst for others. According to the same study there was no difference in terms of decay area in the case of granites.

1.6.2 Salt Contamination Remediation

Soluble salts are one of the main decay agents (see Goudie and Viles 1997) and their treatment might be one of the most troublesome situations. In the case where the salts form efflorescences the substrate generally remains in good condition and if that is the only part affected the best strategy might be a non-intervention policy or the planning of procedures that favour the surface crystallization of the soluble salts, namely promoting a favourable moisture balance according to the model of Hammecker (1995), involving basically less drying conditions or higher capillary moisture migration. Salt efflorescences can cause aesthetic unpleasant stains and in that regard they were considered in the last section. In the cases where salt crystallization leads to erosive processes, salt crystallization needs to be avoided. Caple (2000) proposes for chemical pollutants in general two possible approaches: removal and deactivation.

The removal of salt contamination (desalination) is frequently tried either by immersion in distilled water (Vieweger et al. 1996) or by using poultices, which could be combined with impregnation of the stone elements with distilled water by irrigation (Siedel 1996; Vergès-Belmin 1996). Pel et al. (2010) present a review of the mechanisms involve in desalination by poulticing, pointing out that desalination requires the dissolution of the soluble salts in the pores (and for that it is necessary the supply of a solvent) and its migration towards the poultices. Comparing diffusion and advection, Pel et al. (2010) concluded that desalination by diffusion could attain 100 % salt removal but required extremely long times during which the substrate must be kept water-saturated, while desalination by advection will be faster but limited to the surface portions of the substrate and that salts pockets might remain in the micropores. Voronina et al. (2013) discuss the possible existence and effects of osmotic pressure during poulticing. Several materials can be used for poulticing (Vergès-Belmin and Siedel 2005) including namely clays and cellulose compounds. There are several questions to be considered in the application of poultices including the efficiency of salt extraction (DeWitte et al. 1996; Rager et al. 1996; Siedel 1996; Verges-Belmin 1996; Vergès-Belmin et al. 2011; Pel et al. 2010),

which could be affected by the chemical characteristics of salt pollution (Twilley and Leavengood 2000) with possible selective extraction effects and related changes in the chemistry of the salt solution, also with mobilization and concentration of soluble salts to different positions (Siedel 1996; Simon et al. 1996; Zehnder 1996; Vergès-Belmin et al. 2011). The pore size characteristics of the treated substrate could also have an impact in the effect of the poultices application (Lubelli and Hees 2010; Vergès-Belmin et al. 2011; Pel et al. 2010). Comparing desalination Pel et al. (2010) present theoretical predictions of salt reduction but there are few studies regarding the effectiveness of salt reduction in situ mostly due to the difficulty of assessing the results that required invasive destructive sampling (an example can be seen in Ahmad and Rahman 2010). The preparation of the poultices can also have relevance for the desalination results (Bourgès and Vergès-Belmin 2010).

A similar desalination methodology is the covering of materials with renderings that favour the extraction of soluble salts extraction from the materials (Fassina et al. 2002; Setina et al. 2009; Grave et al. 2011) and that could work as a "sacrificial" layer that is renovated periodically. Comparing laboratory experiments using plaster on different substrates, Petković et al. (2006) concluded that results depended on the relations between the poresize of the plaster in relation to substrate with salt accumulation in the plaster when it had smaller pores than the substrate and salt accumulation in the substrate when the plaster had larger pores. This could help to explain the field observations of Klenz Larsen (2006) that found degraded bricks under an original lime plaster. Lubelli et al. (2006) review and discuss field examples where the application of plasters especially prepared for salt-laden substrates show damage after a few years. A variant will be the substitution of old mortar joints with new mortars that could contribute to absorb the soluble salts (Jeanneau 1996).

Desalination can also be attempted with cycles of washing-and-vacuuming, where it is expected that water spraying will penetrate in the porous material and dissolve the salts, being the salty solution extracted afterwards by the vacuuming (Dragovich and Egan 2011).

Other techniques for desalination include the application of electrical currents (Fauck et al. 1996; Mouton 1996; Palem 1996; Castellote et al. 2000; Fajardo et al. 2006; Ottosen and Rörig-Dalgaard 2008). This approach has been frequently referred for the treatment of concrete attempting to remove chlorides that can promote corrosion of metallic bars due to carbonation of the cementitious paste (Orellan et al. 2004; Sánchez and Alonso 2011). Palem (1996) refers that electrical techniques showed higher efficacy in the removal of salt contamination than the application of poultices. Results can be dependent on the type of salts that are present (Feijoo et al. 2012; Paz-García et al. 2013). Differences in the pore media as they affect solutions migration can also have impact in the desalination results (Feijoo et al. 2012). There have also been laboratory experiments of the application of electrokinetic desalination with clay poultices at the electrodes (Ottosen and Christensen 2012; Rörig-Dalgaard 2013).

Another approach to salt contamination remediation is the use of chemical additives that act as crystallisation modifiers of soluble salts with most of the studies consisting in laboratory tests with sodium chloride and sulphate. Chemical additives

seem to be able to change the moisture balance dynamics under drying and promote the formation of efflorescences (salt crystallisation at the surface) hence with lesser physical damage (Selwitz and Doehne 2002; Rodriguez-Navarro et al. 2002; Lubelli and Hees 2007; Rivas et al. 2010). On the other hand, in a field study Ruiz-Agudo et al. (2010) concluded that the chemical additive used promoted subflorescences (crystallisation inside the pore space) without increasing the damage to the support. Selwitz and Doehne (2002) refer that potassium ferrocyanide was useful for preventing NaCl crystallisation but it did not contribute for enhancing salt dissolution, hence, not being useful for desalination. Rodriguez-Navarro et al. (2002) propose that the application of poultices with ferrocyanides could be useful for desalination. The effect of the chemical additives seems to be affected by the characteristics of the substrate (Lubelli and Hees 2007; Rivas et al. 2010). Lubelli and Hees (2007) indicate that different results could be obtained according to the way the additives were applied with spraying enhancing damage development and these authors suggest that better results could be obtained with an application method that favour slow drying of the substrate and transport of a large amount of salt to the surface.

Biocleaning has also been attempted for remediation of salt contamination. As referred above, biological agents have been used for the conversion of soluble salts and this has been attempted for removal of nitrates (Wilimzig 1996).

Achieving 0 % salt levels might be an utopic and unnecessary and a general question regarding desalination regards the salt level at which desalination can be considered as effective (what Pel et al. 2010 designated as effectiveness of the desalination processes) in the sense that the attained salt levels ceases to cause problems, and one can found recommendations such as "significant" decrease in electrical conductivity (Vieweger et al. 1996) or salt levels of 1 % (Mouton 1996). According to Pinna et al. (2011) the project SCOT proposed threshold values for salt contents in the case of application of protective treatments. One should consider, however, the representativeness of damage thresholds defined in laboratory tests, referring for example the lower destructive efficiency of halite and gypsum in laboratory tests when compared with observations in field studies (Goudie and Viles 1997).

1.6.3 Consolidation

When the alteration processes compromise the physical integrity of materials promoting ongoing erosion (see illustration in Fig. 1.7) that might endanger the aesthetic value of material it might be deemed necessary to restore the physical strength of the material by consolidation. For that end can be used materials that penetrate in the porous media and strengthen them. Doehne and Price (2010) present a general review of different substances that have been applied as consolidants, application techniques and problems.

The dominant products are organic-based products, mostly silanes but also epoxy resins (the review by Doehne and Price 2010 defend that they have been successful in some instances), acrylics, isocyanates, polyurethanes, polyureas and cyclododec-

Fig. 1.7 Physical disruption processes leading to erosion of material in limestone statuary (exemplifying a situation in which consolidation procedures could be considered to avoid the progression of erosion and further loss of detail)

ane. Other products that have been used as consolidant include lime and barium hydroxide. Given the several issues that have been found this is an environmental chemistry line where there is always interest for innovation and there are attempts to develop new products such as the dispersion of lime on alcohol (tested in laboratory and on wall paintings by Giorgi et al. 2000) calcium alkoxides (Favaro et al. 2008) as a way to promote formation of calcium carbonate that so far, to our knowledge, has been tested in laboratory experiments with a porous glass frit. There are proposals for consolidation by promoting apatite formation inside carbonate rocks (Yang et al. 2011). Penetration being generally an important issue in the case of consolidation and one of the alternatives that has been proposed is impregnation by a monomer and "in situ" copolymerization afterwards (Vicini et al. 2002; Cardiano et al. 2002). Of course there have been research studies in the possible application of nanotechnology and some examples include using nanoparticles of calcium and magnesium hydroxide and carbonate (Baglioni and Giorgi 2006; López-Arce et al. 2011) and addition of nanoparticles of silica to polymeric products (Mosquera et al. 2003; Kim et al. 2009; Ferri et al. 2011; Tulliani et al. 2011; Xu et al. 2012) and silica and other oxides (Miliani et al. 2007). There is also a significative research in consolidation promote by micro-organisms by the production of calcium carbonate

in limestones and concrete (Rodriguez-Navarro et al. 2003; Fernandes 2006; Jimenez-Lopez et al. 2007; De Muynck et al. 2010, 2011; Peihao and Wenjun 2011; Rodriguez-Navarro et al. 2012) including the healing of fractures in concrete (Van Tittelboom et al. 2010; Wiktor and Jonkers 2011; Jonkers 2011; Li and Jin 2012).

The main problems for which the diagnostics can provide support are related to the characteristics of the materials (not only its porosity but also the pore size distribution) and the characteristics of the contamination that can affect the substances used for the consolidation.

The question of the penetration of the consolidants is a critical one, namely the relation of this depth to the distribution of the pollutants. The penetration of the consolidants will depend on the characteristics of the consolidant substances (namely viscosity and surface tension) but also the characteristics of the pore space, namely porosity (that would define the available void space that can be filled with the consolidant substance) and the poresize distribution. The pore size distribution controls the migration of the substances on the pore space and hence the absorption of the consolidant in the material (Clifton 1980; Cnudde et al. 2004; Ferreira Pinto and Delgado Rodrigues 2008; Maravelaki-Kalaitzaki et al. 2008; López-Arce et al. 2010) and it has also impact on bioconsolidation treatments (De Muynck et al. 2011). Consolidants can also be used for cracks (Muñoz-Viñas 2005) and in it is also necessary to register the variation of the characteristics of the cracks that would influence the penetration of the consolidant. As noted by Muñoz-Viñas (2005), the required characteristics of the consolidants might vary from one area to another.

The application of consolidants can experience different results depending on characteristics of the application environment such as relative humidity (López-Arce et al. (2011)) and of the substrate such as pore space (Maravelaki-Kalaitzaki et al. 2008), and the presence of moisture and pollutants (López-Arce et al. 2010). The mineralogy of the substrate has also been referred as having a possible impact on the consolidation processes in the case of biomineralization with calcitic substrates offer a higher affinity for bacterial attachment than silicate substrates (Rodriguez-Navarro et al. 2012).

Consolidation procedures face special problems associated with the treatment of salt contaminated materials.. For example, the field experiments of Ashurst of desalination and consolidation (by application of silanes) of columns referred by Watt and Colston (2000) suggest that a consolidation was not successful in very salt-contaminated. Comparing three situations (untreated, treated by poulticing and treated by poulticing and consolidation), Watt and Colston (2000) found that there were “appreciable accumulations of dust ” around the bases of both the treated columns, even if “ marginally less ” than in the untreated column. Thickett et al. (2000) presents observations of ongoing decay after consolidation of salt-contaminated stones. One possible path for this situation could be the improvement of the penetration of the consolidants but given that this would be, at best some cm, and in the case of the creation of an interface promoting salt crystallisation this would imply the loss of greater portions. Hammecker (1995) proposed a model in relation to the pore size distribution that favours the migration and surface crystallization of the soluble

salts. Another proposal is presented in Selwitz and Doehne (2002) for the use of crystallisation inhibitors mixed with concentrated solutions of calcium carbonate and calcium hydroxide for stone consolidation.

Another possible preoccupation in relation to the use of consolidating substances is its possible contribution to the promotion of biological colonization (Warscheid and Braams 2000).

1.6.4 Protective Treatments Against Pollutants

In the case that it is assumed that the materials will be under attack by pollutants it makes sense to applied treatments against these agents in order to avoid the development of the alteration processes. The interventions considered here attempt to protect materials from the ingress of pollutants such as salt pollutants that can promote deleterious processes in the materials such as crystallisation inside pores (another example will be the corrosion of metallic components of concrete).

One general concern in terms of testing of surface treatments (and consolidants) is to keep some significative measure of water-vapour permeability, letting the stone "breathe". While this will be critical when keeping the substrate dry is the goal, it will be not the solution in the case where the substrate is affected by salt pollution, since letting the stone breathe will only mean let the stone dry and hence promote salt crystallization. In the model of Hammecker (1995), based on the established models of porous media drying, a slow drying rate will lead to a lower critical water saturation content, hence a lower amount of salt crystallization inside the pore space; ideally a 0 % critical water saturation will imply that all the salt crystallizes at the surface promoting generally harmless or low harming efflorescences.

A sharp distinction needs to be done between the application of water repellents in the case of atmospheric-sourced pollutants and pollution migration by capillary rising or infiltration since in this last case the pollutants would have invaded globally the porous media.

Zhao et al. 2010a concluded that the application of a coating procedure performed better than penetrant (pore-blocking) treatments since the evaluation was interested in reducing chloride ingress.

Some early protective treatments are reported as having contributed to accelerated degradation by promoting salt crystallization (Pan et al. 2011).

Several polymeric substances can be employed for water-repellence mostly consisting of silane, siloxane, silicon and acrylic products (Mayer 1998; Alessandrini et al. 2000; Alvarez de Buergo Ballester and Fort González 2001; Tsakalof et al. 2007; Toniolo et al. 2002) that can be delivered with organic solvents or in water emulsions with the later being more environmental friendly (Mayer 1998; MacMullen et al. 2012). However in a comparative study Alvarez de Buergo Ballester and Fort González (2001) conclude that products with organic-solvent were more effective than hydric-solvent ones. The polymeric substances can be

mixed with inorganic compounds such as lime (Alvarez de Buergo Ballester and Fort González 2001). Research in fluorinated products seem to indicate that they could be promising products in this respect (Alessandrini et al. 2000; Castelvetro et al. 2002; Tsakalof et al. 2007; Youssef et al. 2008). Other products that have been studied include epoxy-silica polymers (Cardiano et al. 2005; Cardiano 2008), polyurethanes (Vipulanandan and Liu 2005; Doehne and Price 2010; Zhao et al. 2010b), microcrystalline waxes and sodium silicate (Alvarez de Buergo Ballester and Fort González 2001) and Doehne and Price (2010) review the application of colloidal silica as protective agent. Water-repellent procedures could be also be relevant to avoid the continuation of concrete reinforcement corrosion by avoiding moisture migration (Redaelli and Bertolini 2011).

Treatments for salt remediation can also have some protective effect as referred by Ruiz-Agudo et al. (2010) that propose that products for crystallisation modification could have some protective effect against chemical weathering on calcite surface.

Curiously, one of the recent proposals for protective treatment for surface protection has been to promote the formation of calcium oxalate coatings (Doherty et al. 2007; Pinna et al. 2011) that has been assessed on the field and can also apparently have some consolidating effect (Doherty et al. 2007). One can also consider here, in a similar vein, treatments used to stop carbonation of concrete such as realkalinization (Yeih and Chang 2005; Redaelli and Bertolini 2011).

Among protective treatments one can also consider products applied to prevent commonplace human vandal actions such as graffiti (some examples can be seen in Carmona-Quiroga et al. 2010; Licchelli et al. 2011; García and Malaga 2012).

Biomineralisation has also being experimented as protective treatment through the development of layers of calcium carbonate (Dick et al. 2006; De Muynck et al. 2008; Chunxiang et al. 2009; Achal et al. 2010; Anne et al. 2010).

Also among the protective treatments could include the application of biocides that, besides being used in the removal of biological colonisation, aim to avoid the future occurrences of these processes. Diverse products has been used as biocides Warscheid and Braams (2000) including quaternary ammonia compounds and tin organic compounds (Warscheid and Braams 2000), triazines (Gladis et al. 2010), isothiazolinones and carbamates (Barrionuevo and Gaylarde 2011; de los Ríos et al. 2012), benzimidazoles (Barrionuevo and Gaylarde 2011), *p*-hydroxybenzoic acid ethyl ester (PHB, Aseptine A) in combination with silicone resins proposed in 1992 (according to Warscheid and Braams 2000) as a more environmentally-friendly product, compounds with metals such as cupric ethanolamine or cupric sulphate (Warscheid and Braams 2000). Warscheid and Braams (2000) referring that tin organic compounds are not absorb onto material surfaces and are effective over a longer period of time. Young et al. (2008) present a polyphasic approach for prevention of biological colonization, in which besides biocides are used with cell permeabilisers, polysaccharide and pigment inhibitors and a photodynamic treatment. Biocides can also be mixed with consolidants and water-repellents to promote protection against future biological growth (Ditaranto et al. 2010; Barrionuevo and Gaylarde 2011; Khamova et al. 2012; Pinna et al. 2012). There are also examples of

the use of biological such as plant extracts (Afifi 2012). One could as well include among these protective treatments the use of products in woods against insects (Ghosh et al. 2012).

Some examples of different effects depending on the kind of biological colonization (Bastian et al. 2009; Fonseca et al. 2010; Barrionuevo and Gaylarde 2011; de los Ríos et al. 2012) and there could be microbial selection by the use of biocides – see e.g. Bastian et al. (2009).

The use of nanotechnology has also attempted for protection against biological agents and other pollutants with examples of nano-particulates of SiO_2 (Manoudis et al. 2009; Matziaris et al. 2011), organomodified montmorillonite (D'Arienzo et al. 2008; Scarfato et al. 2012), titanium dioxide and zinc oxide (Fonseca et al. 2010; Gladis et al. 2010; Maury and De Belie 2010; MacMullen et al. 2012; La Russa et al. 2012; Quagliarini et al. 2012), copper (Ditaranto et al. 2010) or diamonds (Khamova et al. 2012).

The application of the protective treatment can have different results according to the substrate with references to better results in the more porous materials (Cardiano et al. 2005) while Alvarez de Buergo Ballester and Fort González (2001) propose that the determining factor affecting penetration of the treatment is the porous system (and not the porosity value). De los Ríos et al. (2012) highlights the interest of biocides penetrating on deeper portions of the treated material. In relation to biological colonization, the preventive action of the treatments could be affected by the persistence of microorganisms after treatment (Cámara et al. 2011; de los Ríos et al. 2012) and it is also necessary to consider that some treatments might became nutrient sources for biological recolonization (Warscheid and Braams 2000).

1.6.5 Materials Replacement

When it is considered that the material attained an unacceptable state of degradation from the visual, functional or even security perspective (e.g. an element might have elements in risk of falling over people) the option of substituting the old piece by a new could be considered. This solution is historically exemplified in the Bible when alterations features (in the context representing a plague of leprosy) such "with hollow strakes, greenish or reddish, which in sight are lower than the wall" and if at the seventh day of the visit of the priest "if the plague be spread in the walls of the house" then the stones shall be shall disposed "into an unclean place" and "take other stones, and put them in the place of those stones; and he shall take other morter, and shall plaister the house" (Leviticus 03:014:033-045, King James Bible, http://www.gutenberg.org/files/7999/7999-h/contents.htm). The possible objections related to the preservation of the cultural significance will not be considered here. The substitution of materials will depend strongly on the construction characteristics: Peris Mora (2007) highlights the importance of modular constructions for

easing the repairing action of materials or parts of works without affecting the basic structure of the architectural work.

In replacing a material with a new one it is expected generally (but see the option of a sacrificial layer below) that the new material will behave better than the previous one and hence it should important to select more durable options as well as aesthetic compatible ones. In the case of replacement of natural stone higher durability could be attained through selection of more durable stones, through the previous treatment of the same stone before application or even the use of artificial similar material (Attewell and Taylor 1990; Stefanidou 2010). Recommendations in relation to stone selection were already present in Vitruvius (English translation by Morris Hicky Morgan according to Project Gutenberg file in http://www.gutenberg.org/etext/20239) who refers soft stones that "can be easily worked" but when used in the seacoast "the salt eats away and dissolves them", while "travertine and all stone of that class can stand injury" resulting from weather elements and using field observations in some monuments that "look as fresh as if they were only just finished", as evidence of durability. Jackson et al. (2005) present a study showing the choices of the Romans in terms of stone selection according to use. It is interesting to register that Attewell and Taylor (1990) concluded that the original sandstone was, according to the performed test, more durable than the sandstones that were used as replacement. Cardell et al. (2003) refer that their field observations the development of erosive features in freshly quarried stones in the course of 15 years when place in a marine environment. However, one can refer that, in a strategic perspective, the remediation of the origin of the alteration processes allow the use of the similar stone. Replacement can also be considered in the case of concrete structures by removal of portions affected by carbonation and substitution of new alkaline material (Redaelli and Bertolini 2011).

The treatments can also be applied in stones before application. However, it would be advisable to protect all the faces of the stones and not only the exposed surface, since pollutants can migrate from the inside due to infiltration. In the example presented in Fig. 1.8 the moisture stains observed could have been promoted by the impermeabilization of the exposed surface (moisture having migrated by infiltration). The problems referred above in relation to penetration and performance in already contaminated stones can also be posed for protective treatments and the possible unsuitability of water-repellents for salt affected substrates has been highlighted in the review of Doehne and Price (2010). The notion of a damage threshold in relation to the salt levels has been advanced (Pinna et al. 2011) and the same issues previously raised in relation to safe thresholds could be considered here.

An important research area in this regard concerns the development of new cementicious mixtures. Some examples are the preparation of mortars with bioadditives to improve the strength of cement mortar (Ghosh et al. 2005), with crystallisation inhibitors to improve salt weathering resistance (Lubelli et al. 2010) and with water repellents in order to avoid water migration such as sodium carboxymethylcellulose (Lu and Zhou 2000), a combination of polyacrylamide as a principal raw material and FDN-2 (naphthalene sulfonic acid formaldehyde condensation product) as an additive (Lu et al. 2004), oligomeric organo-siloxane (Maravelaki-Kalaitzaki

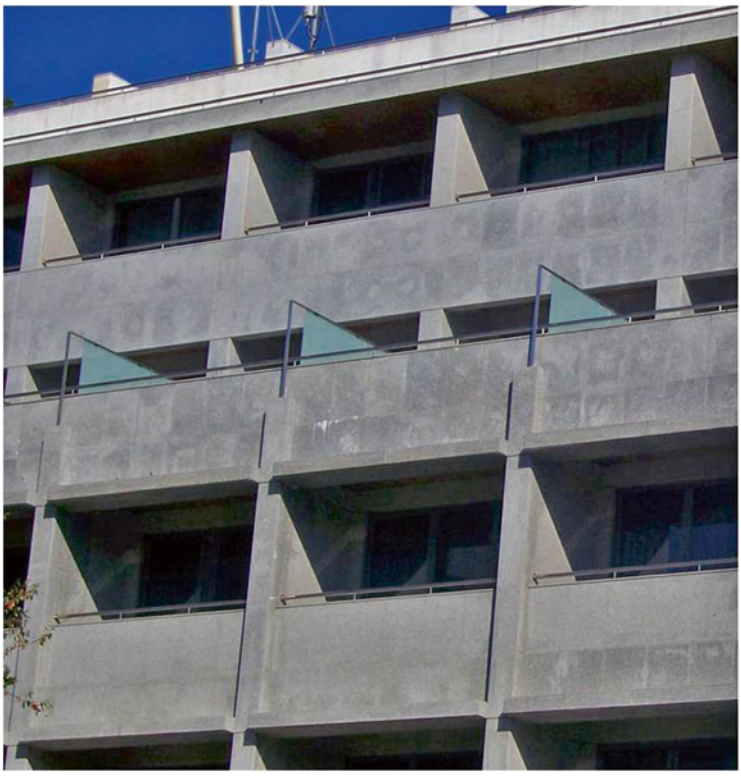

Fig. 1.8 Moisture stains in granite slabs of a façade

2007), sodium oleate and calcium stearate (Izaguirre et al. 2009). The pre-treatment to reduce water migration has also been essayed in bricks (Matziaris et al. 2011). A recent review of mortars specifically for restoration based on the experience on previous interventions can be found in Torney (2012).

Another common perspective in terms of materials replacement is the possible adoption of a sacrificial layer of insignificant value that could be replaced periodically and that will be affected by the alteration processes protecting the substrate (Donovan 2011; Duran et al. 2012) and promote the attainment of moisture equilibrium (Torney 2012). Such option allows the elimination of such layer when altered if causes aesthetical of other kind of damage without causing important damages in the building materials. However, it might be pertinent to refer the study of Cultrone and Sebastián (2008) of salt weathering of masonry models that conclude that the mortars did not act as sacrificial layer also to remember the results discussed above of the laboratory experiments of Petković et al. (2006) and the field observations of Klenz Larsen (2006).

Besides the durability issues, it is advisable also to consider the compatibility of the replacement materials in terms of appearances and possible impacts on the other materials. There is some generally some concern that there must be a visual match between the old materials and new ones (Nijland et al. 2010) and colour thresholds can be

Fig. 1.9 Stains related to alteration of petrographic heterogeneity on granite pavement slabs (the distribution of the stain crossing the slabs edges shows that it develop after emplacement of the stones)

defined for aesthetically compatible replacement of stones on monuments (Concha-Lozano et al. 2012a). However, one can find in the intervention works the opinion that it is desirable to mark the materials that are used in the restoration in a clear distinction from the historical ones. It is advisable to evaluate the compatibility of the physical properties of the new material in relation to the older materials of the structure and this has been much discussed in the case of old mortar formulations (lime mortars) in comparison with hydraulic Portland mortars (Kerstin et al. 2002; Beck and Al-Mukhtar 2010; Nijland et al. 2010). The materials that are to be newly introduced could also be a source of pollutants There is the classical reference of Vitruvius that mortars prepared with sand beach could cause the formation of efflorescences. Other materials could have soluble salts (see Sanjurjo-Sánchez and Alves 2012 for a review) and there are tests to assess the possible salt contaminating potential of materials (e.g. Chin et al. 2010; Sanders et al. 2010). In Fig. 1.9 it is shown an example of reactive heterogeneities of a granite stones in the urban environment causing stains that affect the stone and other nearby (it can be seen that the stain cross several slabs, a proof that the stains development occurred after the slabs emplacement).

1.7 Sustainability Issues

Wood (2003) defines sustainable care as caring for any or all buildings in a sustainable manner including issues such as:

– Low maintenance
– High durability

– Design attuned to use of building
– Adaptability
– Reusability
– Use of appropriate technology

The study of alteration processes of materials in the built environment can be an important source of information on the durability of materials, as recognized early by Vitruvius that recommended that stones be left on the place where they were to be applied to assess its behaviour on those conditions. However, whether the information from the experience in relation to alteration will be considered over other (e.g. aesthetic, economic) considerations is a hope that should be taken with an important portion of sceptical salt as could perhaps be illustrated with the use of very porous soft limestones several centuries after Vitruvius (as seen in Fig. 1.7) and its use still today (for a case study concerning a recent building and the litigious contention afterwards see Hartog and McKenzie 2004). Previous constructions are also natural experiments regarding the implications of architectural design options in relation to alteration processes. The study of alteration process could also contribute to assess the alteration processes that will affect replacement stone and therefore provide indications on the need to take additional measures to remove these sources. These informations could contribute to the utopical aim of the maintenance free object (Wood 2003). Of course in terms of sustainability one needs to consider, following the lines of environmental impact assessment, primary, secondary, tertiary, etc. impacts (Carter 1996).

In general there are found few considerations on the possible impact of the interventions for the conservation of materials in the built environment. In terms of sustainability implications of the intervention procedures one can consider:

– Consumption of natural resources and its associated impacts
– Emissions
– Residues
– Positive effects of the interventions

Interventions required generally consumption of natural resources directly (water, geological substances) or indirectly (energy, manufactured materials). In relation to the direct impacts, it deserves special attention the case of the materials substitution since it requires the preparation of new material. In that sense the use of less durable materials could be considered a less sustainable option given that it will imply substitution and new consumption in some cases of scarce or valuable (for other reasons materials). Al-Agha (2006) presents an example where there it is proposed the adoption of protective measures in relation to the aggressive environment (Gaza Strip) in order to minimize the consumption of sand from geological formations that constitute the recharge area of the regional aquifers. Another example is the study of Dias et al. 2008 assessing the possible use of offshore sands in concrete in order to preserve river sands. But it is necessary to ponder the different direct and indirect impacts. For example Wood (2003) refers that high

durability materials might be non-renewable resources or that require a high amount of energy for extraction or one whose extraction has a high environmental impact. Peris Mora (2007) highlights that ephemeral materials could be use in transcendent construction providing that maintenance needs are taking in consideration in the project. Among other impacts that can be associated with the preparation of the new materials one can highlight the case of mortars replacement where besides the impacts related to the extraction of the raw materials it is necessary to consider the resulting CO_2 emissions. It is also necessary to include in these considerations the long-term effectiveness of the interventions, since interventions that require repeatedly application (as illustrated in Fig. 1.4) imply periodic consumption of resources but this must be compared with the possible effects of more durable interventions.

A growing concern in relation to the intervention procedures is related to the destiny of the cleaned substances and the residues of the cleaning material (Doehne and Price 2010). Several intervention procedures can cause emissions of unwelcomed substances as is the case of cleaning operations such as residual waters with the removed products and substances used for cleaning, gaseous emissions from the products, abrasive particles, and particles resulting from physical removal procedures. Two illustrative examples are the possible health impact of the solvents used in the preparation of polymeric products has promote the use of emulsions with water (Mayer 1998; Doehne and Price 2010; MacMullen et al. 2012) and the concern in relation to health hazards in relation to laser cleaning (Kusch et al. 2003). Some substances can cause emissions after the application and a case for which diverse research studies can be found concerns the leaching of the toxic substances used in biocides (Warscheid and Braams 2000; Bester and Lamani 2010; Burkhardt et al. 2012; Coutu et al. 2012). One of the advantages referred by Young et al. (2008) in relation to the BIODAM approach discussed above was the reduction of the concentration of biocide required. It has been argued (e.g. Graef et al. 2005; Geweely and Afifi 2011) that biocleaning can be considered more environmentally friendly than chemical cleaning (this of course means that it is considered that there are not biorisks). This could be even more worrisome in the case of approaches such as the proposed in Cámara et al. (2011) of testing biocides in abandoned quarries since these could be located in the natural environment.

Several of the procedures can left residues that need to be treated considering in general the questions presented above. It is also necessary to ponder on the treatment of the residues resulting from materials replacement. As can be seen in older monuments stones can have a great potential for reusability and recyclability. In the case of affected stones it could be pondered the removal of the altered stone its laboratory treatment to remediate the present problem and the application of protective treatments to avoid the redevelopment of new problems.

In the light of the previous comments one can see that the popular option of the sacrificial layer could imply several sustainability issues when it implies periodic production of residues and consumption of further material with the impact associated with its production (e.g. CO_2 emissions in the case of lime).

In terms of sustainability one can also consider the possible positive effects of some interventions, namely does that attempt to reduce the moisture of materials given that it has been proposed that the protective treatment of walls can improve thermal isolation (MacMullen et al. 2012) and hence reduce energy consumption.

Another perspective to be considered in terms of sustainability in relation to the treated object is presented in Muñoz-Viñas (2005), where the intervention should consider the meaning of the object after the intervention for the future generations, i.e. to take in consideration how they will look upon the intervention.

1.8 Final Considerations

The review presented here has attempted to show the diversity of procedures that can be used to address the alteration processes affecting materials in the built environment as well as the main elated issues.

The assessment of the short-term effectiveness of the interventions concerning the treatment of the existing materials depends on the intrinsic aspects of the interventions. In the case of outside-sourced processes the measures pass through surface intervention. A more complex situation corresponds to the situation where the alteration agents had pervaded the inside of the porous materials as it is necessary to proceed to its removal. There is a trivial assessment for consolidation in whether materials regain consistency. In the case of the cleaning operations there would be an immediate result that can be evaluate qualitatively or quantitatively using, e.g., chromatic measurements. For protection against water (water-repellence) there could be measurements of permeability and it will be necessary to expect that the reduction in water permeability will be enough to avoid penetration in the material in a significant way (i.e. such that will not promote alteration). In a similar light, in desalination procedures what can be assessed is the salt content while the real goal is to stop the alteration effects of the salts. Potentially deactivation by chemical additives cannot be assessed in the short-term and neither can be biocide treatments. Material substitution can be considered a "chirurgical" operation where the short term result is trivially effective (in the sense that the new materials do not have the alteration features of the replaced ones).

While one can consider that regarding cleaning there is a presently a good knowledge state in relation to short-term effectiveness since the removal of any surface coating or stain is essentially a matter of time, financial and technical adjustment (as well as previous evaluation of the impact on the substrate), results concerning desalination, consolidation and protection are still very much open to discussion (which will of course be related to the issues mentioned in the previous paragraph). Current hot research topics concern nanotechnology and biotechnology in several of the treatments of materials but we did not find any application of nanotechnology to desalination procedures. One can speculate, however, that the use of nanomaterials could eventually develop pore space modifications that could promote superficial

salt crystallisation and contribute to desalination according to the existing models (Hammecker 1995; Pel et al. 2010).

Long-term effectiveness will be frequently more dependent on the global intervention strategy and the conditions under which the alteration processes develop considering spatial and temporal components and there is danger of recurrence unless measures are undertaken towards the inactivation of the alteration processes including the isolation from the sources of the alteration agents. As was signalled in the previous paragraphs, diverse intervention process had the risk of contributing to long-term alteration processes. In that regard an illustrative example is presented in Fig. 1.10 where it is suspected that a previous protection treatment could had contribute to further decay given that the alteration agents (soluble salts) were inside the porous materials and a surface treatment could favour its crystallisation in the pore space.

Another final reflection concerns the, perhaps expectable, trend in research for laboratory controlled essays of small clean specimens but the consequence is that there is poor information on the behaviour of the product on real-scale objects under field conditions, a feature that adds to the previous one on the need for long-term assessments. Additionally, a trend that must not be favoured, at least in stony materials, is the comparison of procedures based on one specimen by treatment given the variations of properties on these materials.

However, it is not foreseeable that these trends would change much in the present short-term research-grant environment. In our opinion the solution for a significative turning point in this respect could only happen through legislative measures that mandate a detailed record of the interventions (substances use, contact times, local of application) that should be made public available after a certain moratorium period. Until that there is a massive waste of potential study of real-scale experimentation corresponding to the diverse interventions around the world.

Fig. 1.10 Suspected application of protective treatment that is thought could have contributed to further erosive processes (resulting from salt crystallisation at interface with substrate)

In terms of sustainability, there are several direct and indirect impacts to be considered and it is advisable to use the tools classically available in environmental impact assessment. In this regard were highlighted the concern in relation to the possible leaching of products used in biocide treatment and the problems associated with one of the popular choices in terms of interventions which is the use of a sacrificial layer that would imply the impacts associated with the preparation of the replacement mortars (such as periodic consumption of resources and CO_2 emissions). The sustainability analyses needs to consider the balance between periodicity of applications and the effects of more permanent solutions. In general there is scarce reflection on the sustainability implications of these procedures for conservation of materials.

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Chapter 2
Nanotechnology in Water Treatment

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Abstract Industrialization and excessive use of pesticides for boosting agricultural production has adversely affected the ecosystem, thus polluting natural water reserves. Remediation of contaminated water has been an area of concern with numerous techniques being applied to improve the quality of naturally available water to the level suitable for human consumption. Most of these methods however generate byproducts that are sometimes toxic. Heterogenous photocatalysis using metal oxide nanostructures for water purification is an attractive option because no harmful byproducts are created. A discussion on possible methods to engineer metal oxides for visible light photocatalysis is included to highlight the use of solar energy for water purification. Multifunctional photocatalytic membranes are considered advantageous over freely suspended nanoparticles due to the ease of its removal from the purified water. An overview of water remediation techniques is presented highlighting innovations through nanotechnology for possible addressing of problems associated with current techniques.

Keywords Water treatment • Photocatalysis • Desalinisation • Capacitive deionization • Water filtration • Heavy metal • Zeolite • Alginate • Charcoal • Activated carbon • Chlorination • UV • Ozonation • Nanofiltration

2.1 Introduction

The rapid pace of population growth has resulted in severe environmental contamination in air, water and soil. The world population is estimated to increase from the current figure of about 6.5 billion to an alarming nine billion by the middle of the twenty-first century (Wiesner et al. 2006). Attempts to deal with the issues related to population has adversely affected our ecosystem leading to health problems resulting from environmental pollution. Large-scale deforestation to accommodate the burgeoning population has resulted in dearth of agricultural land, bringing down the agricultural yield. The production level of food grains has been showing a consistent downward trend over the last decade. To boost agricultural production, farmers started relying on the extensive use of chemical pesticides which has resulted in the contamination of ground water (Baruah and Dutta 2009a; Sugunan and Dutta 2008). At the same time, industrial growth is leading to increased consumption of the available natural resources and contamination of water resources.

Water is an essential requirement for life and its availability in pure form is important for different life sustaining activities like human consumption, agriculture, to name a few. Human activities have affected nature's very own water recycling and purification mechanism and have totally disturbed the balance between the consumption and natural purification processes resulting in a shortage of drinkable water. Almost all of the natural sources of drinking water have been found to be contaminated with a wide variety of toxic materials and pathogenic microorganisms (Baruah and Dutta 2009a). Almost 12 million people die every year from

water related diseases, as per a World Health Organization (WHO) report (http://www.who.int/infectious-disease-report/pages/textonly.html). Impure water is the cause of about 90 % of all diseases occurring in developing countries. There are over four billion reported cases of diseases resulting from consumption of impure water globally. Pure low carbon content water through novel methods is of utmost necessity for the healthy existence of human being as well as the eco system.

Drinking water is currently being disinfected through the use of physical as well as chemical techniques. Conventional water disinfection processes has certain restrictions resulting in apprehensions about their applications at a mass scale. Disinfection using UV light is effective against most of the harmful microorganisms but is incapable of inactivating certain disease causing microbes like *Cryptosporidium, Giardia lamblia*, etc. Chlorination is generally accepted as an effectual water disinfection technique as it is robust, cheap and has prolonged post treatment outcome. Chlorine readily reacts with natural organic materials present in water thereby producing halogenated Trihalomethanes (THMs) like chloroform, bromoform, and bromodichloromethane and Haloacetic acids (HAA), which are carcinogenic in nature (Baruah and Dutta 2009a; http://www.who.int/infectious-disease-report/pages/textonly.html). Chlorine, by itself, is a noxious gas and even very low concentrations (0.1 % in air by volume) can be fatal to humans. Ozonation is another alternative disinfection technique that effectively removes many of the disease causing microbial contaminants from water. However, this technique has a setback as ozone is an unstable gas and therefore requires on site production. Another drawback is that the effect of ozonation is not enduring and it is not possible to guarantee water safety to the end users.

Heterogeneous photocatalysis shows promise as a water purification technique as compared to other conventional methods as this process does not generate harmful byproducts (Baruah and Dutta 2009a; Sugunan and Dutta 2008). It can break up complex long chained organic molecules, which are mostly toxic, into benign fragments as well as immobilize microbial cells by fracturing the cell walls. Nanotechnology is a disruptive technology that can make an impact in the area of water purification as nanostructures offer large surface to volume ratios ideal for surface reactions (Hornyak et al. 2008). The possibility of preparing photocatalytic membranes by growing semiconducting nanostructures on conventional membranes makes this technique even more attractive (Baruah et al. 2008, 2010). Replacement of fossil fuel with renewable energy like solar energy can present a cleaner and more efficient way of water purification, even in isolated rural sites. Point of use water purification systems can be designed using antimicrobial nanomaterials like silver (Ag), zinc oxide (ZnO), etc (Li et al. 2008). The concept of decentralized water treatment systems is being taken into consideration following reports of deterioration of water quality as it flows through old distribution networks coupled with the escalating transportation costs. Membranes are increasingly being used in the fields of drinking water and waste water treatment (Marcucci et al. 2003). Active functional membranes incorporated with antimicrobial or photocatalytic nanomaterials will be capable of accomplishing multiple treatment targets in a single course of action, at the same time minimizing fouling (Li et al. 2008). Loss of nanomaterials into water during treatment is a matter of concern for human health and the ecosys-

tem and therefore proper attachment of nanomaterials to supports is of importance (Hirano 2009; Wiesner et al. 2006).

2.2 Sources of Natural Water for Human Consumption

Surface Water: Naturally available fresh water on the earth's surface in rivers, lakes or wetlands is called surface water. Natural replenishment of surface water takes place through precipitation. Surface water is depleted through natural processes like evaporation, discharge to seas and oceans and sub surface seepage. Human activities can have severe detrimental effects on the quality and availability of surface water. The upper limit of human consumption is restricted by the rate of precipitation within a watershed. Pulling in water from other watersheds through canals or pipelines can increase natural surface water in a particular watershed. Surface water is more prone to pollution from various human actions and needs extensive treatments to make it suitable for human consumption.

Ground Water: Fresh water stored in the pores in soil and rocks is commonly known as ground water. Water in aquifers flowing under the water table can also be considered as ground water. Often referred to as sub surface water, ground water is comparable to water on the earth's surface considering the source (input), the storage and the exit (consumption and evaporation). However, due to slow rate of exit, the storage of water is much more in sub surface water as compared to surface water. This disparity makes it convenient for humans to unsustainably use subsurface water for extended periods without adverse consequences. Seepage from surface water is the natural source for ground water with the natural outputs as springs and seepage into the seas and oceans. With rapid evaporation of the surface water, salts can seep into the ground water thereby making it saline. Consumption of ground water near coastal areas can also lead to reverse flow of water from the sea, resulting in increase in salinity. Rapid use of chemicals as pesticides has resulted in the pollution of the ground water making it unusable for human consumption.

2.2.1 Lakes and Reservoirs

Lakes and reservoirs are considered as standing waters and vary from gravel pits, ponds and canals to big natural lakes. In lakes (either natural or man made), water levels do not fluctuate much. Reservoirs are man-made open water storage facilities mostly serving as public utility water supply sources. Reservoirs are also used to store water for irrigation along with river corridors (http://www.euwfd.com/html/lakes_and_reservoirs.html). The quality of water in lakes and reservoirs depends partly on the amount being drained in and partly on the speed at which the water moves in (http://www.unep.or.jp/ietc/publications/short_series/lakereservoirs-1/5.

asp). Considerable changes in the characteristics of river water take place owing to a decrease in velocity resulting in sedimentation. A change in the structure of the biological specimens could also be observed with ideal conditions for the growth of phytoplankton and the onset of hypertrophication (ecosystem reaction to the accumulation of substances like nitrates and phosphates, mainly through fertilizers or sewage) (Schindler and Vallentyne 2004). Reservoirs usually receive more particulate materials as compared to lakes, but flushing rate is also higher due to greater water inflows. So, pollution in reservoirs is less as compared to lakes (http://www.unep.or.jp/ietc/publications/short_series/lakereservoirs-1/5.asp). The quality of lakes and reservoirs on the basis of transparency, nutrients present, concentration of dissolved minerals, oxygen concentration and pH. Toxic chemical wastes released by industries into lakes and reservoirs, apart from killing aquatic organisms and adversely affecting irrigated crops, can be a source of water borne diseases resulting from uncontrolled growth of bacteria, viruses, etc. Water purification using novel techniques is therefore of utmost necessity.

2.2.2 Rivers and Canals

A river is actually a natural fresh watercourse, which normally terminates in an ocean, a sea, lakes or other rivers (http://www.merriam-webster.com/dictionary/river). In rare occasions, a river completely dries up before reaching another water body or it just flows into the ground. Rivers are part of the hydrological cycle. Water gets collected in rivers from precipitation, surface run off as well as from ground water. Numerous other names are used for smaller rivers like stream, rivulet, creek, brook, tributary, etc. No official definitions exist for generic terms, such as river, with reference to geographic features (Impacts Consortium 2004), although in certain places a stream may be defined by its size. Sometimes a river is said to be larger than a creek (http://www.observatorynano.eu/project/document/2013/), however, this is not always valid (http://geonames.usgs.gov/domestic/faqs.htm). The water flowing in a river is usually restricted to a conduit composed of a streambed between banks. A wider floodplain exists for larger rivers normally shaped by floodwaters over flowing the conduit. Rivers and lakes have been used as a source of water for human consumption by water authorities. The water however needs to go through numerous treatments to take them to a level that is safe for human consumption.

2.2.3 Rain Water Harvesting

Rainwater harvesting is the collection and storing of rainwater for human use like drinking, water for irrigation and livestock, etc. Rainwater harvesting can guarantee a self-sufficient water supply during water limitations. Water from rainwater harvesting is usually of acceptable quality for household use. This process

produces long term beneficial effects through reduction of storm water runoff and is also comparatively economical (White 2009). Rainwater harvesting systems are simple, both in installation and operation with very low running costs. In the urban scenario, rainwater harvesting is significant as excess runoff during storms can lead to release of raw sewage creating problems to treatment plants. A process of pulling down rainwater deep inside the ground through shafts is used; however this practice normally carries surface pollutants into ground water. Rainwater can get polluted as it passes through the atmosphere contaminated with volatile organic compounds released through industrial activities. Rainwater can also be a storehouse of disease causing microbial contaminants and as such proper treatment need to be carried out to make rainwater suitable for human consumption (White 2009). Rainwater harvested from roofs of houses can contain animal and bird excretions, mosses and lichens, particulate contaminants like dust and airborne urban pollutants, pesticides, inorganic ions like Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, SO_4^{2-} etc. and dissolved gases like NO_x, SO_x and CO_2. Alarming levels of pesticides have been recorded in rainwater in parts of Europe (Impacts Consortium 2004). The maximum concentrations of contaminants were observed in the first rain following a dry spell. A commonly practiced method of purifying rainwater is through the use of parabolic solar cookers prior to consumption (Frasier and Lloyd 1983).

2.2.4 Sea Water Desalination

Desalination or desalinization refers to artificial processes that remove some amount of salt and other minerals from saline water (mostly sea water) (http://dictionary.reference.com/browse/desalination) for human use. The most commonly used desalination processes are distillation (Alkhudhiri et al. 2012) and reverse osmosis (Misdan et al. 2012; Singh et al. 2012). Another recent development in the field of water desalination is the brackish water desalination using Capacitive Deionization (CDI) technique. This method relies on electrochemical control to remove ions from saline water upon by electrostatically attracting them towards electrically charged electrodes (Alkhudhiri et al. 2012). The process of converting saline water to fresh water is an expensive process and only a minute fraction of the total human consumption is met through desalination. However, for arid countries, where there is scarcity of fresh water, desalination is looked upon as an attractive option. The most extensive use of desalination is in the Persian Gulf (Henthorne 2009). Desalination has high-end applications in ocean liners and submarines. Like recycled waste water, this is another example of water source not relying on rainfall. Large-scale desalination involves enormous energy requirements apart from specialized, expensive infrastructure, making it a more expensive process than harnessing rivers or ground water (Fiscetti 2007).

As per a study by the International Desalination Association, 14,451 desalination plants were in operation worldwide in 2009, producing 59.9 million m^3 of water per

day (Henthorne 2009). The volume increased to 68 million m^3 in 2010 and is estimated to be 120 million m^3 by 2020. The Middle Eastern countries and North Africa alone contributes to more than 50 % of the world's desalination capacity (http://www.h2ome.net/en/2012/02/opportunities-aplenty/). Saudi Arabia takes the top position in the world in desalinated water production with 17 % of the global capacity, closely followed by the United Arab Emirates at 14 %. The world's largest desalination plant is the Jebel Ali Desalination Plant in the United Arab Emirates (http://www.h2ome.net/en/2012/02/opportunities-aplenty/). Muscat in the Sultanate of Oman, has of late commissioned a 76,000 m^3/day Membrane Bioreactor plant that treats water to low levels of suspended particles, transparency and biological oxygen demand (BOD) and for irrigational and industrial uses (http://www.h2ome.net/en/2012/02/opportunities-aplenty/).

2.3 Water Remediation Processes

2.3.1 Filtration

Filtration is the process of removal of solids from water by allowing the water to pass through a medium that blocks the particulate contaminants. The porous medium can be a porous physical barrier, a chemical or a biological process (Baker and Taras 1981). This process is capable of removing mainly macroscopic particles. Microscopic particles and microbial specimens cannot be efficiently filtered out using standard filtration methods. However, innovative filtration technologies like microfiltration, ultrafiltration, nanofiltration, etc. have emerged to handle these issues. In water treatment, filtration is also used to depict some biological processes in which undesirable constituents are removed by absorption into a biological film present in the filter medium.

2.3.1.1 Sand Filtration

Sand has been in use as a filtration medium for ages. There are three main types: gravity sand filters, upflow sand filters and slow sand filters all of which are widely used. Slow sand filters can produce good quality water even without the use of chemical flocculants, which the other two types do require (Rushton et al. 2000). The particulates are either captured on a permeable membrane (surface filters) or within a porous body material (depth filters). Further, arrangements leading to solid-liquid separation like hydrocyclones, centrifuges, settling tanks and self cleaning filters are widely used (Rushton et al. 2000). Sand bed filters use granular loose media and are typically used to separate minute amounts (less than 10 parts per million) of fine solids with physical dimensions less than 100 μm (μm) and are often employed in seawater and waste water treatments (Coulson et al. 1991).

2.3.1.2 Microfiltration

Microfiltration is a membrane filtration process where the pores in the membrane range in size between 0.1 and 10 μm. Microfiltration does not require a pressurized system, unlike reverse osmosis and nanofiltration, where pressure is applied on water to force it to move against the density gradient (http://www.water.siemens.com/en/products/membrane_filtration_separation/microfiltration_membrane_systems/). Microfiltration can be carried out in different configurations; the filters can be totally submerged in water or can be used in a pressurized vessel. They are available in various configurations like flat sheets, spiral wound, tubular, hollow fibers, to name a few (http://www.water.siemens.com/en/products/membrane_filtration_separation/microfiltration_membrane_systems/). These porous filters allow water, monovalent ionic species like Na^+, Cl^-, etc., colloidal particles, dissolved organic matter and viral colonies through them but blocks particulate contaminants, sediment, algae or large bacteria though microfiltration membranes are capable of removing all types of bacteria from water. A fraction of viruses also get immobilized in the process, even though viruses are smaller than the pores of a micro filtration membrane but viruses get adsorbed on to the bacterial film (http://www.lenntech.com/microfiltration-and-ultrafiltration.htm). Microfiltration is also used for pretreatment of water for reverse osmosis and nanofiltration.

2.3.1.3 Ultrafiltration

Ultrafiltration is a type of membrane filtration which utilizes hydrostatic pressure to force the water through a semipermeable membrane (http://www.lenntech.com/microfiltration-and-ultrafiltration.htm; http://www.seccua.de/download/press/2010_04_WCP_Seccua_4922.pdf). Low molecular weight suspended solutes pass through the membrane along with water while the high molecular weight solutes and suspended particles are blocked. With a much higher reliability compared to other processes coupled with the ability to remove nanometric sized particles, ultrafiltration has of late become the most sought after treatment technology in the field of drinking water treatment (http://www.seccua.de/download/press/2010_04_WCP_Seccua_4922.pdf). Fundamentally there is not much difference between ultrafiltration and nanofiltration (to be discussed later in the chapter) with the difference only in the size of the particles and molecules that is retained. Another attractive feature of ultrafiltration is it can do away with the need for clarifiers, especially for wastewater treatment. A properly designed ultrafiltration system employ submergible, back flushable and air scoured ultrafiltration/microfiltration membranes that offers improved performance in water purification (http://www.lenntech.com/microfiltration-and-ultrafiltration.htm).

Table 2.1 Maximum contaminant level (MCL) of heavy metals in surface water and their toxicities

Heavy metals	Toxicities	Maximum effluent discharge standards (mg/L)		
		[a]EPA (2004)	[b]PCD (2004)	[c]EPD (2004)
		USA	Thailand	Hong Kong
Chromium (IV)	Headache, vomiting, diarrhea, nausea	0.05	0.25	0.05–0.10
Chromium (III)		0.10	0.75	
Zinc (II)	Lethargy, depression, neurologic signs	1.00	5.00	0.60–1.00
Copper (II)	Liver damage, insomnia, Wilson disease	0.25	2.00	0.05–0.1
Cadmium (II)	Renal disorder, kidney damage	0.01	0.03	0.001–0.05
Nickel (II)	Nausea, chronic asthma, coughing, dermatitis	0.20	1.00	0.10–0.20

[a]EPA (Environmental Protection Agency), USA
[b]PCD (Pollution Control Department), Thailand
[c]EPD (Environmental Protection Department), Hong Kong

2.3.2 Heavy Metal Adsorption

Industries are constantly dumping heavy metal ions into lakes, rivers and reservoirs, thereby polluting them. Heavy metals are broadly defined as materials whose density is above 5 g/cm^3 (Barakat 2011). Common heavy metals present in aqueous streams include chromium, mercury, lead and cadmium (Bailey et al. 1999). Table 2.1 (Kurniawan et al. 2006a) shown below describes the maximum contaminant level of heavy metals in surface water and their toxicities.

Heavy metal removal from water is crucial as these metals are non-biodegradable and can cause various health risks to both human and animal life (Argun and Dursun 2008; Babel and Kurniawan 2003). A variety of techniques can be applied to remove these metals from water which include chemical precipitation (usually used for inorganic effluents and not much effective for trace amount of solvents) (Bose et al. 2002; Wang et al. 2004), coagulation and flocculation (higher cost and lower efficiency) (Kurniawan et al. 2006b; Semerjian and Ayoub 2003; Ayoub et al. 2001), reverse osmosis (effective but expensive) (Ozaki et al. 2002; Qdais and Moussa 2004; Eddy 2004), electrodialysis (effective for concentrated solution only) (Eddy 2004; Bhattacharyya and Gupta 2008), ion exchange (sophisticated and expensive) (Bose et al. 2002; Wang et al. 2004) and adsorption and filtration (efficient and cost effective) (Bose et al. 2002; Kurniawan et al. 2006b).

Heavy metal adsorption is a well-known process that utilizes mass transfer technique to remove adsorbates by depositing them on the surface of adsorbent. It can be applied at lower concentrations for both continuous and batch operations. Ease of access and cost effectiveness are other advantages of this technique

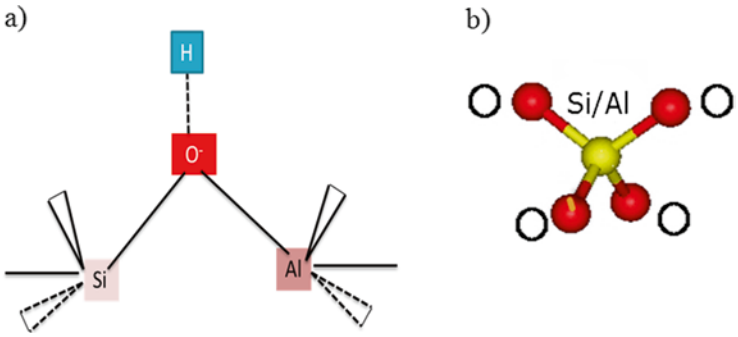

Fig. 2.1 (**a**) Chemical structure of zeolite. (**b**) Primary binding unit of Zeolite

(Bhattacharyya and Gupta 2008; Mohanty et al. 2006). Two kinds of forces may act during adsorption namely, physisorption and chemisorption (Rouquerol 1999). Physisorption is normally a weak force of attraction between molecules; it is non-specific and molecules can move freely from one surface to another (Sawyer et al. 1994). This weak force of attraction can be dipole-dipole attraction and hydrogen bonding (KeleSolgu 2007). Chemisorption is based on very strong electrostatic forces and a chemical bond forms between adsorbent and adsorbate, which is normally covalent or electrostatic bonding.

Many naturally occurring materials and industrial residue from different processes can be suitable adsorbents due to their cost effectiveness (Bailey et al. 1999). A low cost adsorbent is normally the material which is abundantly available, no further or very little purification is required or is a waste or by product of some industrial process (Bailey et al. 1999). A lot of research is going on to study the properties of these adsorbents. Some common adsorbents used in water treatment to adsorb heavy metal ions are zeolites, alginates and activated charcoal.

2.3.2.1 Zeolites

Zeolites are naturally occurring materials which can also be produced synthetically (Ming and Dixon 1987). There are more than 40 natural zeolite occurring species with clinoptilolite being the most abundantly available. Zeolite have three dimensional crystal structure containing negative charge which is produced by replacement of Al^{3+} ions with Si^{4+} ions in a tetrahedron structure (Bailey et al. 1999) as shown in Fig. 2.1 below.

Enhanced adsorption capacities of zeolites are due to their higher ion exchange capabilities. Zeolite structure consists of large channels and cavities where the ion exchange takes place and ion exchange selectivity of zeolite results in charge separation. Figure 2.2 shown below describes the selectivity of zeolite for both reactant and products depending on channel.

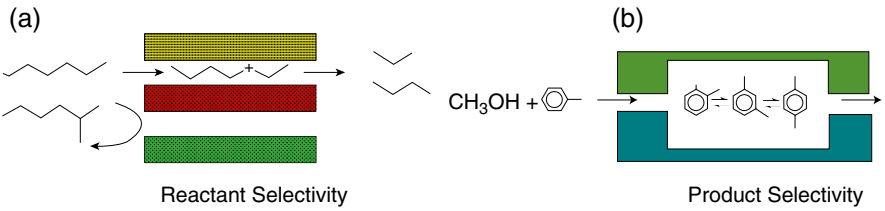

Fig. 2.2 Reactant and product selectivity of zeolite through channels (Stöcker 2005)

Table 2.2 Adsorption aptitudes of some naturally arising zeolites (mg/g)

Type of zeolite	Lead (Pb)	Chromium (III)	Chromium (IV)	Mercury (Hg)	Cadmium
CETYL-modified zeolite			0.65		
EHDDMA-modified zeolite			0.42		
Zeolite	155.4	26.0		150.4	84.3

Potassium, sodium, calcium and other positively charged ions present in the channel are exchangeable and get replaced by heavy metal ions. Heavy metals present in wastewater (chromium, mercury, lead and cadmium) are effectively adsorbed on zeolites. Clinoptilolite is a widely used zeolite for wastewater treatment due to its higher selectivity and ion exchange capability to remove heavy metal ions including strontium and cesium (Grant et al. 1987). Vaca Mier et al. (2001) studied the selectivity of zeolite for the removal of various heavy metals and observed that zeolites show higher selectivity for lead ions followed by cadmium, copper and cobalt. Table 2.2 (Bailey et al. 1999) shows the some of the reported adsorption capacities of zeolites.

It can be seen from Table 2.2 that zeolite has higher adsorption capacity for heavy metal ions then amended composite materials of zeolites. Maliou et al. (1992) also showed that zeolite has better selectivity for lead and chromium which are the most toxic metals present in waste water streams. Erdem et al. (2004) used natural zeolite as adsorbent to remove heavy metals from waste water and authors report that Zeolite has highest selectivity for cobalt as compared to copper, zinc and manganese. Adsorption in aqueous solution decreases with increase in metal ion concentration because less thermodynamic energy favorable places are vacant for absorption. The authors attributed this to crystal structure of zeolite as during ion exchange process ions have to move through crystal lattice and pores and channels of zeolite. Diffusion is faster in pores then in channels. Hui et al (2005) used zeolite synthesized from fly ash to study the adsorption of different heavy metals including Cr^{3+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Cu^{2+}. The Authors observed that adsorption process has high pH dependency and copper showed maximum affinity to zeolites. The adsorption efficiency was reported to increase with increasing pH and with mass of zeolites.

Dwairi and Al-Rawajfeh (2012) studied the cobalt and nickel removal from aqueous stream using zeolites. These studies by the authors showed that Zeolite are

useful materials to remove heavy metal from waste water. Chojnacki et al. (2004) studied the mercury removal from waste water using natural zeolites and explored the reaction mechanism for both ion exchange and adsorptions. Zeolite showed good cation exchange properties to be used as a good adsorbent. It was reported that zeolite can remove mercury as well as other heavy metals. The most important property for an adsorbent to adsorb material from aqueous stream is its hydrophobicity (Blocki 1993). Zeolites due to its Si/Al ratio showed good hydrophobic properties to adsorb heavy metals selectively from waste stream. Large specific surface area along with higher ion exchange capabilities make zeolite as an ideal adsorbent (Ahmaruzzaman 2008).

Guan et al. (2009) removed trivalent chromium ions from waste water using synthetic zeolites. It was observed that zeolite can selectively adsorb chromium even in the presence of other alkali and alkaline earth metal cations including sodium and potassium. Xie et al. (2012) stated that if zeolites are modified with chitosan by forming a monolayer of chitosan on zeolite surface their adsorption capacities increases and they can preferentially adsorb phosphorous along with many other heavy metals from waste water. It was attributed to the increased porous structure of zeolites and non-zeolite fraction of different oxides. Monolayer of chitosan acted as binding material for adsorption of different heavy metal ions.

Han et al. (2009) coated zeolite with iron oxide to form a composite adsorbent for removal of copper from waste water streams. This study showed that composite zeolites are efficient materials for waste water removal and also desorption of copper is easy. The column where reaction takes place can be reused. Zeolites have certain limitations as well as due to the high sludge and waste production; heavy metals are not fully removed and considerably higher energy are required leading to high operational costs (Aklil et al. 2004; Cochrane et al. 2006) that is leading scientists to study other alternative adsorbents such as bio adsorbents etc.

2.3.2.2 Alginates

Bio-adsorbents are becoming increasingly popular for heavy metal removal from aqueous streams because of their effectiveness on treating dilute waste water containing heavy metals. Presence of many functional sites unlike ion exchange resins (having only one functional site) and their relatively lower costs have led to widespread use of these materials (Fu and Wang 2011; Wang and Chen 2009). Bio sorbents can be derived from many sources such as from algal biomass, non-living sources including shrimp, squid, crab etc. and microbial mass such as yeast, fungi and bacteria (Apiratikul and Pavasant 2008). Extensive research has been carried out by many researchers to study these bio-sorbents. Alginate is a polysaccharide that can be easily obtained from brown seaweeds. Alginates are biodegradable materials and good biocompatibility makes them useful material for various applications (Ahmaruzzaman 2008). Brown seaweed Algae abundantly available in the world has attracted the attention of many researchers due to its renewable natural biomass source and easy availability, low cost, higher metal affinity for adsorption

and comparable removal efficiency (Fu and Wang 2011; Apiratikul and Pavasant 2008). Vijaya et al. (2008) stated that at trivial conditions physiochemical properties e.g. porosity, degradability of alginates can be altered very easily, which can improve efficiency of heavy metal removal.

Alginates are biopolymers and these have higher binding affinity for heavy metals making them suitable material for higher metal uploads (Volesky 2003). Bailey et al. (1999) stated that alginates contain calcium ions which are replaced with heavy metals to form metal alginates by adsorption process. Cochrane et al. (2006) compared alginate (*Fucus vesiculosus)* and other commercially available adsorbents to study their selectivity and efficiency for copper removal from waste water. Microalgae showed more than 95 % removal efficiency for copper which is comparable to other low cost adsorbents and due to their low cost can prove to be a good alternative. Araújo and Teixeira (1997) removed trivalent chromium using calcium alginate, where they studied the relationship between amount of chromium adsorbed and calcium replaced and the effect of initial concentration of alginate on adsorption processes. Park and Chae (2004) used different types of alginates for removal of heavy metal from waste water. Alginate gel, alginate beads and alginate capsules were used as adsorbent in the study. Alginate capsule showed higher adsorption of lead. Alginates capsule has high binding capacity to lead because of presence of xanthan gum in alginate solution. Aderhold et al. (1996) studied the ability of heavy metal removal of different alginates and also the effect of presence of more than one heavy metal on removal efficiency. Holan et al. (1993) stated that bio sorbents can swell and disintegrate which can restrict their use for heavy metal removal. Thus alginate composites can be a good solution. Alginate composite beads can be formed to remove heavy metals from waste water streams. Composite beads of sodium, chitosan can be synthesized to increase adsorption capacity. Different composites remove different heavy metals depending upon their selectivity and more work needs to be carried out to find a clear correlation (Wang and Chen 2009). Adsorption capacities of different adsorbent was compared by Choi et al. (2009) modeling using Langmuir adsorption isothermal model (Eq. 2.1) and three stage kinetic model as shown in Eqs. (2.2) and (2.3) below.

$$S = \alpha\beta C \,/1 + \alpha \mathrm{C} \tag{2.1}$$

where S and C are adsorbed and aqueous concentrations and α and β are coefficients related with binding energy.

$$\frac{C(t)}{C(o)} = \frac{(1-\xi 1)(1-\xi 1-\beta\xi 2)}{(1-\xi 1-\beta\xi 2\ \ EXP[-\gamma t])} \tag{2.2}$$

$$\xi 1 = \frac{MS1(\infty)}{VCo}; \xi 2 = \frac{MS2(\infty)}{VCo}; \gamma = \frac{(1-\xi 1-\beta\xi 2)\alpha}{\beta\xi 2} \tag{2.3}$$

Table 2.3 Langmuir adsorption isotherm parameter for different heavy metal ions

Alginates gel				Activated carbon		
	Maximum absorption capacity qm (mg/g)	Affinity coefficient b (L/mg)	Coefficient of correlation r^2	Maximum absorption capacity qm(mg/g)	Affinity coefficient b(L/mg)	Coefficient of correlation r^2
Pb^{2+}	526	0.0349	0.968	149	0.0121	0.976
Cu^{2+}	208	0.0361	0.973	74	0.0346	0.969
Cd^{2+}	159	0.0257	0.993	<5	–	–
Zn^{2+}	77	0.0149	0.990	12	0.0535	0.972
Mn^{2+}	53	0.0193	0.985	<5	–	–

In Eq. (2.2),*C(t)* and *C(o)* is the aqueous concentrations at time t and initial stage, V is the solution volume, *M* is the mass of adsorbent, S1 (∞) and S2 (∞) are the adsorbed concentrations for both the types of kinetic models respectively. Mass transfer rate from solution in both the types is shown by α and b is defined as a limiting factor for S2(1) with its range $0 < \beta < 1$ (Choi et al. 2009).

Park et al. (2007) calculated Langmuir adsorption isotherms parameters for various heavy metals comparing both alginates and activated carbon shown in Table 2.3 (Park et al. 2007).

Table 2.3 shows that alginates have higher adsorption capacity for various heavy metals than activated carbon.

2.3.2.3 Activated Charcoal/Activated Carbon

Water purification using charcoal is a very old practice. Use of charcoal to treat wastewater dates back centuries when charcoal was primary material to remove odour and taste from waste water (Gupta and Suhas 2009). Water was kept in vessels open to sunlight and then filtered through charcoal. Modern use of charcoal is after oxidizing it, often called activated charcoal or activated carbon. Charcoal is oxidized at high temperature using different activation agents to increase surface area and porosity, which are essential for adsorption of heavy metals. Charcoal can be activated by both physio thermal and chemical methods. In physio thermal process all the volatile matter removed by heating excessively at 500–600 °C and gasification is done at milder conditions to develop pores in the crystal structure. Activated carbon is a basic form of graphite and has an amorphous structure containing pores of various sizes (Mohan and Pittman 2006). Chemical activation is carried out by carbonization and metallic additives such as zinc chloride added prior to carbonization (Allen et al. 1998). Basic scheme of producing activated carbon is shown in Fig. 2.3 (Gupta and Suhas 2009).

Figure 2.3 shows that physical activation process is generally longer and carried out at high temperature as compared to chemical activation. Exhaustive washing is required in chemical activation process due to use of chemicals. The major sources

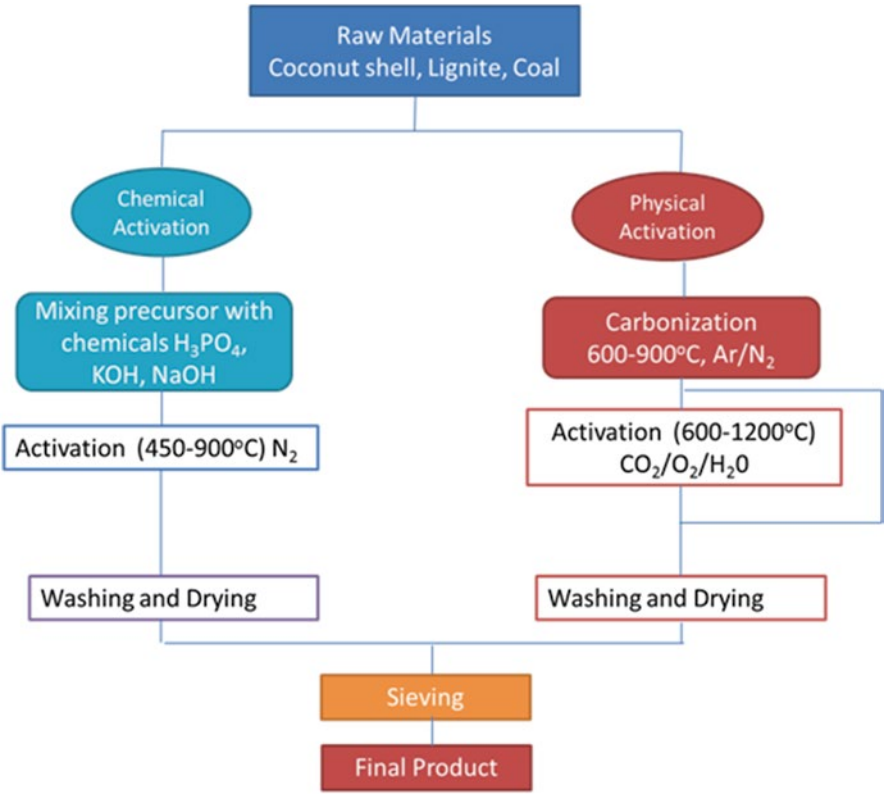

Fig. 2.3 Synthesis of activated carbon both by physical and chemical activation (Gupta and Suhas 2009)

or raw material for activated carbon include, wood char, petroleum coke, sawdust, carbon black, peat, coconut shells etc. (Pollard et al. 1992). Source of activated carbon plays a major role in its selection as adsorbent. High cost of activated carbon restricts its use for various environmental applications. Natural sourced activated carbons are low cost and widely used for heavy metal removal. Activated carbon can be classified into four types, i.e. granular, powdered, fiber and cloth activated carbon depending upon size and shape and type of raw material used (Kurniawan et al. 2006a). Activated carbon can be used for a variety of purposes and is a very good adsorbent material due to its porous structure for removing heavy metals from wastewater. Natale and Huang et al. (2007; Huang and Wu 1977) successfully removed chromium (IV) from water using activated carbon. Reaction mechanism involved reduction of chromium (IV) to chromium (III) and subsequent adsorption on activated carbon. pH of the water was noted to be one of the most important parameter for adsorption efficiency. Different research groups (Ranganathan 2000; Lee et al. 1995) have also used activated carbon to remove chromium from wastewater. The studies revealed that carbon could be reused after adsorption efficiently.

Corapcioglu and Huang (1987) studied the removal of Cu(II), Pb(II), Ni(II) and Zn(II) from waste water using hydrous activated carbon. They observed that a complex is formed during the adsorption reaction and hydrogen bonding provides all the energy needed for reaction.

Huang and Blankenship (1984) studied mercury removal from water by using activated carbon as adsorbent. They observed that at pH 4–5 almost 99 % mercury removal was achieved using various types of activated carbons. The removal mechanism was found to occur by adsorption followed by reduction processes. Chang and Ku (1994) used activated carbon along with chelating agents such as Ethylenediaminetetraacetic acid (EDTA) to remove cadmium from wastewater by adsorption process. Authors observed that efficiency of adsorption process depends upon the distribution of cadmium chelate species and pH. Anirudhan and Sreekumari (2011) used activated carbon synthesized from waste coconut buttons to study the adsorption efficiency for removal of copper, lead and mercury from waste water. Authors also studied the effect of pH on adsorption efficiency. Lead showed higher adsorption efficiency as compared to other metals at pH 6. Kobya et al. (2005) synthesized activated carbon from apricot stone and studied the removal of different heavy metal ions Co (II), Cd(II), Ni(II), Pb(II), Cr(III), Cu(II) and Cr(VI) from waste water using adsorption technique. Authors observed that adsorption process for the metals are pH dependent. Different metals showed higher efficiency at different pH. Paajanen et al. (1997) removed heavy metals using activated carbon synthesized from peat, coconut shell and coal. Uzun and Guzel (2000) compared efficiency of activated carbon with other low cost adsorbent such as agar and chitosan for heavy metal removal from waste water. Authors concluded that one material can be a good adsorbent for certain metals but it may not be suitable for another one. All adsorbent showed comparable efficiency but order of selectivity for different materials was different depending on surface properties, solution pH and many other factors.

Kurniawan et al. (2006a) reviewed and compared various low cost adsorbents for heavy metal removal and studied their efficiency by studying the effect of source, solution pH, cost, initial metal concentration, metal dosage and adsorption capacity. The authors observed that agricultural based activated carbons after treatment are most effective for removal of heavy metals i.e. hazelnut shell activated carbon, orange peel and citric acid modified activated carbons have the highest removal capacity for heavy metals such Cr (IV), Ni, and Cu(II) etc. as compared to activated carbon synthesized from coal, calcined phosphate and other synthetic materials (Ranganathan 2000).

It can be concluded that among all three types of adsorbents discussed above agriculture based activated carbon after heat treatment has shown outstanding adsorption properties as compared to other low cost adsorbents such as zeolites and alginates. It also should be noted that effectiveness of adsorbents also depends upon local conditions e.g. countries with less agriculture cannot use agriculture based adsorbent due to higher cost of logistics (Kurniawan et al. 2006a).

2.3.3 Disinfection

Microorganism present in water and wastewater can cause a variety of diseases to humans. Microorganisms that are responsible for diseases are known as pathogens. Various water treatment technologies are available to inactivate these pathogens. Inactivation of pathogen is usually called as disinfection of water (Sobsey 1989). Disinfection is the term used for removal of only pathogens, it doesn't remove all the microorganisms present in water as few useful microorganisms are also present in water (Ellis 1991). Different techniques are applied for disinfection including chlorination, ultraviolet light treatment and ozonation.

2.3.3.1 Chlorination

Chlorination is a widely used method for water treatment for centuries. Initially chlorine was used for odour removal but in late nineteenth century chlorination progressed as an effective disinfection technique (Tzanavaras et al. 2007). Chlorination is effective against bacteria and viruses but it is not effective against protozoan cysts which restrict its use for some applications (Burch and Thomas 1998). Mechanism for chlorination involves damage to cell wall of microorganisms, where chlorine penetrates in to its cell to unsettle respiration and DNA activity. Chlorination is normally carried out by liquefied chlorine gas, chlorine dioxide, calcium hypochlorite particles, sodium hypochlorite solution etc. (World Health Organization 2004). Use of chlorine gas can cause organic matters such (Fulvic acids) etc. prevailing in water to form halogenated hydrocarbons or TMH which are health hazards if consumed (Huang et al. 1997; Lykins and Griese 1986). Chlorine dioxide has better disinfection properties than chlorine because less organoleptic interference is produced. Chlorine dioxide (ClO_2) gas is neutral intricate of chlorine gas with IV^+ oxidation state. ClO_2 is a highly volatile compound and in aqueous solutions found as free radical (Tzanavaras et al. 2007). ClO_2 is soluble in water at very low temperatures and due to its one electron transfer mechanism it is one of the most versatile compound for disinfection (Hoehn et al. 1996). ClO_2 can be easily removed from water by de-aeration. Huang et al. (1997) compared ClO_2 and Cl_2 for disinfection of bacteria in water and found that ClO_2 is better disinfectant than its counterpart chlorine gases. Disinfection by ClO_2 depends upon pH of solution, disinfectant loading and contact time.

Chlorination can be used for both pre-treatment and post-treatment of wastewater and surface water. Pre-treatment removes Iron, manganese, controls biological, algal growth and also removes taste, colour and odour from water. Post-treatment involves disinfection of pathogens. Mechanism of disinfection involves formation of hypochlorite ions (OCl^-) and hypochlorous acid (HOCl) which disinfect the chlorinated water from pathogens (http://www.safewater.org/PDFS/resourcesknowthefacts/What+is+Chlorination.pdf). When chlorine is added to water, it reacts to form a equilibrium mixture (pH dependent) of chlorine, hypochlorous acid and hydrochloric acid: (Nakagawara et al. 1998).

$$Cl_2 + H_2O \rightarrow HOCl + HCl \tag{2.4}$$

Depending on the pH, hypochlorous acid dissociates partly to hydrogen and hypochlorite ions:

$$HOCl \rightarrow H^+ + ClO^- \tag{2.5}$$

In acidic solution, the major species are Cl_2 and HOCl while in alkaline solution effectively only ClO^- is present. Very small concentrations of ClO^{2-}, ClO^{3-}, ClO^{4-} are also found. The amount of hypochlorous acid and hypochlorite ions determine the amount of free Chlorine which is powerful disinfectant because of its strong oxidation potential.

2.3.3.2 Ultra Violet (UV) Light Treatment

Since the inception of UV treatment of water in the 1970s (Bukhari et al. 1999), it has been widely used for microorganism reduction as it produces no harmful by products (Hijnen et al. 2006). The UV treatment technique involves a low pressure UV lamp at a wavelength from 200 to 300 nm (Zhou and Smith 2002). UV lamps don't affect the biological stability of water as it happens with chemical treatment. UV dose is very important parameter for the efficacy of this process. UV dose is defined as the rate of total incident radiation per unit area from all the directions and at all wavelengths and the exposure time (Zhou and Smith 2002; Bolton 1999).

UV light treatment utilizes physical mechanism instead of addition of any chemicals used by other techniques. UV light penetrates the structure of microorganism by absorption. Dosage of UV light is very important and at higher dosages of UV light, proteins absorb the light and damage the cell wall leading to death of the cell. At lower concentration of UV light, it is absorbed by DNA and RNA to inactivate the cell (Zhou and Smith 2002). Rate of inactivation depends upon amount of light absorbed by microorganisms i.e. UV dosage which is the intensity of UV light and time of exposure (Gyürék et al. 1999). Another important parameter for UV treatment performance is the surface area of microorganisms and their distribution (Loge et al. 1999). The inactivation by UV varies considerably for different organisms (Karanis et al. 1992). The efficiency of UV treatment system thus strongly depend upon type of water being used (Zhou and Smith 2002). A UV reactor designed by Mamane et al. for water treatment in low head recirculating aquaculture systems is shown in Fig. 2.4 (Mamane et al. 2010).

Clancy et al. (1998) studied the efficacy of UV irradiation for treatment of Cryptosporidium and Giardia and observed that UV irradiation has more degradation efficiency than with other chemical treatment techniques. This pioneering work spearheaded further research and use of UV in drinking water treatment. Von Sonntag et al. (2003) studied the degradation of protozoan cysts using UV light and observed that UV light prevents replication and transcription of DNA/RNA thus preventing the cell or virus from reproducing. Severin et al. (1983) studied the effect

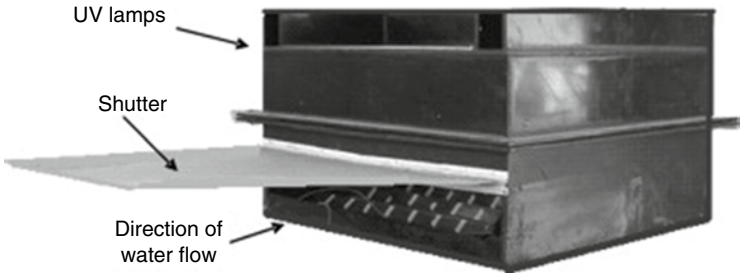

Fig. 2.4 Photograph of a UV reactor for water purification (Mamane et al. 2010)

of process conditions on the efficiency of UV light degradation and observed that UV disinfection is independent of process parameters such as temperature, pH and reactive organic matter as compared to chemical treatment where these parameters play a major role in disinfection efficacy. Hijnen et al. (2006) studied the factors which effect the efficiency of disinfection process includes majorly physiological state of microorganisms and reflection, adsorption, and refraction of UV light through the water and intensity of lamp. Normally the UV sensitivity of microorganism is related with rate of inactivation. Higher UV sensitive microorganism would have higher rate constant and lower effect of process parameters such as reflection, refraction and adsorption of UV light.

2.3.3.3 Ozonation

Ozone is a colourless and very unstable gas that consists of three oxygen atoms. Ozone is readily converted back to oxygen by forming one free oxygen atom or radical during transition stage. The free radical of oxygen is short-lived and very reactive. At ambient conditions free radical of oxygen survives only for a few milliseconds. Due to its high oxidation potential, ozone is a good candidate for disinfection of water streams. Ever since the use of ozone for water disinfection in 1886 by De Meritens, considerable research has taken place especially in recent times for replacing chlorine with ozone as it does not form TMH and organochlorine during the disinfection process (Camel and Bermond 1998). Ozone when added in wastewater rapidly is consumed because organic matter and inorganic salts require it. After the initial ozone demand is satisfied it leads to disinfection much faster than what is achieved with chlorination.

Ozone reacts directly with organic matter as oxidizing agent and also decomposes instantly to form a complex mechanism which produces free hydroxy radicals during the process further enhancing the disinfection process (Hoigné and Bader 1976). The efficiency of ozonation process strongly depends upon type of water being disinfected. Ozone due to its highly unstable nature produces very low amount of by products (Glaze and Kang 1988). A major disadvantage of ozonation is the fact that ozone needs to be generated at the point of use because it cannot be transported due to its highly unstable nature (EPA 1999).

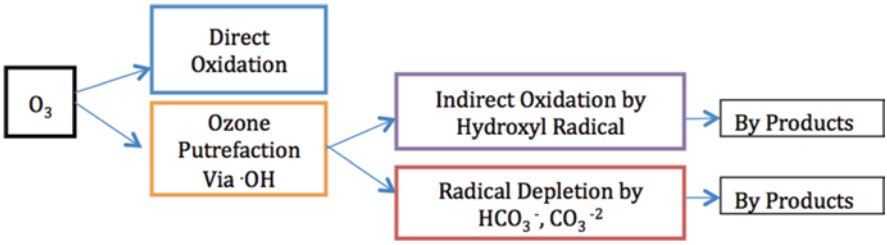

Fig. 2.5 Oxidation reactions of ozone during water disinfection (Hoigné and Bader 1976)

Figure 2.5 shows two pathways of ozonation of water competing for oxidation, direct oxidation is slower than hydroxy radical mechanism but concentration of aqueous ozone is higher as compared to hydroxy radical where ozone concentration is less in indirect oxidation process (EPA 1999).

DeMers and Renner (1992) compared ozone disinfection with that of chlorine and observed that ozonation is better than chlorination but due to its inability to sustain left over in distribution system it should be used with chlorination or other disinfectants for thorough disinfection. Reaction parameters such as pH, temperature, organic matter content can play a major role in increasing the efficiency of the process. Farooq et al. (1977) studied the effect of pH on ozonation reaction and observed that it had very little effect on reaction efficiency. Katz (1980) showed that temperature decreases the solubility and stability of ozone in water but did not affect the rate of disinfection. Kinman (1975) also demonstrated that rate of disinfection is independent of temperature. Ozonation is widely used for inactivation of bacteria (Domingue 1988), removing protozoan cysts (Domingue 1988), and for virus inactivation (Bablon 1991). All the disinfection techniques stated above have certain advantages and disadvantages but UV treatment and Ozonation have greater potential for being used as viable disinfection processes to replace chlorination. Chlorine gas is very effective disinfectant but due to production of harmful byproducts, its use as disinfectant is diminishing very fast.

2.3.4 Harmful Effects of Water Remediation Schemes

Disinfection by-products (DBPs) are generally formed when disinfectants, during water purification, react with naturally occurring organic matter and also anthropogenic contaminants like bromides and iodides (Hebert et al. 2010). Chlorination is a well-accepted method for water treatment but it has its share of disadvantages. Excessive amounts of chlorine can be toxic for human beings and can be a cause of irritation to the eyes, the nasal passage and respiratory system (Medina-Ramón et al. 2005). Use of chlorinated drinking water can be related to the risk of cancer, especially bladder and colorectal cancers, which can be attributed to the presence of THM (Cantor et al. 1998, 1999; Hildesheim et al. 1998). Chlorination is not a useful disinfectant for the removal of protozoan cysts (http://www.inchem.org/documents/ehc/ehc/ehc216.htm#SectionNumber:1.3). The remarkable biocidal properties of

chlorine is negated due to the formation of DBPs during the chlorination process, which are detrimental to human health. A drawback of chemical treatment, especially for drinking water, is the bad taste of the water. Further, the process is potentially harmful for people with thyroid disease or iodine allergy (http://www.who.int/water_sanitation_health/dwq/gdwq0506.pdf).

A report released by the national environmental health group Women's Voices for the Earth (WVE) (http://www.womensvoices.org/wp-content/uploads/2010/05/Disinfectant-Overkill.pdf) linked disinfectant chemicals with chronic illnesses and conditions such as asthma, hormone imbalance, and immune system problems in a report titled "Disinfectant Overkill: How Too Clean May Be Hazardous to Our Health," which cited more than 40 peer-reviewed reports and scientific studies. According to the report Triclosan and Triclocarban, two antibacterial disinfectants may have hormone-disrupting effects: Triclosan adversely affects communication between cells in the brain and the heart while Triclocarban appears to amplify testosterone in the body (http://www.womensvoices.org/wp-content/uploads/2010/05/Disinfectant-Overkill.pdf). The physical and chemical properties of disinfectants and their byproducts can influence their behavior in drinking water.

Ozone can react with bromide to form brominated ozone DBPs like bromate ion (BrO_3^-). In the presence of natural organic matter, ozonation leads to the formation of non-halogenated organic DBPs such as carboxylic acids, aldehydes and ketoacids. In presence of both natural organic matter and bromide, ozonation forms hypobromous acid and this can form brominated organohalogen compounds. Halobenzoquinones (HBQs) have recently been considered a disinfection byproducts (DBPs) of toxicological relevance as it can be the cause of bladder cancer (Zhao et al. 2012). The problem with ultraviolet treatment is that even though it is capable of immobilizing a wide variety of disease causing bacteria, the effect is temporary. UV treated water should not be stored for long as the bacteria again revives after the UV source is removed (http://www.drinking-water.org/html/en/Treatment/Chemical-Disinfection-Oxidants-technologies.html). The flow rate of water is crucial in a UV treatment process as a high flow rate may lead to insufficient UV exposure and a slow rate may lead to heat buildup and subsequent damage to the bulb. (Gadgil 1997)

2.4 Nanotechnology in Water Purification

Nanotechnology, which relates to materials and devices with physical dimensions comparable to or less than 100 nm, shows immense promise as a viable means of treating both persistent and emerging water contaminants (Baruah and Dutta 2009a; Brame et al. 2011). This emerging technology is capable of positively affecting technologies such as desalination of seawater to increase fresh water supply. Engineered nanomaterials could also have adverse effects on the ecosystem by contributing to water contamination (Baruah and Dutta 2009a; Baruah et al. 2009). Here, we discuss both the positive properties as well as implications of nanomaterials in water treatment.

2.4.1 Photocatalysis

The Report from the Workshop on Nanotechnologies for Environmental Remediation (http://www.who.int/infectious-disease-report/pages/textonly.html) identifies solar photocatalysis as the main technology breakthrough for water treatment and purification, particularly in developing regions. Initial pilot projects are now being carried out. Photocatalytic systems may also complement existing techniques in the removal of trace contaminants. Such systems are commercially available e.g. for the disinfection of swimming pools.

Photocatalysis, using nanostructures of metal oxide semiconductors like zinc oxide (ZnO), titania (TiO_2), tungsten oxide (WO_3), zinc stannate (Zn_2SnO_4), etc. can be an attractive way of water purification as it is capable of removing chemical as well as biological contaminants (Baruah and Dutta 2009b, c; Baruah and Dutta 2011). A good photocatalyst should absorb light efficiently preferably in the visible or near UV part of the electromagnetic spectrum. Sufficient electron vacant states need to be present to inhibit recombination of electron hole pairs upon light exposure. As a lot of work is going on using photocatalysis in the agriculture and microbiology fields, it is important that the photocatalysts should be biologically inert and non-toxic. Nanostructured photocatalysts offer large surface to volume ratios allowing higher adsorption of the target molecules. Intensive research over the past decade for its implementation in the purification of drinking water can be found in the literature (Baruah and Dutta 2009a; http://www.inchem.org/documents/ehc/ehc/ehc216.htm#SectionNumber:1.3). Efficacy of photocatalysis, in the detoxification of a wide range of industrial and agricultural effluents is also well documented (Gaya and Abdullah 2008). Another interesting aspect of photocatalysis is the potential utilization of sunlight, which could allow energy efficient treatment in remote locations.

The underlying mechanism of heterogeneous photocatalysis is schematically represented in Fig. 2.6. It involves a wide band gap semiconductor photocatalyst, which upon irradiation with light of energy higher than the band gap energy of the material, electron-hole pairs (excitons) are created. The photogenerated electron moves up to the conduction band while the hole drifts to the bottom of the valence band. Majority of these photogenerated charge carriers undergo wasteful recombination, while escape recombination and initiate redox reactions in molecules adsorbed at the surface of the photocatalyst and thereby degrading them. The photogenerated electrons and holes have been found to degrade almost all types of organic, inorganic, and microbial contaminants (Gaya and Abdullah 2008), owing to their high redox potentials.

The fundamental process during photocatalysis is given by

$$\mathrm{MO} + h\nu \rightarrow \mathrm{MO}\left(\mathrm{e}^- + \mathrm{h}^+\right) \tag{2.6}$$

where MO represents a metal oxide photocatalyst like TiO_2, ZnO etc. Photogenerated electrons lead to the formation of superoxide anions ($\bullet O_2^-$), hydrogen-peroxide molecules (H_2O_2), hydroxyl radicals (•OH), hydrogen dioxide anion

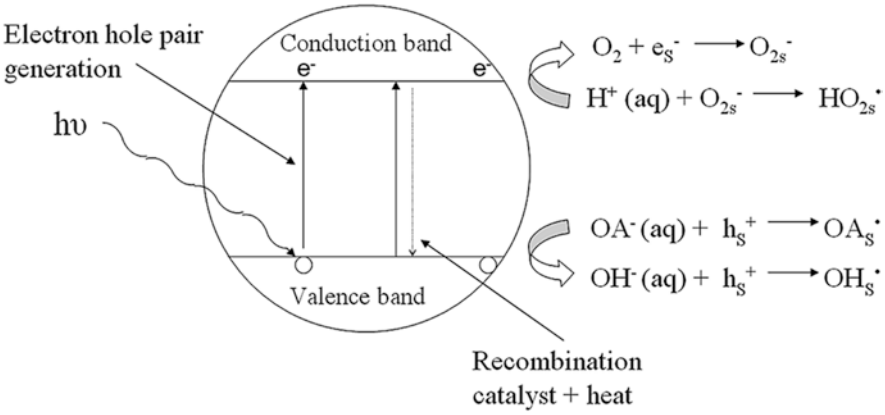

Fig. 2.6 Schematic diagram explaining photocatalysis on semiconducting surface (Baruah and Dutta 2009a)

(HO_2^-) and the hydroperoxy radicals ($^{\bullet}HO_2$) (Baruah and Dutta 2009b; Banerjee et al. 2006)

$$MO\left(e^-\right) + O_2 \rightarrow MO + {}^{\bullet}O_2^-, \tag{2.7}$$

$$MO\left(e^-\right) + {}^{\bullet}O_2^- + 2H^+ \rightarrow MO + H_2O_2, \tag{2.8}$$

$$MO\left(e^-\right) + H_2O_2 \rightarrow MO + {}^{\bullet}OH + OH^-, \tag{2.9}$$

$${}^{\bullet}O_2^- + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2, \tag{2.10}$$

$${}^{\bullet}O_2^- + H^+ \rightarrow {}^{\bullet}OH_2, \tag{2.11}$$

$$MO\left(e^-\right) + {}^{\bullet}OH_2 \rightarrow MO + HO_2^-, \tag{2.12}$$

$$HO_2^- + H^+ \rightarrow H_2O_2, \tag{2.13}$$

$$2\,{}^{\bullet}OH_2 \rightarrow O_2 + H_2O_2. \tag{2.14}$$

While the oxidation reactions initiated by the photo-generated holes are:

$$MO\left(h^+\right) + H_2O \rightarrow MO + {}^{\bullet}OH + H^+, \tag{2.15}$$

$$MO\left(h^+\right) + H_2O \rightarrow MO + {}^{\bullet}OH + H^+, \tag{2.16}$$

$$MO\left(h^+\right) + OH^- \rightarrow MO + {}^{\bullet}OH. \tag{2.17}$$

The reactions are terminated as:

$$^{\bullet}OH + H^{+} + 2e^{-} \rightarrow H_2O, \tag{2.18}$$

$$1/2\,O_2 + 2H^{+} + 2e \rightarrow H_2O. \tag{2.19}$$

Nanostructures consist of a large number of low coordination number atoms at edge and corner sites of the crystal lattice providing numerous catalytically active sites. A lot of work is going on to remove the harmful effects of chemical contaminants from groundwater mainly through photocatalysis using nanoparticles of metal oxides like TiO_2 and ZnO (Cantor et al. 1999; Zhao et al. 2012). Another nanostructured semiconductor that is receiving attention from researchers as a stable photocatalyst is the ternary oxide zinc stannate (Cantor et al. 1998; http://www.drinking-water.org/html/en/Treatment/Chemical-Disinfection-Oxidants-technologies.html). Rahman and Muneer (2005) studied the degradation kinetics of two pesticides dichlorvos and phosphamidon using Degussa 25 (commercially available TiO_2 nanoparticles) and observed that the addition of electron acceptors like hydrogen peroxide (H_2O_2) enhances the degradation rates of the pollutants. Solar photocatalysis was also successfully used for degrading aldrin with three transformation products, dieldrin, chlordane and 1,2-hydroxy dieldrin (Bandala et al. 2002). Dichlorvos, which has been classed as a Restricted Use Pesticide (RUP) due to its toxicity, has been successfully degraded using photocatalysts like TiO_2 and ZnO (Evgenidou et al. 2005). It was observed that the addition of electron acceptors like hydrogen peroxide (H_2O_2) or potassium persulphate ($K_2S_2O_8$) increases the degradation rate in presence of TiO_2 but retards it in presence of ZnO. Mahalakshmi et al. (2007) has used total organic carbon (TOC) analyzer to confirm the complete mineralization of carbofuran which is an extremely toxic carbamate pesticide that is extensively used as an insecticide in a wide variety of field crops, including soyabean and potatoes. Water soluble pesticides have also been mineralized at the pilot plant scale using two well defined systems exploiting solar UV light for heterogenous photocatalysis using TiO_2 nanoparticles (Oller et al. 2006). Nearly 100 % mineralization was obtained with total removal of pesticides like cymoxanil, dimethoate, methomyl, oxamyl, pyrimethanil and telone (Oller et al. 2006).

Water purification agents should be capable of removing not only chemical, but also microbial contaminants like bacteria, fungi, virus, molds, etc. Photocatalytic inactivation of microorganisms is a complex process and the rate of inactivation varies with the type, concentration and the physiological state of the microbes (Lonnen et al. 2005; Rincon and Pulgarin 2004). The nature, morphology, concentration, and state (slurry or immobilized) of the catalyst material, also, have a great influence on the microbial inactivation rates (Cantor et al. 1999; Baruah and Dutta 2009c). Among the various bacterial species, *Escherichia coli (E. coli)* which causes diarrhea have been extensively tested to optimize photocatalytic processes as well as for testing newly designed photo reactors (Krishna et al. 2008). Apart from *E. coli* in pure water, the photocatalytic inactivation of other *coliform bacteria*

has also been reported in the literature (Gelover et al. 2006). TiO_2 nanoparticles (Degussa P25) have been used to successfully inactivate different genera of bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Enterobacter cloacae* (Ibanez et al. 2003). Reports of photocatalytic inactivation of model microbes like *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *Aspergilus niger spores* have been reported for palladium (Pd) doped TiO_2 and tin dioxide (SnO_2) films grown on glass substrates (Erkan et al. 2006). The use of TiO_2 nanoparticles (Degussa P25) to inactivate bacteria (*E. coli, Pseudomonas aeruginosa*), fungi (*Candida albicans, Fusarium sloani*), and protozoa (the trophozoite stage of *Acanthamoebe polyphaga*), spores (*Bacillus subtilis*), and cysts under solar light irradiation are also available in the literature (Lonnen et al. 2005)

2.4.2 Nanofiltration

Nanofiltration, a relatively new entrant in the group of membrane filtration processes, used mostly with surface water and fresh groundwater having fewer amounts of dissolved solids. The objectives of nanofiltration are the softening (polyvalent cation removal) of water and removal of DOB precursors like natural and synthetic organic matter (Letterman 1999; Hillie and Hlophe 2007). The type of materials that can be filtered out depends upon the pore sizes of the filtration membranes. Nanofiltration, which is a cross-flow filtration technology, can be placed in between ultrafiltration and reverse osmosis. The pore size of the nanofiltration membrane can go down to about 1 nm. Figure 2.7 shows some contaminants that can be removed using membranes with different pore sizes ranging from 0.5 to 1000 nm (http://www.techneau.org/fileadmin/files/Publications/Publications/Deliverables/D5.3.4b.pdf). Figure 2.8 shows a schematic representation of water softening using nanofiltration membrane where the water is forced through the membrane using high pressure.

The selectivity of a nanofiltration membrane is governed by two different parameters: retention and permeability. The retention is a function of the solute size. In nanofiltration membranes, retention and permeability are also a function of electric charge and the valency of the salts and compounds in the solution (http://www.fumatech.com/EN/Membrane-technology/Membrane-processes/Nanofiltration/). Monovalent ions of mild concentrations can mostly pass through a nanofiltration membrane unobstructed while most of the multi-valent ions like sulfates and carbonates are blocked. Cation retention using a nanofiltration is minimum for protons and for other commonly present cations, retention increases following an order as sodium, potassium, calcium, magnesium, copper and iron. As for anions, the retention capability of nanofiltration membranes increases in the following order: nitrates, chlorides, hydroxides, sulfates, carbonates and phosphates (http://www.fumatech.com/EN/Membrane-technology/Membrane-processes/Nanofiltration/).

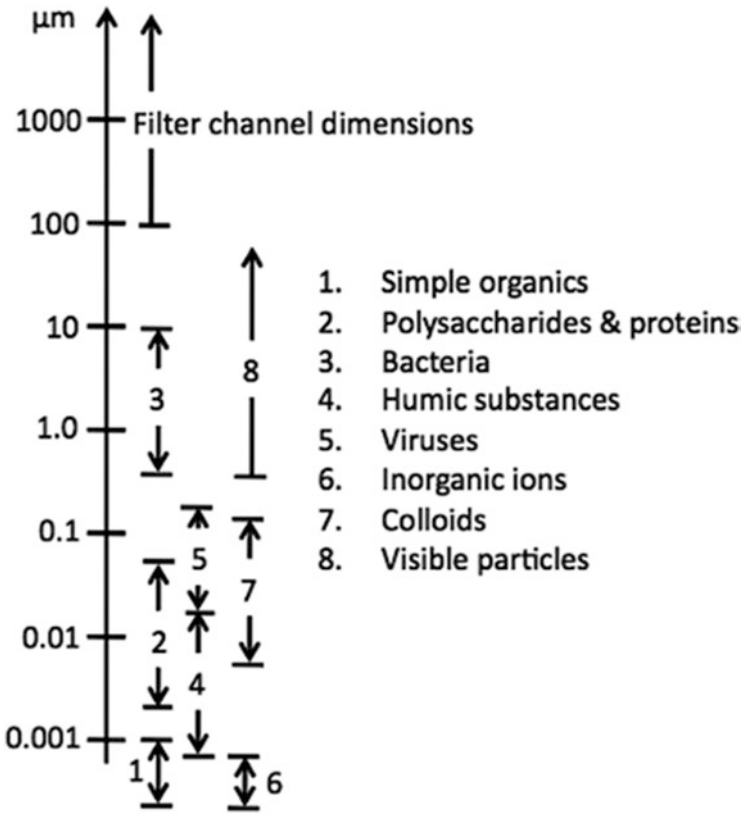

Fig. 2.7 An overview of membrane filtration. The pores sizes of the filtration membranes lie in the range between 0.5 and 1000 nm

The membrane pore size is a major factor that determines whether a particular solute will pass through the membrane or not. Nanofiltration is a type of crossflow filtration where the majority of the feed flow travels tangentially across the surface of the filter media, rather than through it (Koros et al. 1996). An organization of crossflow filtration techniques is given in Table 2.4, which shows that membranes are available with pore sizes in the range of about 0.5 nm to about 5 µm. Dalton is a general unit of molecular weight in membrane filtration and expressed in g/mole (http://www.techneau.org/fileadmin/files/Publications/Publications/Deliverables/D5.3.4b.pdf).

From Table 2.4, it is obvious that the nanofiltration process is capable of removing almost all type of solutes from natural surface waters. However, if the source water is seawater, brackish water or ground water, reverse osmosis is a better option. Even then, nanofiltration techniques are widely used for softening and natural organic matter may be a matter of apprehension here. Natural organic matter can add undesirable color to the water and also foul the membranes. Nanofiltration and reverse osmosis techniques are efficient in softening hard water for which high

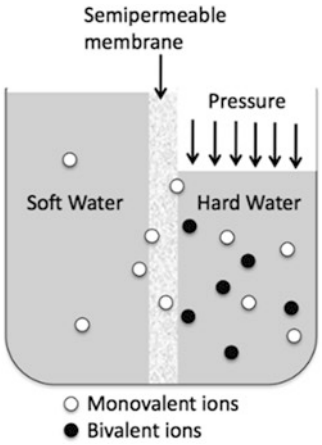

Fig. 2.8 Schematic representation of water softening using nanofiltration membrane

Table 2.4 Properties of different crossflow filtration techniques

Method	Pore size (nm)	Molecular weight	Pressure (bar)	Permeation
Reverse osmosis	<0.6	<500	30–70	Water
Microfiltration	50–5000	>500 kDa	0.5–2	Water + low molecular solutes + macromolecules + colloids
Ultrafiltration	5–50	2–500 kDa	0.5–10	Water + low molecular solutes + macromolecules
Nanofiltration	0.6–5	500–2000 kDa	10–40	Water + low molecular solutes

operating pressures are needed. To address scaling and fouling issues, modifiers such as anti-scalants become necessary (http://www.techneau.org/fileadmin/files/Publications/Publications/Deliverables/D5.3.4b.pdf).

2.4.3 Future Prospects

Nanotechnology can usher in a revolution in the domains of water treatment and distributed water reuse. It is capable of precluding concerns related to the formation of harmful disinfection by products associated with conventional water treatment methods. Nanomaterials are endowed with unique properties like high surface to

volume ratios, enhanced surface related activities like catalysis and antimicrobial properties, property of self assembling on substrates to form films, high conductivity that can be effectively used in capacitive deionization method for desalination, high fluorescence for detection, etc. Nanomaterials can be engineered to effectively act as a visible light photocatalyst so that water purification can be carried out using solar energy, which is available freely. Another area of concern that can possibly be addressed by nanotechnology is the degradation of the quality of water as it moves through distribution networks. Using nanotechnology, point of use water purification systems utilizing solar energy could be designed, which can be ideal for disaster prone areas (Baruah et al. 2012). Nanotechnology is capable of exploiting alternative water sources for drinking and agricultural keeping energy consumption to a bare minimum. Nanotechnology can especially impact the developing countries, which are more prone to degradation of water quality. High performance innovative water treatment technologies have now become a necessity. Future water treatment systems in developing countries will most likely opt for nanotechnology based water monitoring, treatment and reuse systems that can efficiently immobilize a wide variety of water pollutants coupled with affordability and ease of operation.

2.5 Conclusion

Nanotechnology is likely to make a tremendous impact in the area of drinking and waste water purification and reuse. Nanomaterials possess unique properties as compared to their bulk counterparts like increased surface area to volume ratios, higher surface reactivity, band tunable semiconductivity, to mention a few. Wideband semiconducting nanostructures can be used to degrade harmful contaminants and microbes through photocatalysis using solar radiation. Nanofiltration, using membranes with pore sizes in the nanometer regime, can successfully convert hard water into soft water by blocking mono and bivalent ions as the water passes through the semipermeable membranes. Nanotechnology can potentially improve all the current disinfection, purification and desalination techniques and usher in an era of point of use water purification systems harnessing solar energy.

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Chapter 3
Biodegradation of Organophosphate and Pyrethroid Pesticides by Microorganims

Natália Alvarenga, Willian Garcia Birolli, and André Luiz Meleiro Porto

Contents

Abstract Major methods for the biodegradation of organophosphate and pyrethroid pesticides are reviewed in this chapter. Although these methods are very promising, it is not easy to avoid fully the release of metabolites into the environment. Therefore, serious problems of soil, water and even foods contamination still exist. Despite the great benefits of pesticides to agricultural productivity, they also cause serious problems of contamination and increasingly need studies, especially in the search of compounds that are less harmful to the environment. Knowledge of the biodegradation route of pesticides and the development of new techniques that allow the improvement of these degradation pathways are essential, therefore, this chapter presents studies about the biodegradation of organophosphate and pyrethroid pesticides by biological processes, focussing on the development of new enzymatic methods, especially those using bacteria and fungi. Other methods

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of biological degradation of organophosphate and pyrethroid pesticides are also described.

Keywords Pyrethroid pesticide • Organophosphate pesticide • Acetylcholinesterase • Microbial degradation • Phosphotriesterases • Fungi

3.1 Introduction

The use of pesticides is crucial to the increase of agricultural production in the world, and thus to human survival. This productivity increase was essential for livestock, such as cattle, pigs, poultry and fish, maintaining the food production chain. The rise in food production was necessary to support the population growth and reduce hunger, especially in poor countries.

Food production is insufficient in many populous countries that have little fertile land or are unfit for agriculture. There are countries with large areas of land, which are deserts, mountains or glaciers, and therefore prevent a suitable food production even with modern techniques. Even in countries with large territories, such as Brazil, the expansion of cultivated areas is problematic, since it would require the exploitation of conservation areas, causing the destruction of biomes, especially the Amazon rainforest, Brazilian savannah, Atlantic forest and Pantanal (Dov et al. 2005; Brazil Biomes). This deforestation for food production may cause several environmental problems, such as climate change, freshwater shortages and extinction of animals, plants and microorganisms species (Londres 2011).

The most appropriate way to produce more food is the sustainable use of land, aiming a higher productivity per hectare. There is no doubt that, over the years, pesticides have brought great benefits for agricultural production. However, the genetic improvement of crops is imperative, as well as the development of new technologies, since many problems have arisen of the excessive use of pesticides. One of the first chemical class of pesticides synthetized was the organochlorine, which have contaminated large tracts of soil and drinking water because of their high toxicity and persistence in the environment. Despite the banning of this pesticide class for agricultural use, the problems have continued over the years and were certainly responsible for the death of many living organisms as well as the extinction of endemic species.

New classes of pesticides, such as organophosphates and carbamates, which are less persistent and recalcitrant than organochlorines, have become a viable choice for use in agriculture and households. However, these pesticides have also led to serious environmental problems and caused many cases of poisoning, due to their excessive use and high toxicity to humans and animals. For these reasons, it is necessary to develop new pesticides that are more efficient and less toxic to non-target species. The pyrethroids are currently widely used, especially as insecticides, although they are less toxic than the other classes of pesticides, these substances can damage the environment when used in large quantities (Stoytcheva 2011).

While the use of pesticides in agriculture and livestock rearing is necessary to control several types of pest, the challenge is to seek ways to eliminating them from the environment. Among the various methods of degradation, there is no doubt that the biological techniques are the most efficient and appropriate. Microorganisms, especially fungi and bacteria, have contributed significantly to the elimination of several types of contaminants produced by humans, however, these bioprocesses have limitations since reactions catalyzed by living organisms only tolerate restricted ranges of pH, temperature and concentration, but it still represents the most promising way for the degradation of organopollutants and their metabolites released into the environment by anthropogenic activities (Mudhoo and Mohee 2012).

Organophosphate and pyrethroid pesticides were the two most sold chemical class of pesticide in 2007, when approximately 1.8 million euros of organophosphate pesticides were commercialized, while 1.0 million euros of pyrethroids were sold (Wirtz et al. 2009). Although pyrethroids insecticides have been available for decades, pyrethroids are increasingly being used as replacements for organophosphate insecticides, since regulatory restrictions have been imposed in some countries (You et al. 2004).

The aim of this chapter is to present some studies described in the literature about the biodegradation of organophosphate and pyrethroid pesticides, especially those using bacteria and fungi focusing in the development of new enzymatic methods. Summarily, is also described other methods of biological degradation of organophosphate and pyrethroid pesticides. It is hoped that this issue arouses the interest of many researchers to study new and alternative enzymatic routes that minimize the impact of excessive use of pesticides in environment, as well as the study in development of new pesticides, which may be less toxic to human beings and more specific against target insects, contributing to greater food productivity.

3.2 Biodegradation of Organophosphate Pesticides

3.2.1 Introduction

Organophosphate pesticides (OPs) constitute a heterogeneous category of chemicals with a broad spectrum of application for crops and animal protection as well as for public health (Kumar et al. 2010; Mansee et al. 2005). Unfortunately, volatile compounds such as sarin, soman, tabun, cyclosarin and VX, have been used as chemical warfare agents (nerve agents), due to their high toxicity to the human nervous system (Fig. 3.1) (Faria 2009). The OPs have been widely used to replace organochlorine compounds because of their low cost, easier synthesis, increased biodegradability (compared to organochlorines) and limited accumulation in living organisms (Santos et al. 2007). However, the OPs have an acute toxic effect on humans and other mammals, since they are inhibitors of acetylcholinesterase, which is essential for nerve impulse transmission (Galli et al. 2006).

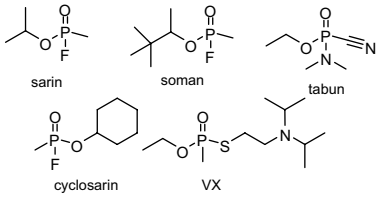


Fig. 3.1 Chemical structures of organophosphates nerve agents

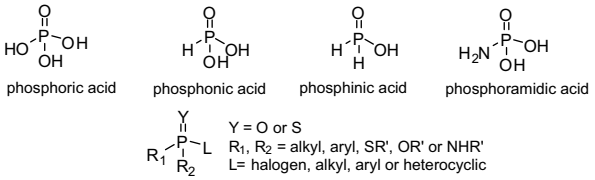


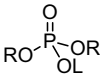
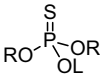
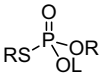
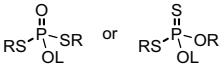
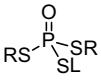
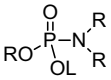
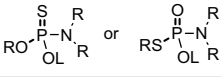
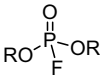
Fig. 3.2 General structure of organophosphate pesticides and its derivatives of phosphoric acid

The OPs of major commercial interest are esters or thioesters derived from phosphoric acid, phosphonic acid, phosphinic acid or phosphoramidic acid. The mostly used derivatives are phosphates, phosphorothioates (S=) and phosphorothioates (*S*-substituted). The general chemical structure of the OPs is a phosphate center with three ester linkages (Fig. 3.2) (Sogorb and Vilanova 2002; Bigley and Raushel 2013).

The phosphorus atom may be double-bonded with oxygen and sulfur and the R_1 and R_2 groups are usually alkyl, aryl, halogen or heterocyclic substituent. These groups may be directly bonded to the phosphorus atom (phosphinates) and through an oxygen or sulphur atom (phosphates or phosphothioates). In some cases, one of the groups is directly bonded to the phosphorus and the other is bonded through an oxygen or sulphur atom (phophonates or phosphonotioates), also one of these substituent may be an amino group (phosphoramidates). The "-L" group (called as *leaving group*) may be halogen, aliphatic, aromatic or heterocyclic groups, but it is most commonly a phenol or a thiol with straight, branched or aromatic chain, which is bonded to the phosphorus atom through an oxygen or sulphur and released when the OP is hydrolyzed by phosphotriesterases enzymes. The general structures of different OPs are shown in Fig. 3.2 and Table 3.1 (Sogorb and Vilanova 2002; Bigley and Raushel 2013; Bleecker 2008).

Despite of being a widely used pesticide, organophosphates are falling into disuse owing to their high toxicity to mammals. As a percentage of total pesticides used in the USA, organophosphates decreased from 70 % in 1990 to 36 % in 2007 (EPA 2011). In 2003, OPs were responsible for 24.7 % of the global pesticides market share (Elbert et al. 2007). Data shows an estimated expenditure for OPs around 1.25 billion euros in 2011, while the global market predicts a drastic decrease in commercialization of this class of pesticide, with expenditure about 0.8 billion euro in 2018 (Wirtz et al. 2009).

Table 3.1 General structure and various types of OPs Bleecker (2008)

Type	Chemical structure	Examples
General structure of organophosphates		
Phosphates		Chlorfenvinphos, dichlorvos, monocrotophos, tri-*o*-cresyl phosphate
Phosphonates		Trichlorfon
Phosphinates		Glufosinate
Phosphorothioates (S=)		Bromophos, diazinon, fenthion parathion, pirimiphos-methyl
Phosphonothioates (S=)		EPN, leptophos
Phosphorothioates (*S*-substituted)		Demeton-*S*-methyl, ecothiopate
Phosphonothioates (*S*-substituted)		VX
Phosphorodithioates		Azinphos-ethyl, azinphos-methyl, dimethoate, disulfoton, malathion, methidathion
Phosphorotrithioates		
Phosphoramidates		Fenamiphos
Phosphoramidothioates		Methamidophos, isofenphos
Phosphorofluoridates		Diisopropyl phosphorofluoridate (DFP)
Phosphonofluoridates		Cyclosarin, sarin, soman

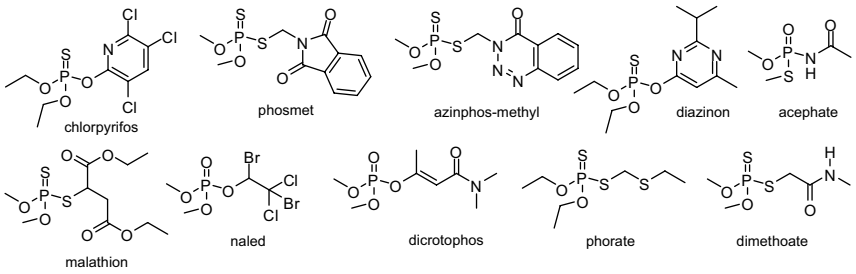


Fig. 3.3 Chemical structures of some organophosphate pesticides

According to the Environmental Protection Agency (EPA), in 2007, among the pesticides most commonly used as conventional active ingredients of the agricultural market sector, only two OPs entered into a ranking of 25 pesticides. Chlorpyrifos occupied the 14th position and acephate the 21st position in this ranking. Malathion occupied the 7th and 9th position in home and garden market sector and industry/commercial/government market sector, respectively (EPA 2011). These data show that although OPs are still widely used in the world, there is a decrease in the use of this class and it is believed that in the next few years they will be, in the most part, replaced by pyrethroids and neonicotinoids pesticides (Wirtz et al. 2009).

Organophosphate pesticides are used in agricultural and residential applications as insecticides, herbicides, fungicides and rodenticides (Jauregui et al. 2003). The main OPs used as active ingredients in the USA were, in 2007, chlorpyrifos, phosmet, azinphos-methyl, diazinon, acephate, malathion, naled, dicrotophos, phorate and dimethoate (Fig. 3.3). Among the main utilized products, chlorpyrifos occupies prominent postion, since approximately 800 registered products in the market contain this compound. These products are used for several purposes, including pest control for a variety of food crops, turf and ornamental plants, greenhouses, indoor pest control, structural pest control and pet collars (Lee et al. 2007). In agriculture, it is applied to control a variety of pests (flies, caterpillars, mites, aphids) that attack crops such as corn, soybeans, wheat, sorghum, beans, coffee, cotton, citrus, apple, banana, potato, carrot, cabbage, tomatoes and tobacco (Pena et al. 2003). Another important pesticide, malathion, is a nonsystemic wide-spectrum organophosphosphate insecticide and acaricide used to control household and agricultural pests. This pesticide is suitable for the control of sucking and chewing insects on field crops, fruits, vegetables and livestock. It is also extensively used to prevent mosquitoes, flies, household insect, animal parasites and body lice as a substitute for the banned organochlorine pesticide, DDT (dichlorodiphenyltrichloroethane) (Ramadevi et al. 2012; Kim et al. 2005).

OP compounds are sprayed onto agricultural land all over the world and, therefore represent a human health risk through the contamination of drinking water and food. Although some OPs levels may be reduced by biodegradation and hydrolysis

(mostly under alkaline conditions), repeated applications followed by watering allow the pesticides to run off into the groundwater (Romyen et al. 2007).

OPs present a short half life in the environment and act fast in their target species; however, because of their non-specific toxicity, non-target wildlife is also affected when exposed. Besides poisoning birds, which is a sensitive class of animals affected by OPs, these compounds are also involved in acute human poisonings (Jauregui et al. 2003). The World Health Organization reported that exposure to these pesticides results in roughly three million cases of severe poisoning and 220,000 deaths worldwide, furthermore, more than 0.7 million cases of poisoning could be assigned to occupational and incidental exposure to OPs (Mansee et al. 2005).

The OPs exert their main toxicological effects by non-reversible phosphorylation of esterases in the central nervous system, whereas the acute toxic effects are related to inhibition of acetylcholinesterase (AChE) (Sogorb and Vilanova 2002). AChE is a very efficient enzyme, since one unique molecule hydrolyses approximately 5,000 acetylcholine (ACh) molecules per second (Schofield and Dinovo 2010). ACh is a chemical mediator, needed for transmission of nerve impulses, present in mammals and insects (Santos et al. 2007).

AChE inhibition by OPs causes rapid ACh flooding, resulting in over-stimulation of nicotinic and muscarinic acetylcholine receptors (Schofield and Dinovo 2010). The hydrolysis catalyzed by AChE depends on the attack of a serine residue at the carbonyl group in ACh, but in the presence of organophosphates, serine is readily phosphorylated. The hydrolysis mechanisms inhibition involves the capture of a proton from the serine residue by a histidine at the active site of the AChE, increasing the nucleophilic character of serine, which then attacks the eletrophilic phosphorus atom, and releases the leaving group (L). The phosphorylated enzyme reacts slowly with water, allowing the dealkylation of the alkoxy substituent (R_2) attached to the phosphorus atom. Finally, a strong bond between the protonated histidine residue and the negatively charged oxygen atom of the inhibitor is formed in the catalytic site, preventing the histidine residue to act as a general base catalyst for the hydrolysis of the phosphorylated enzyme, which is a necessary step for the reactivation of the AChE (Fig. 3.4) (Santos et al. 2007).

Inhibition of AChE is irreversible, preventing the substrat (ACh) from reacting with the esterase site. Consequently, accumulation of ACh results in all symptoms of acetylcholine poisoning caused by organophosphates (Santos et al. 2007). Symptoms of intoxication appear after approximately 50 % of the AChE is inhibited and the typical effects are agitation, muscle weakness, muscle fasciculations, hypersalivation and sweat. Severe poisonings may cause respiratory failure, unconsciousness, confusion, convulsions and/or death (Sogorb and Vilanova 2002; Duysen et al. 2012).

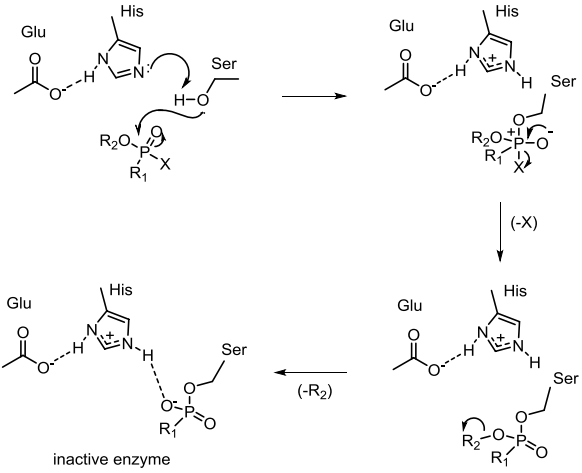


Fig. 3.4 General inhibition mechanism of acetylcholinesterase by organophosphate pesticides (Santos et al. 2007)

The process of OPs biodegradation in soil, surface water and groundwater depends on both physical and chemical characteristics of the pesticides as well as the soil and water conditions. Microbial degradation can be influenced by pH, temperature, organic matter content and other factors (Flores et al. 2004). Hong et al. studied some factors that have influenced the degradation of the pesticide fenitrothion in the presence of the bacteria *Burkholderia* sp. FDS-1 isolated from contaminated site, by evaluating the characteristics of the soil in which the pesticide showed optimal rate degradation. In soil, in the presence of the bacteria FDS-1, fenitrothion and its metabolite (3-methyl-4-nitrophenol), the optimum degradation characteristics were pH 7.5 (as in the high pH fenitrothion undergoes alkaline hydrolysis followed by accumulation of metabolite) and optimum temperature was 30 °C. However, these values may vary with the pesticide and microorganism involved (Hong et al. 2007). The biodegradation can also be affected by the production of surfactants by soil microorganisms. Wattanaphhon et al. studied the effect of surfactants produced by the bacterium *Burkholderia cenocepacia* BSP3 in the biodegradation of pesticides parathion and methyl parathion, noting that these biosurfactants can enhance pesticide solubilization for environmental remediation (Wattanaphon et al. 2008).

Laboratory-scale studies in degradation of pesticides are very important, since they allow the simulation of the behavior of pesticides in conditions that can be applied on field-scale. Mulbry et al., have developed a biofilter capable of handling about 15,000 dip-liter batches of dip waste containing the organophosphate pesticide, coumaphos. This biofilter was able to reduce the concentration of coumaphos from 2000 to 10 ppm in approximately 14 days at 25–29 °C, demonstrating the importance of the field-scale studies (Mulbry et al. 1998).

Since biodegradation is one of the most important techniques for the removal of pesticide residues, the following sections present studies on the biodegradation of organophosphate pesticides mainly by fungi and bacteria.

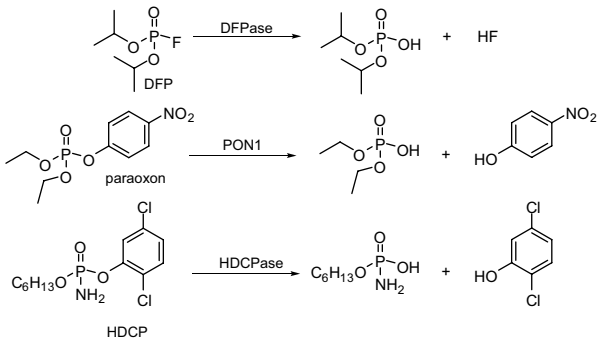


Fig. 3.5 Hydrolysis of organophosphate pesticides by phosphotriesterases (Sogorb and Vilanova 2002)

3.2.2 Microbial Degradation of Organophosphate Pesticides

Microbial degradation of organophosphate pesticides is of particular concern because this class of pesticide is highly toxic to mammals (Malghani et al. 2009). The OPs are mainly detoxified by oxidation and hydrolysis reactions. The main enzymes involved in the hydrolysis of these compounds are phosphotriesterases (PTEs) and carboxylesterases (CbEs), although the detoxification of OPs by PTEs is more efficient than by CbEs. The presence of PTEs has been described in a large variety of biological tissues of mammals, fish, bird, mollusk and bacteria (Sogorb and Vilanova 2002).

A wide variety of enzymes has been found with phosphotriesterase activities, including organophosphate hydrolase (OPH), methyl parathion hydrolase (MPH), organophosphorus acid anhydrolase (OPAA), diisopropylfluorophosphatase (DFP) and paraoxonase 1 (PON1) (Bigley and Raushel 2013). The PTE catalyzes the hydrolysis of OPs, which is the most significant step in detoxifying organophosphate compounds. The enzymatic hydrolysis of OP by PTEs is considered a detoxifying reaction because the resulting metabolites are usually less toxic than the original pesticides (Sogorb and Vilanova 2002). The Fig. 3.5 shows the enzymatic hydrolysis of diisopropylfluorophosphate (DFP), paraoxon and *O*-hexyl-*O*-2,5-dichlorophenyl phosphoramidate (HDCP). Usually, PTEs are called after the name of their substrate.

According to Bigley and Raushel, the OPs hydrolysis reactions proceed through an enzymatic mechanism at the active site of the phosphotriesterase. All of the PTEs require a divalent metal ion, and the active site presents three binding sites, which are generally hydrophobic and allocate the leaving group and the others substituent to position the phosphorus center for catalysis (Bigley and Raushel 2013).

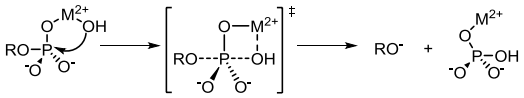


Fig. 3.6 Metal-dependent catalysis of enzyme phosphotriesterases (Gani and Wilkie 1995)

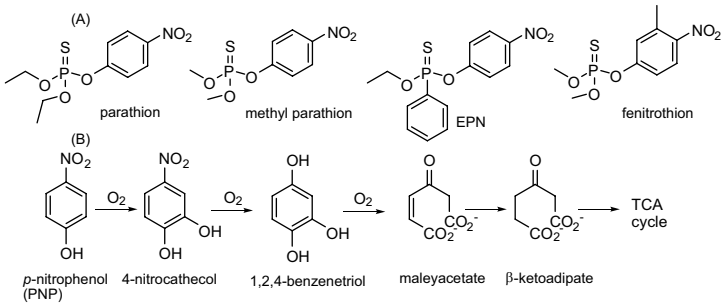


Fig. 3.7 (**a**) OPs containing the *p*-nitrophenol substituent. (**b**) Biodegradation of PNP by *Arthrobacter* sp. (Lei et al. 2004)

The OPs hydrolysis rate can be accelerate when the metal ion coordinates with the oxygen atom of the phosphoryl group, increasing the electrophilic character of the metal. It is suggested that the transition state involves the binding of the metal ion and a distortion of the phosphorus center in order to bring it closest to the hydroxide, yielding a pentavalent phosphorus intermediate (Bigley and Raushel 2013; Gani and Wilkie 1995; Domingos et al. 2003). Figure 3.6 shows how a metal ion can promote the reaction by correct positioning of the nucleophile to attack the phosphorus atom. This arrangement promotes an electrophilic increasing of the phosphorus atom by coordination of the metal ion to a phosphate oxygen atom (Domingos et al. 2003).

The hydrolysis of OPs by PTEs produces compounds that may be further degraded. Lei et al. studied the total degradation of *p*-nitrophenol (PNP), a substituent group present in OP derivatives paraoxon, parathion, methyl parathion, *O*-ethyl-*O*-4-nitrophenyl phenylphosphonothioate (EPN) and fenitrothion. According to that study, PNP was oxidized by the bacterium *Arthrobacter* sp., which degrades PNP by consuming the molecular oxygen (Fig. 3.7) (Lei et al. 2004).

The ability of particular microorganisms to hydrolyze OPs depends on the enzyme activity produced by these strains. Genetic engineering has been used to improve the capacity of bacterial strains to degrade OPs by increasing its enzyme activity. Several studies have followed this approach, since the engineered strain would be expected to generate a more stable enzymatic activity than the regular genes. Zheng et al. achieved an increased OP degradation by genetically engineered *Escherichia coli* cells expressing both OPHs and CbEs intracellularly. These enzymes were presented as a powerful degrading agent of OPs in residues of contaminated vegetables. It was demonstrated that washing the parathion-contaminated vegetables with the enzyme extract of the engineered *Escherichia coli* cell powder

produced a better detoxification (97 %) than that achieved by washing with commercially available enzyme product (94–86 %) (Zheng et al. 2007).

The two main approaches for pesticide detoxification by microorganisms are the use of whole cells or isolated enzymes. Enzymes can be immobilized and used in enzyme reactors for the detoxification of OPs. Both native and recombinant OPHs can be immobilized on nylon, porous glass and silica beads. In some cases, the enzyme extraction and purification can be difficult or expensive, so it becomes necessary to immobilize whole cells (Richins et al. 1997). However, the use of immobilized cells in a bioreactor does also have disadvantages, such as the outer membrane acting as a permeability barrier, preventing OPs from interacting with OPH enzymes residing within the cell (Yang et al. 2008).

The degradation depends on the pesticides transport into the cells, where the degrading enzymes reside. Even in recombinant strains with a high intracellular activity of pesticide-degrading enzymes, the overall detoxification rate may be limited by the transport mechanism. Therefore, OPH has been expressed at the cell surface using various anchoring strategies, thereby eliminating the need for enzyme purification and eliminating the transport limitation (Chen and Mulchandani 1998).

A developed method to anchor enzymes at the cell membrane of microorganisms was described by Richins et al. as a gene fusion system, in which the signal sequence and first nine amino acids of the lipoprotein (Lpp) were joined to a membrane domain from the outer membrane protein A (OmpA), which was fused with the enzyme of interest. This system was first used as anchor for β-lactamase at the cell surface. Using the same Lpp-OmpA fusion system as for β-lactamase, OPH was successfully anchored and displayed at the surface of *Escherichia coli* cells. In these studies, it was demonstrated that *E. coli* cultures with surface-expressed OPH degraded parathion and paraoxon very effectively, without the transport limitation observed in cells expressing OPH intracellularly (Richins et al. 1997; Chen and Mulchandani 1998). *E. coli* whole cells with surface-expressed OPH had seven times higher activity than whole cells expressing similar amounts of intracellular OPH, and it was more stable and robust than purified OPHs, retaining 100 % activity over a period of 1 month when incubated at 37 °C (Chen and Mulchandani 1998).

3.2.2.1 Bacterial Degradation of Organophosphate Pesticides

Organophosphate bacterial degradation has been more studied than fungal degradation. The most studied enzyme capable of hydrolyzing organophosphates pesticides is OPH, which is encoded by the *opd* (organophosphate-degrading) gene of *Flavobacterium* sp. ATCC 27551 and *Pseudomonas diminuta* MG (Zhang et al. 2008; Malghani et al. 2009; Schofield and DiNovo 2010). The native OPH enzyme contains two Zn^{2+} ions, but both divalent cations can be replaced by Cd^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+} without loss of catalytic activity (Benning et al. 2000; Scott et al. 2008). The proposed catalytic mechanism of this enzyme is shown in Fig. 3.8. As mentioned before, the transition state involves bringing the phosphorus center close to the hydroxyl group from an amino acid residue at the active site and the formation

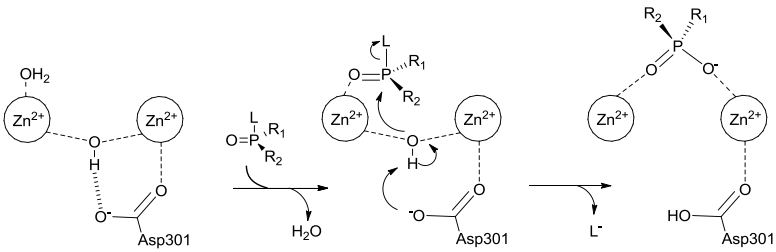


Fig. 3.8 Mechanism of OPH containing Zn^{2+} (Bigley and Raushel 2013)

of a pentavalent phosphorus species, by the formation of a complex between zinc and the organophosphate substrate (Bigley and Raushel 2013).

OPH has broad organophosphate hydrolase activity and is able to cleave various phosphorus-ester bonds (P-O, P-CN, P-F, P-S) (Schofield and DiNovo 2010). Another very similar enzyme has been identified in the soil bacterium, *Agrobacterium radiobacter* (OpdA). Like the OPH, OpdA displays extraordinary catalytic efficiency for OPs; for instance, the k_{cat}/K_m of OpdA for the pesticide methyl parathion is in the order of 3×10^6 s^{-1} M^{-1} (Scott et al. 2008; Bigley and Raushel 2013).

Researchers have found bacterial species, such as *Pseudomonas diminuta*, *Flavobacterium* sp. and *E. coli*, possessing the organophosphate hydrolase enzymes playing an important role in OPs degradation, since the enzymatic hydrolysis rates are 40–2450 times faster than chemical hydrolysis by 0.1 N NaOH at 40 °C and the activity of these enzyme are stable at temperatures up to 45–50 °C (Malghani et al. 2009; Richins et al. 1997).

Although the enzymatic hydrolysis reaction, in most cases, reduces the toxicity of OPs by converting them into less toxic metabolites, these metabolites also present a potential source of contamination to the environment. A very interesting mechanism of complete pesticide degradation was proposed by Mattozzi et al. describing the metabolic engineering of *Pseudomonas putida* strain to hydrolyze paraoxon and mineralize the hydrolysis products into sources of carbon and phosphorus. Previous studies reported that the hydrolysis of paraoxon to *p*-nitrophenol (PNP) and diethyl phosphate (DEP), by an OPH from *Flavobacterium* sp. strain ATCC 27551, reduces the toxicity of the pesticide 100-fold (Mattozzi et al. 2006).

Although PNP is much less toxic than paraoxon, it is still classified as a persistent and toxic contaminant. In the environment, PNP is degraded by few microorganisms, mainly by the bacteria *Moraxella* sp. and *Pseudomonas* sp., while the metabolite DEP has been ignored because it is relatively inert in the environment. The complete mineralization of paraoxon with degradation of its hydrolysis products involves a complex enzymatic route and it initializes with the hydrolysis of the pesticide by organophosphate hydrolase from *Flavobacterium* sp. into the two primary intermediates, DEP and PNP. Diethyl phosphate is further converted to orthophosphate by an enzymatic reaction with phosphodiesterase (Pde) from *Delftia acidovorans,* followed by a reaction with an alkaline phosphatase (PhoA) from *Pseudomonas aeruginosa*. PNP is converted to β-ketoadipate by a five-enzyme process (encoded

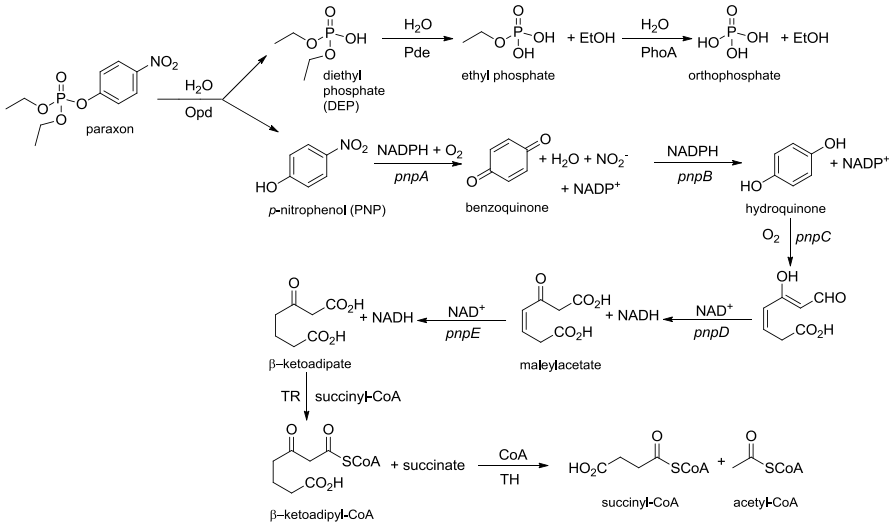


Fig. 3.9 Biodegradation of paraoxon by engineered bacteria (Mattozzi et al. 2006)

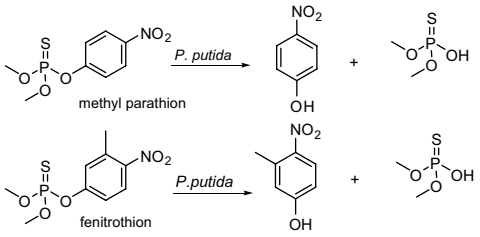


Fig. 3.10 Degradation of methyl parathion and fenitrothion by *P. putida* JS444 (Yang et al. 2008)

by *pnpA* to *pnpE*) from *Pseudomonas* sp. strain ENV2030. β-Ketoadipate enters the tricarboxylic acid cycle as succinyl-CoA and acetyl-CoA after conversion by *Pseudomonas putida*'s native β-ketoadipate, succinyl-CoA transferase (TR) and β-ketoadipyl-CoA thiolase (TH) (Fig. 3.9) (Mattozzi et al. 2006).

Thus, it has been observed that by different pathways, bacterial enzymes are able to completely degrade OP pesticides. There are other bacterial enzymes capable of degrading this class of pesticides, which include methyl parathion hydrolase (MPH) and organophosphate anhydrase (OPAA). MPH has an OP degrading gene called *mpd*, which was isolated from a methyl parathion-degrading *Plesiomonas* sp., showing no homology to the known *opd* genes (Yang et al. 2008).

Yang et al. reported experiments in which MPH was anchored onto the surface of *Pseudomonas putida* JS444. In this study, the *P. putida* JS444 cells with surface-displayed MPH showed higher activity for dimethyl OPs, such as methyl parathion and fenitrothion, than OPH-displaying cells, demonstrating that *P. putida* JS444 with surface-expressed MPH may be a good low cost option for the bioremediation of pollution caused by dimethyl OPs (Fig. 3.10) (Yang et al. 2008).

There is a wide range of bacteria capable of degrading different types of organophosphate substrates, providing environmentally-friendly decontamination strategies at low cost and effective methods for pesticide degradation. Thus, bacteria are promising sources of enzymes capable of promoting the biodegradation of OP pesticides.

3.2.2.2 Fungal Degradation of Organophosphate Pesticides

Fungi are known to degrade a wide variety of compounds. In this process, known as mycodegradation, the compound is broken down to smaller molecules (which may be toxic or non-toxic) or may be removed by a simple absorption or adsorption mechanism (Ramadevi et al. 2012). Bacterial degradation studies have proposed possible mechanisms for the degradation of OP (Krzysko-Lupicka et al. 1997), however, the mechanism of fungal degradation of these compounds is less well established than the bacterial one, since there are few studies reporting OP degradation by fungi.

Filamentous fungi of the genus *Aspergillus* have been used in OPs biodegradation. *Aspergillus niger* showed high biodegradation activity for malathion pesticide (Ramadevi et al. 2012), while *Aspergillus flavus* and *Aspergillus sydowii* were capable of degrading the pirimiphos-methyl, pyrazophos and malathion even at high concentrations (1,000 ppm) and utilizing them as sole phosphorus and carbon sources by releasing the phosphate moieties by the action of their phosphatases. In these studies, the fungal species presented both acid and alkaline phosphatases, yielding the hydrolytic detoxification of these pesticides (Fig. 3.11) (Hasan 1999).

As demonstrated for bacteria, there are studies in which fungal species are engineered to promote increased rates of OP degradation. Tang et al. studied 247 transformants of *Trichoderma atroviride* T23 strain for dichlorvos degradation. The dichlorvos degradation rates of the transformants ranged from 81 to 96 %, compared to 72 % of the parent strain (Fig. 3.12). Among the transformants, eight were improved by over 30 % in their degradation ability, showing that genetic engineering strategies can also be applied to OP biodegradation by fungi (Tang et al. 2009).

The enzymatic mechanism for OP transformation by various white-rot fungi was studied by Jauregui et al., showing that parathion was totally consumed by 18 strains of white-rot ligninolytic fungi. In addition, the three best strains were selected (*Bjerkandera adusta* 8258, *Pleurotus ostreatus* 7989, *Phanerochaete chrysosporium* 3641) to degrade parathion, terbufos, azinphos-methyl, phosmet and tribufos. All the OPs tested were depleted by the three fungal strains, even those containing thioether bonds, which make the pesticide resistant to bacterial biotransformation. Microsomal fractions obtained from *P. ostreatus* 7989 were used in biodegradation of several OPs (azinphos-methyl, phosmet, malathion, terbufos, trichlorfon) and their products were determined by GC-MS (Fig. 3.13). Although some OPs had been transformed by bacteria as discussed before, these bacterial enzymes have a limited capacity to cleave the thioether bond present in several OPs. For this and

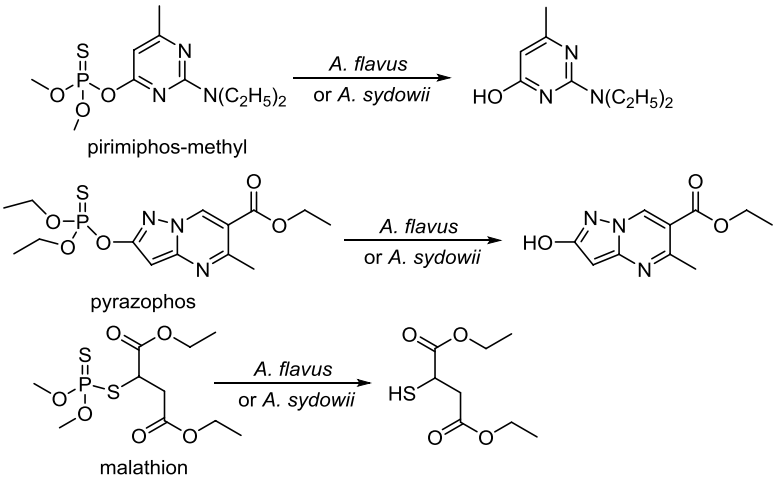
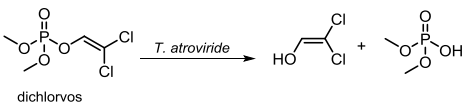


Fig. 3.11 Possible degradation of pirimiphos-methyl, pyrazophos and malathion by *A. flavus* or *A. sydowii*

Fig. 3.12 Degradation of dichlorvos by *T. atroviride* T23



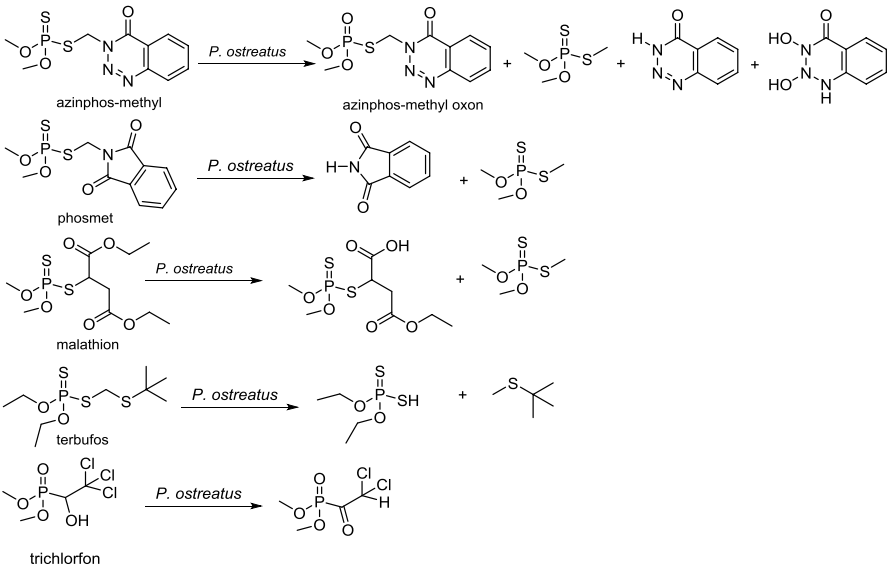


Fig. 3.13 Biodegradation of OPs by microsomal fraction from *Pleurotus ostreatus* 7989 (Jauregui et al. 2003)

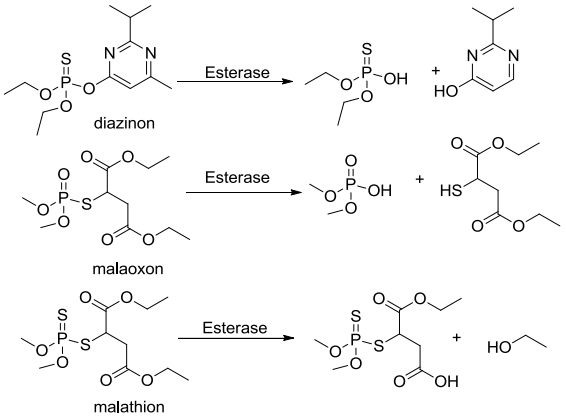


Fig. 3.14 Possible biodegradation of OPs by esterases

other classes of pesticides, fungal biotransformation seems to be a more promising strategy than bacterial transformation (Jauregui et al. 2003).

3.2.2.3 Other Types of Biodegradation of Organophosphate Pesticides

In addition to degradation by bacteria and fungi, there are enzymes from other organisms capable of degrading OPs. In the family of carboxylesterases, esterase 3 (E3) is an α,β-hydrolase fold enzyme obtained from the sheep blowfly, *Lucilia cuprina*. The species *Lucilia cuprina* and *Lucilia sericata* have become primary ectoparasites of sheep in farms all around the world, thus organophosphate insecticides has been widely used to control them, however, detoxification-mediated resistance was developed by these species. The resistance has been attributed to recently occurring E3 mutations, which affect the enzyme activity and is associated with diazinon and malathion resistance. The mutation causing diazinon resistance bestows a new OP hydrolase activity on the enzyme. The mutation also abolishes the native carboxylesterase activity of the enzyme, which then has greater activity towards diethyl-substituted phosphotriesters, as in diazinon, than dimethyl substituents, with a k_{cat} up to 0.05 min^{-1}. In contrast, the mutation causing malathion resistance confers a lower level of OP hydrolase activity and it has much less effect on carboxylesterase activity, with particular activity towards dimethyl substituted OPs such as malaoxon and malathion, presenting a k_{cat} up to 0.061 min^{-1} (Fig. 3.14) (Scott et al. 2008; Hartley et al. 2006).

Paraoxonase/arylesterase from human serum and other mammalian species catalyzes the hydrolysis of organophosphates, aromatic carboxylic acids and possibly carbamates (Primo-Parmo et al. 1996). Paraoxonase (PON1) is so called because paraoxon is the substrate commonly used to measure its enzyme activity (Mackness et al. 1998). The crystal structure of PON1 family of calcium-containing

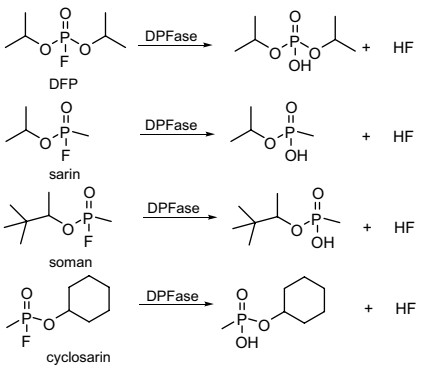


Fig. 3.15 Hydrolysis reaction of P-F bond by DFPase

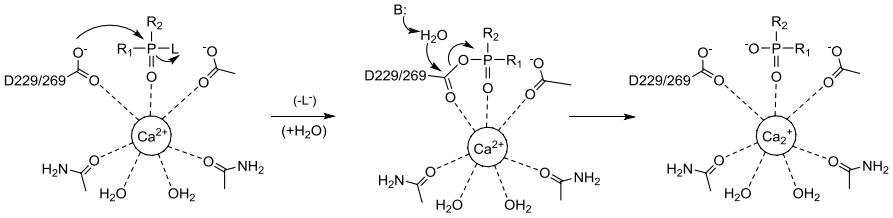


Fig. 3.16 Proposed mechanism for reaction catalyzed by PON1 and DFPase (Bigley and Raushel 2013)

hydrolases has a six-bladed β-propeller fold and broad substrate specificity, being able to hydrolyze γ- and δ-lactones, various aryl esters and a wide variety of organophosphates. These enzymes are also able to hydrolyze nerve agents as sarin, soman, tabun, cyclosarin, VX (Fig. 3.1). Although PON1 hydrolyzes a variety of substrates, investigations suggested that the native activity of this class of enzymes is the hydrolysis of lactones (Durrington et al. 2001; Bigley and Raushel 2013).

The diisopropylfluorophosphatase (DFPase) is found in cephalopod nerve, hepatopancreas and saliva from squid (Anderson et al. 1988). Structurally similar to PON1, the DFPase also is a calcium-containing hydrolase with a six-bladed β-propeller fold and readily hydrolysis the organophosphate fluoride, diisopropyl-fluorophosphate (DFP), as well as the nerve agents sarin, soman and cyclosarin, in addition to the cyanide-containing OP, the nerve agent tabun (Fig. 3.15). DFPase was initially found to hydrolyze P-F and P-CN bonds and to be inert toward P-O or P-S bonds (Bigley and Raushel 2013).

The proposed mechanism for phosphotriester cleavage by PON1 and DFPase is shown in Fig. 3.16. According to Bigley and Raushel, these enzymes present two calcium ions located in the core of the structure, however, only one participate in the active site. This is the only mechanism of phosphotriesterases where a single active site metal ion is employed in the catalysis. The pH profiles for both enzymes indi-

cate that a single amino acid side chain carboxylate group must be deprotonated at a *pKa* of 7 and the supposition for this mechanism is a simple attack by an activated water or hydroxide, as observed in OPH. Then, the catalytic deprotonated base promotes a nucleophilic attack on the phosphorus atom. In Fig. 3.16 the R_1 and/or R_2 are ester-linked alcohol or methyl groups. L is a leaving group, which is fluoride for DFPase and a fluoride, phenol or thiol for PON1 (Bigley and Raushel 2013).

Studies of OP biodegradation *in vivo* have been developed in mice. As reported by Duysen et al., the mouse blood contains four esterases capable of detoxifying organophosphorus compounds: carboxylesterase, butyrylcholinesterase, acetylcholinesterase and paraoxonase-1. Since OPs exert their toxicity by AChE inhibition, the CbE binds and inactivates OP poisons, reducing the number of OP molecules available for AChE inhibition. The mice (homozygous plasma CbE deficient $ES1^{-/-}$ mice and wild-type littermates) were treated with subtlethal doses of OP, and it was found that wild-type mice were protected from the toxicity of 12.5 mg/kg parathion, since they present a higher rate of CbE activity than the $ES1^{-/-}$ mice. However, both genotypes responded similarly to paraoxon, cresyl saligenin phosphate, diisopropylfluorophosphate, diazinon, dichlorvos, cyclosarin thiocholine, tabun thiocholine and carbofuran, demonstrating that plasma carboxylesterase has a minor role in protection against the toxicity of these pesticides. Unlike to the other pesticides, CbE showed to be harmful rather than protective to chlorpyrifos and chlorpyrifos oxon (Duysen et al. 2012).

Resistance to OP associated with natural occurring enzymes in animals provides in vivo detoxification, as well as different sources of enzymes capable of degrading this pesticide. These researches are important to a better study the mechanism of OPs poisoning in living beings, in order to determine the enzymes and genetic mutations involved in this process. In these studies, together with the bacterial and fungal biodegradation, it was described that there are several pathways and organisms capable of degrading organophosphate compounds in different levels, from the hydrolysis until complete mineralization of the pesticide, providing various strategies for the decontamination of this class of compounds.

3.3 Biodegradation of Pyrethroid Pesticides

3.3.1 Introduction

Synthetic pyrethroids have been developed to improve the specificity and activity of the natural insecticide pyrethrin, which are present in pyrethrum, an extract from the flowers of *Chrysanthemum cinerarifolium.* Pyrethroid pesticides have achieved remarkable effectiveness and set new standards for contact insecticides through improvements made during decades of research. Therefore, several structural modifications have been introduced over time in order to increase photostability, air stability and insecticidal activity (Sogorb and Vilanova 2002).

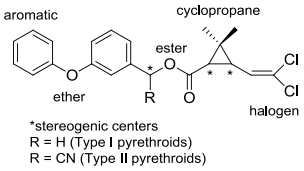


Fig. 3.17 Structure of cypermethrin showing distinct groups

According to Casida and Quistad, the most important pyrethroid improvements achieved so far were: "((*a*) photostability without compromising biodegradability; (*b*) selective toxicity conferred by target-site specificity and metabolic degradation; (*c*) modification of every part of the molecule with retention of activity; (*d*) maintenance of high insecticidal potency while minimizing fish toxicity; (*e*) development of compounds effective as fumigants and soil insecticides and (*f*) optimization of potency conciliated with the reduction of environmental contamination" (Casida and Quistad 1998).

One of these modifications was the addition of an α-cyano group to the alcohol moiety (cyanohydrin), considered a milestone in the development of synthetic pyrethrin analogs on account of its critical role in providing enhanced insecticidal activity. Pyrethroids containing the α-cyano group are called Type II, while compounds lacking this group are classified as Type I. Further modifications were performed, such as insertion of various halogens and hydrophobic chemical groups, besides the stereochemical arrangement of the molecule (Figs. 3.17 and 3.18). Currently, there are several structures, some of them are very different from the original structure of the pyrethrins, including structures without the dimethylcyclopropane ring and the central ester bond (Wolansky and Harril 2008; Soderlund et al. 2002; Sogorb and Vilanova 2002).

Insect resistance caused field failures of carbamate and organophosphate insecticides during the 1980s, thus generating the need for a new class of insecticides at the same time the pyrethroids were developed, showing good results in terms of effectiveness, low dose rates and fast action. Consequently, the use of pyrethroids grew rapidly in the 1980s, reaching around 20 % of the total insecticide market in 1986 and remaining at around 17 % until today, making them the third largest class of chemical insecticides, with a market value of 1,300 million dollar and 320 million hectares of treated agricultural area in 2007 (Housset and Dickman 2009).

Pyrethroids are used in all regions of the world, on almost all crops and all types of pests because they are effective, used at very low dose rates, have a broad spectrum of action, low cost (since only a small amount of product is applied) and relative safety for operators and consumers. In addition, this class of pesticide will continue to offer substantial benefits in modern agriculture, especially in the major emerging markets, where the demand for low-cost solutions will remain strong (Housset and Dickman 2009; Wirtz et al. 2009).

In general, synthetic pyrethroids are degraded by both abiotic and biotic pathways. Even though residues of synthetic pyrethroids have frequently been detected in soils, sediments, natural waters and agricultural products (Wang et al. 2011). The pyrethroid insecticides have low mammalian toxicity and persistence in

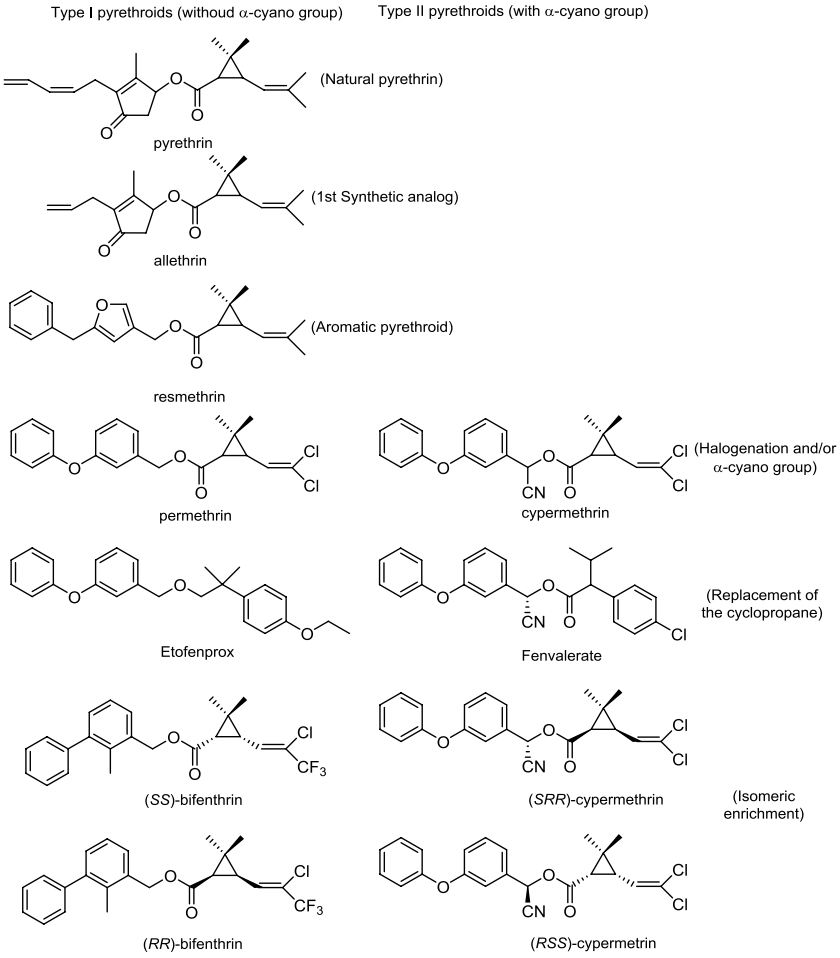


Fig. 3.18 Structure of natural pyrethrin and representative Type I and Type II pyrethroids

the environment. However, pyrethroids do affect the environment because they are active over a broad spectrum of insects and toxic to fish and aquatic organisms, causing a decrease in the natural control of pests and increasing the need for chemical control after initial applications (Kunz and Kemp 1994).

Pyrethroid insecticides have several types of effects on target and non-target species. For example, they induce alterations in the hematological profile of the fish *Channa punctatus* (Saxena and Seth 2002), in the reproduction and physiology of the carp *Cyprinus carpio* (Ayadin et al. 2005), in the ion conductance of *Bufo arenarum* nerve-cell membranes (Salibian and Marazzo 1995), in the increase in transmembrane sodium influx and inhibition of ion-dependent ATPases in the nervous tissues of insects, squid and toads (Berlin et al. 1984), in the induction of apoptosis in *Physalaemus biligonigerus* tadpoles (Izaguirre et al. 2000), and in testicular

tissues of rats (El-Gohary et al. 1999). Kumar et al. noted that all of these cellular processes are directly or indirectly regulated by host genes that might be interacting with pyrethroids, which are suspected of having genotoxic/carcinogenic potential (Kumar et al. 2008).

Pyrethroids have been reported to be carcinogenic in various models. However, the results of these studies are still controversial. According to Rusieck et al., permethrin had no association in insecticide applicators with all malignant neoplasms combined, melanoma, non-Hodgkin lymphoma, leukemia or cancer of the colon, rectum, lung or prostate, although a small number of cases of myeloma suggest further evaluation (Rusiecki et al. 2009). It is important to emphasize that cypermethrin showed increased sister chromatid exchange and chromosomal aberrations, showing cytotoxic and cytostatic effects in peripheral blood lymphocytes (Kocaman and Topaktaş 2009), while cyfluthrin caused an increase in chromosomal aberrations in human lymphocytes and bonemarrow cells of rats (Lla et al. 2008). There is strong evidence supporting an action mode of metofluthrin-induced hepatocellular tumors in male and female rats. However, this mode of action is similar to that demonstrated for phenobarbital, a drug with a strong epidemiological data for lack of carcinogenicity in humans. Consequently, the data strongly suggest that this mode of action would not promote tumors in humans exposed to metofluthrin (Yamada et al. 2009).

According to Bradberry et al. pyrethroids are 2,250 times more toxic to insects because of the high sodium channel sensitivity, small body size and low body temperature, whilst mammals are protected by low dermal absorption and rapid metabolism of these compounds. The mechanisms of pyrethroid toxicity are complex and become more complicated when they are combined with either piperonyl butoxide or organophosphorus insecticides, or both, as these compounds inhibit pyrethroid metabolism. Pyrethroids modify the gating characteristics of voltage-sensitive sodium channels to delay their closure, generating a protracted sodium influx and causing repetitive firing. At high pyrethroid concentrations, the sodium influx may be sufficiently great to prevent further action potential generation and cause 'conduction block'. Type II pyrethroids also decrease chloride current through voltage-dependent chloride channels and at relatively high concentrations, they can also act on GABA-gated chloride channels (Bradberry et al. 2005).

The characteristic symptoms of human poisoning by Type I pyrethroids are tremors, hypersensitivity, hyper-excitability, muscle cramps and seizures, while the main symptoms of poisoning by Type II pyrethroids are choreoathetosis, salivation, lachrymation, nasal hyper-secretion, hypersensitivity, cutaneous sensory disturbances (tingling, numbness and burning sensation), skin irritation, headache, loss of appetite, fatigue, dizziness, loss of consciousness, muscle cramps and seizures (Appel et al. 1994; Barrot 1996).

As described in this brief introduction, the pyrethroid pesticides are widely used and although these pesticides are useful, they are dangerous to living organisms. Therefore, the study of the degradation pathways (biotic or abiotic) is very important to prevent a harmful environmental impact and contamination of ecosystems, as occurred in the case of excessive use of organochlorine pesticides.

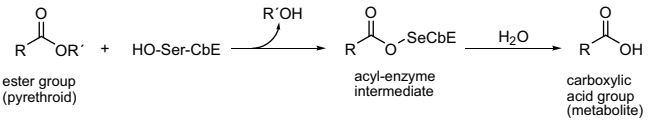


Fig. 3.19 Hydrolysis of carboxylic esters by CbE (Sogorb and Vilanova 2002)

3.3.2 Microbial Degradation of Pyrethroid Pesticides

Many species of pyrethroids-pesticide-degrading microorganisms have been isolated and characterized, including bacteria and fungi. The degradation pathways of some of these pesticides have been elucidated and enzymes responsible for breaking the central ester bond of the insecticide have been characterized (Wang et al. 2011). Structurally, the pyrethroids are carboxylic esters, so, the first step of biodegradation can be hydrolysis by carboxylesterases (CbEs), a reaction that eliminates the insecticidal activity. The target species and incidentally exposed microorganisms have generated new or enhanced existing enzymatic routes for the efficient degradation of many insecticides (Russel et al. 2011).

According to the systematic enzymes classification the International Union of Biochemistry and Molecular Biology, the hydrolytic enzymes, hydrolases, are included in group EC 3, and esterases are classified as subgroup 1 of hydrolases. The different subtypes of esterases are defined on the basis of the different types of ester bonds hydrolysed; thus CbEs are included within subgroup EC 3.1.1, which are able to hydrolyse carboxylic esters and are named as EC 3.1.1.1 (Sogorb and Vilanova 2002). The hydrolysis by CbEs is based on the reversible acylation of a serine residue. This acylation releases the alcohol moiety and generates an acyl-enzyme intermediate, which is later hydrolyzed by a water nucleophilic attack, releasing the carboxylic acid moiety and the regenerated enzyme (Fig. 3.19). After the hydrolysis, several steps are performed to metabolize the correspondent alcohol and carboxylic acid produced (Sogorb and Vilanova 2002).

Kinetic experiments have shown that the biodegradation of synthetic pyrethroids is well described by first-order decay kinetics, in both fungi and bacteria (Eq. 3.1).

$$c = c_{t=0} e^{-kt} \tag{3.1}$$

where k is the first-order rate constant, c is the concentration of the pyrethroid at time t and c ($t = 0$) is the initial concentration of the pyrethroid. The rate constant can be used to compare the speed of pyrethroids degradation by different microorganisms and to calculate half-life time (Liu et al. 2005; Chen et al. 2011a, b, c, d).

3.3.2.1 Bacterial Degradation of Pyrethroid Pesticides

A small number of bacteria capable of degrading synthetic pyrethroids have been isolated and characterized, including *Achromobacter* sp. (Maloney et al. 1988), *Bacillus cereus* (Maloney et al. 1993), *Pseudomonas fluorescens*, *Pseudomonas pseudoalcaligenes* (Halden et al. 2000), *Pseudomonas stutzeri* (Saikia et al. 2005), *Acidomonas* sp. (Paingankar et al. 2005), *Stenotrophomonas acidaminiphila* (Liu et al. 2005*), Micrococcus* sp. (Tallur et al. 2008), *Ochrobactrum tritici* (Wang et al. 2011), *Streptomyces aureus* (Chen et al. 2011a), *Stenotrophomonas* sp. (Chen et al. 2011b), *Sphingobium* sp. (Guo et al. 2009), *Achromobacter* sp. (Chen et al. 2011c), *Streptomyces* sp. (Lin et al. 2011) and *Ochrobactrum anthropi* (Zhai et al. 2012).

The bacterial biodegradation rate has been investigated by Chen et al., unfortunately, very few biodegradation first-order rate constant data are available in the literature. However, half-lives for the bacterial degradation of several synthetic pyrethroids in mineral salt medium have been published. It was observed that a pyrethroid that is most easily degraded by a given microorganism is not necessarily the same for other microbial species. Half-lives times were between 0.80 and 4 days (Table 3.2) (Chen et al. 2011a, b, c).

Some studies have shown that diastereoisomeric and enantiomeric selectivity is a common phenomenon in biodegradation of chiral pyrethroids by bacteria (Maloney et al. 1988; Liu et al. 2005; Wang et al. 2011). For example, the incubation of *Aeromonas sobria*, *Erwinia carotovora*, and *Yersinia frederiksenii* with permethrin and *Stenotrophomonas acidaminiphila* with bifenthrin showed that both *trans*-diastereoisomers were selectively degraded over the *cis*-diastereoisomers. In addition, the (*SS*)-*cis* enantiomers were preferentially degraded over the (*RR*)-*cis* enantiomers of these pyretroids (Fig. 3.20) (Liu et al. 2005). Similarly, *Bacillus cereus*, *Achromobacter* sp. and *Pseudomonas fluorescens* preferentially catalyzed the biodegradation of the *trans*-isomer of permethrin over the *cis*-isomer (Maloney et al. 1988). However, *Ochrobactrum tritici* could degrade the *cis*- and *trans*-permethrin at approximately the same rate and displayed approximately equal hydrolytic activity toward the two enantiomers of fenpropathrin (Wang et al. 2011).

The degradation pathways of cypermethrin, deltamethrin and fenpropathrin have been studied. The fenpropathrin metabolism pathway of *Achromobacte*r sp. (Fig. 3.21a) was proposed by Wang et al., it was suggested that fenpropathrin was hydrolyzed to 2,2,3,3-tetramethylcyclopropanecarboxylic acid and 3-phenoxybenzaldehyde, which was later converted to 3-phenoxybenzoic acid. The phenoxybenzoic acid was transformed to 3-(4-hydroxyphenoxy)benzoic acid, which was oxidized to 3,4-dihydroxybenzoic acid (protocatechuate) and *p*-hydroquinone. The protocatechuate was further oxidized through an *ortho*-cleavage pathway, and *p*-hydroquinone was degraded via the metabolite benzene-1,2,4-triol (Wang et al. 2011).

On the other hand, the transformation of 3-phenoxybenzoic acid to 3-(4-hydroxyphenoxy)benzoic acid was not detected in the degradation pathway of *Sphingobium* sp. JZ-2 studied by Guo et al., where 3-phenoxybenzoic acid split directly into protocatechuate and phenol (Guo et al. 2009). Similarly, the

Table 3.2 Half-lives (days) of several pyrethroids degraded by bacteria[a]

Bacteria	Deltamethrin	Cyfluthrin	Bifenthrin	Fenvalerate	Fenpropathrin	Permethrin	Cypermethrin	Cyhalothrin
Streptomyces aureus[b]	0.80	0.87	0.90	1.08	1.32	1.56	1.57	–
Stenotrophomonas sp.[c]	1.30	2.00	–	1.20	–	–	1.90	4.00
Achromobacter sp.[d]	1.30	2.50	–	1.80	–	–	2.00	3.00

[a] Half-life (days) determined from $c = c_{t=0}e^{-kt}$
[b] Chen et al. (2011a)
[c] Chen et al. (2011b)
[d] Chen et al. (2011c)

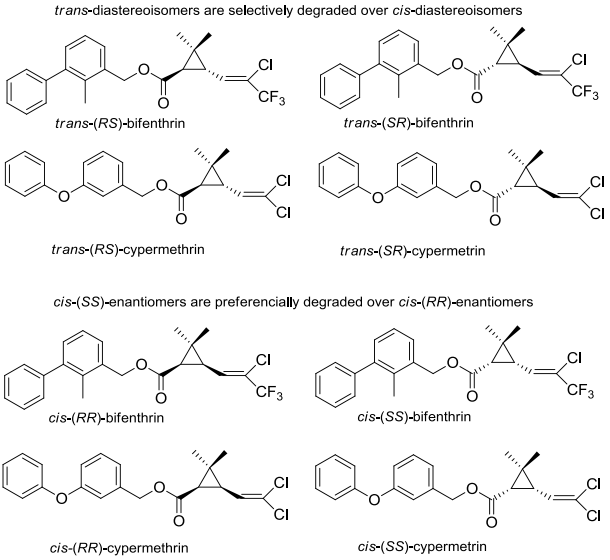


Fig. 3.20 Stereoisomers of the bifenthrin and cypermetrin (Liu et al. 2005)

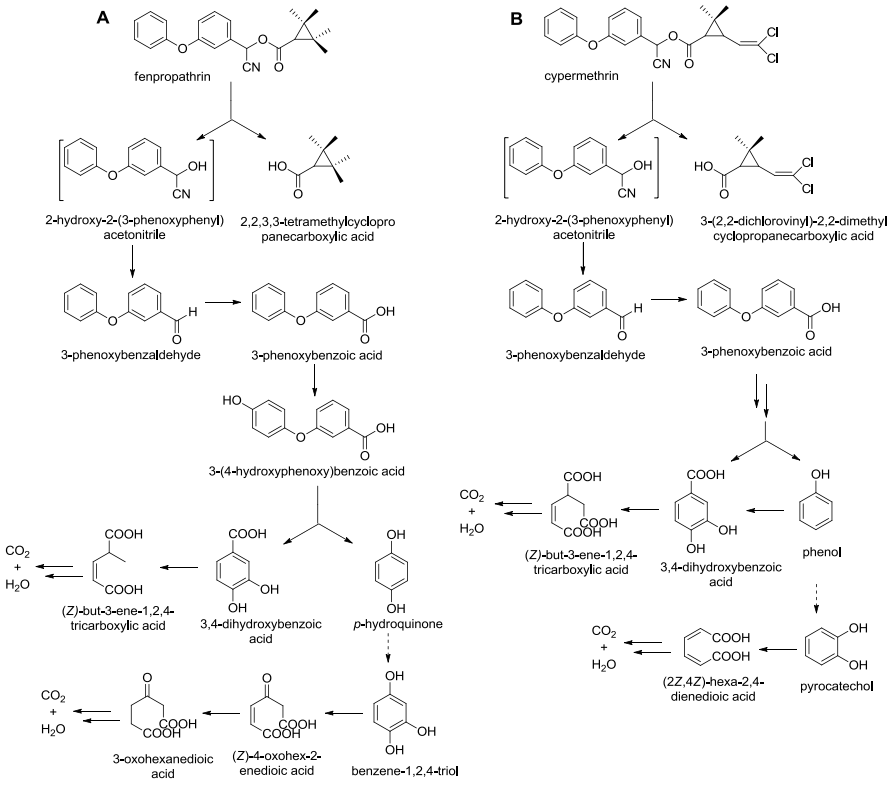


Fig. 3.21 (**a**) Biodegradations of fenpropathrin by *Achromobacte*r sp. (Wang et al. 2011) and (**b**) cypermethrin by *Micrococcus* sp. (Tallur et al. 2008)

cypermethrin degradation pathway by *Micrococcus* sp. (Fig. 3.21b) starts with the hydrolysis of the ester linkage to yield 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid and 3-phenoxybenzaldehyde. The 3-phenoxybenzaldehyde was oxidized to 3-phenoxybenzoic acid, which was cleaved to yielded protocatechute and phenol (Tallur et al. 2008).

Chen et al. showed that deltamethrin was degraded by hydrolysis of the carboxylic ester bond to produce α-hydroxy-3-phenoxy-benzeneacetonitrile and 3-phenoxybenzaldehyde. These intermediates were degraded by oxygenolysis to form 2-hydroxy-4-methoxybenzophenone and 1,2-benzenedicarboxylic acid mono esters, finally resulting in complete detoxication (Chen et al. 2011c).

Interesting studies were performed to evaluate the degradation of pyrethroids on soil, Chen et al. showed the degradation kinetics of fenvalerate and betacypermethrin by *Ochrobactrum* sp. DG-S-01 isolated from pyrethroids contaminated sludge. The results showed that the degradation followed first-order kinetics and the half lives of degradation were shortened by 1.5–30.4 h when compared with respective controls (Chen et al. 2011e). In another study, the biodegradation in various soils revealed that the bacterium *Stenotrophomonas* sp. strain ZS-S-01 could degrade 50 mg kg^{-1} of fenvalerate and 3-PBA with rate constants and half-lives ranged from 0.1418 to 0.3073 d^{-1} and 2.3 to 4.9 d, respectively. Compared with controls, half-lives of fenvalerate were reduced by 25.6 and 16.9 d, respectively, for sterilized and non-sterilized soils. In this study, the efficiency of fenvalerate degradation by the introduced cells was equal to 65.6 % degradation in sterilized soil and 65 % degradation in non-sterilized soil, respectively (Chen et al. 2011b).

Yu et al. performed another interesting work, in which fenvalerate-degrading ability of *Sphingomonas* sp. F-7 was examined in tea garden soil. It was observed that 16.9 mg kg^{-1} of fenvalerate was degraded in uninoculated fresh soil after 10 days. Whereas, 44.4 mg kg^{-1} of fenvalerate was removed from fresh soil inoculated with strain F-7. It is noteworthy that fenvalerate degradation was faster in non-sterilized soils than in sterilized soils indicating a contribution of indigenous flora to fenvalerate removal (Yu et al. 2013).

At the time of writing four pyrethroid-degrading enzymes of bacterial origin have been characterized, permethrinase from *Bacillus cereus* SM3 (Maloney et al. 1993), pyrethroid-hydrolyzing esterase (EstP) from *Klebsiella* sp. ZD 112 (Wu et al. 2006), pyrethroid hydrolase (PytH) from *Sphingobium* sp. strain JZ-2 (Guo et al. 2009) and pyrethroid-hydrolyzing carboxylesterase (PytZ) from *Ochrobactrum anthropi* YZ-1 (Zhai et al. 2012). These enzymes catalyzed the first step in the catabolic pathways. The molecular weights of PytZ and PytH were approximately 25 kDa and 31 kDa, respectively. These enzymes are smaller than the permethrinase (61 kDa) and pyrethroid hydrolase EstP (73 kDa). PytZ, PytH and EstP showed the same optimum pH of 7.5, while the permethrinase optimum pH was 7.0. Slightly different optimal temperatures were observed for PytZ (35 °C), PytH (40°), EstP (40°) and permethrinase (37 °C) (Maloney et al. 1993; Guo et al. 2009; Wu et al. 2006; Zhai et al. 2012).

PytH has greatest homology with members of the α/β-hydrolase fold superfamily of proteins and is likely to be a serine hydrolase, since it is inhibited by diethylpyro-

carbonate and phenylmethylsulfonyl fluoride and contains a conserved serine/aspartate/histidine motif. Similarly, PytZ also shows homology with members of the α/β-hydrolase superfamily and is likely to be a serine hydrolase, as well as inhibition studies suggested for *Bacillus cereus* permethrinase. On the other hand, EstP has no close homologues of known function in the sequence databases, and the lack of conserved serine hydrolase motif in its primary sequence suggests that it may operate by a different mechanism from that of PytH, PytZ and the *Bacillus cereus* permethrinase (Maloney et al. 1993; Guo et al. 2009; Wu et al. 2006; Zhai et al. 2012; Russel et al. 2011).

EstP and PytH are kinetically similar, with extremely low K_m values (in the range of 10 nm–1 μm) for a wide range of pyrethroid substrates. PytH has somewhat higher k_{cat} values than EstP, 0.4–3 s^{-1} for PytH compared to 0.01–1 s^{-1} for EstP. EstP was active with a broad range of ester containing substrates, albeit with K_m values 1,000 to 10,000 times greater than for pyrethroids. According to Russel et al., this observation may suggest that EstP has evolved specifically towards a pyrethroid hydrolase activity (Russel et al. 2011).

Just as previous studies showed, isomer selectivity was a common phenomenon in biodegradation of pyrethroids in soil, as observed for the permethrinase enzyme, which has a strong preference for the *trans*-isomer of permethrin. However, EstP, PytH and PytZ appear to have no isomer specificity (Maloney et al. 1993; Guo et al. 2009; Wu et al. 2006; Zhai et al. 2012; Russel et al. 2011).

There have been more biodegradation studies of pyrethroid pesticides by bacteria than by fungi; consequently, the biodegradation pathway for bacterial degradation is better understood, since some metabolites and even biodegradation pathways have been elucidated. However, the next section reviews the researches results on fungal biodegradation of these pesticides.

3.3.2.2 Fungal Degradation of Pyrethroid Pesticides

Microorganisms are the main agents for biodegradation of molecules that generate environmental concern, since bacteria and yeasts seem to be the dominant degraders in aquatic ecosystems, while filamentous fungi and bacteria are the primary decomposers in soils. However, only a few examples of fungi capable of degrading pyrethroids have been isolated and characterized, such as *Trichoderma viride* (Saikia and Gopal 2004), *Aspergillus niger* (Liang et al. 2005), *Sepedonium maheswarium* (Mukherjee and Mittal 2007), *Aspergillus oryzae* (Liu et al. 2011) and *Cladosporium* sp. (Chen et al. 2011d).

Cladosporium sp. cultures performed by Chen et al. showed the degradation of 90 % of a mixture of pyrethroids (fenvalerate, fenpropathrin, β-cypermethrin, deltamethrin, bifenthrin, and permethrin). The concentration of fenvalerate (100 mg. L^{-1}) decreased over time and completely disappeared after 5 days and simultaneously yielding 3-phenoxybenzaldehyde, 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile and 2-(4-chlorophenyl)-3-methylbutanoic acid. The intermediate 3-phenoxybenzaldehyde was the major metabolite. However, both metabolites were

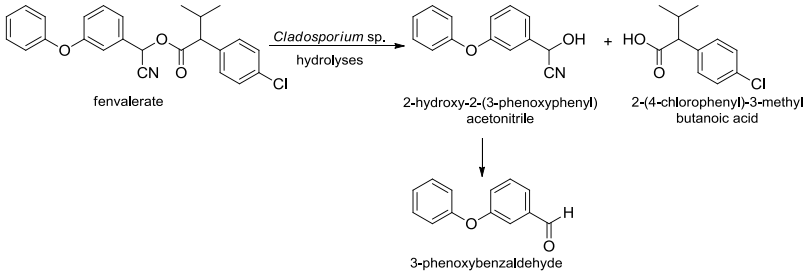


Fig. 3.22 Biodegradation of fenvalerate by *Cladosporium* sp. (Chen et al. 2011c)

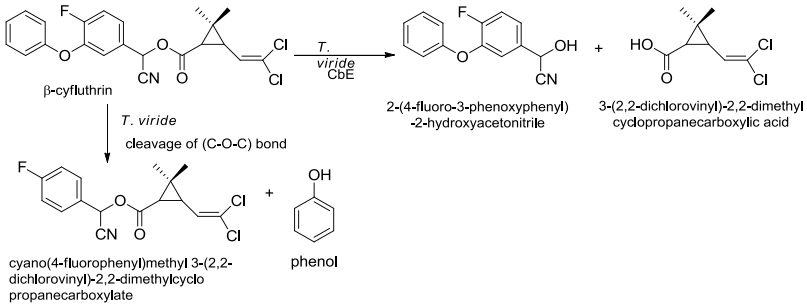


Fig. 3.23 Biodegradation of β-cyfluthrin by *T. viride* (Saikia and Gopal 2004)

transient, evidencing their total degradation. Optimization of the culture conditions for fenvalerate degradation by *Cladosporium* sp. was performed, rendering the optimum conditions of 26.2 °C and pH 7.2 (Fig. 3.22) (Chen et al. 2011d).

Cultures of *Trichoderma viride* catalyzed the biodegradation of β-cyfluthrin, producing 2-(4-fluoro-3-phenoxyphenyl)-2-hydroxyacetonitrile and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid, as a result of a carboxylesterase activity. In addition, the cleavage of the ether bond was also observed by Saikia and Gopal, resulting in cyano(4-fluorophenyl)methyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate and phenol. The ester cleavage metabolite was produced in more significant yield, since ether bonds are not easily cleaved, even with chemical agents (Fig. 3.23) (Saikia and Gopal 2004).

Very few kinetic data on fungal biodegradation are available, but Chen et al. showed that the half-lives ($T_{1/2}$) of fenvalerate, fenpropathrin, β-cypermethrin, deltamethrin, bifenthrin and permethrin were 0.99–1.54 days, when degraded by carboxylesterase from *Cladosporium* sp. For *Trichoderma viride* the $T_{1/2}$ of β-cyfluthrin was 7 days, while half-lives for *Aspergillus nidulans* and *Sepedonium maheswarium* were 11 and 19 days, respectively (Mukherjee and Mittal 2007; Chen et al. 2011d; Saikia and Gopal 2004).

To date, just one pyrethroid-degrading from fungi enzyme have been isolated and studied. Liang et al. have isolated and characterized the hydrolase from *Aspergillus niger*, which catalyzed the hydrolysis of permethrin, possessing

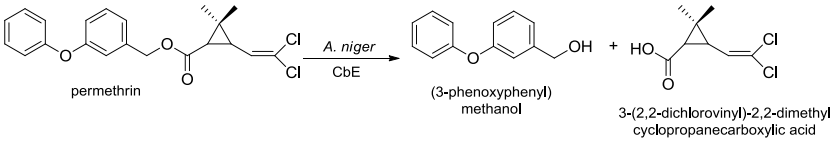


Fig. 3.24 Biodegradation of permethrin by *A. niger* (Liang et al. 2005)

approximately equal activity toward both isomers (*cis* and *trans*) and producing equimolar amounts of (3-phenoxyphenyl)methanol and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (Fig. 3.24). The hydrolase was significantly affected by sulfhydryl oxidant metals (Hg^{2+}, Ag^{+}) and thiol-modifying reagents such as *p*-chloromercuribenzoate, suggesting that sulfhydryl groups may be involved in the catalytic center of the enzyme, substrate binding and/or recognition site. EDTA and 1,10-phenanthroline did not affect activity, indicating that divalent cations are not required for enzyme activity. The purified enzyme not only hydrolyzed various *p*-nitrophenyl esters of short-medium chain fatty acids (*p*-nitrophenyl acetate, propionate, butyrate, valerate, caproate, capryate and laurate), but also degraded many pesticides with similar carboxylester bonds, such as cypermethrin, permethrin, fenvalerate, deltamethrin, and malathion. This last is an organophosphorus pesticide, indicating that the hydrolase of *Aspergillus niger* is an esterase with broad specificity (Liang et al. 2005).

The biodegradation of pyrethroid pesticides by fungi is poorly studied in the literature; consequently, the biodegradation pathways are not well elucidated, since few metabolites have been identified. The study of the produced metabolites is very important because they can also be toxic compounds, like 3-phenoxybenzaldehyde, which is recalcitrant to microbial degradation, has higher mobility than the parent compounds and causes widespread contamination in soil. Furthermore, 3-phenoxybenzaldehyde is classified as an endocrine-disrupting chemical, owing to its antiestrogenic activity. Therefore, effective methods must be developed to eliminate the pesticides and their metabolites generated in the environment (Chen et al. 2012). An interesting property of pyrethroids fungal biodegradation is the ether cleavage revealed by Saikia and Gopal, proving that the ester cleavage is not the only first step of the biodegradation pathway (Saikia and Gopal 2004).

3.3.2.3 Other Types of Biodegradation of Pyrethroid Pesticides

As reported by Russel et al., carried out metabolic studies showed that pyrethroids are mainly metabolized by oxidation and ester cleavage, which are mediated by cytochrome isoforms and carboxylesterases, respectively (Russel et al. 2011). The biodegradation of synthetic pyrethroids is involved in the resistance of insects, in addition carboxylesterases, cytochromes P450 (CYPs) and GSTs (glutathione-*S*-transferases) have all been implicated in this resistance. Intense bands of carboxylesterase activity in nondenaturing polyacrylamide gel had been associated with resistance to synthetic pyrethroids in various species, however, resistance

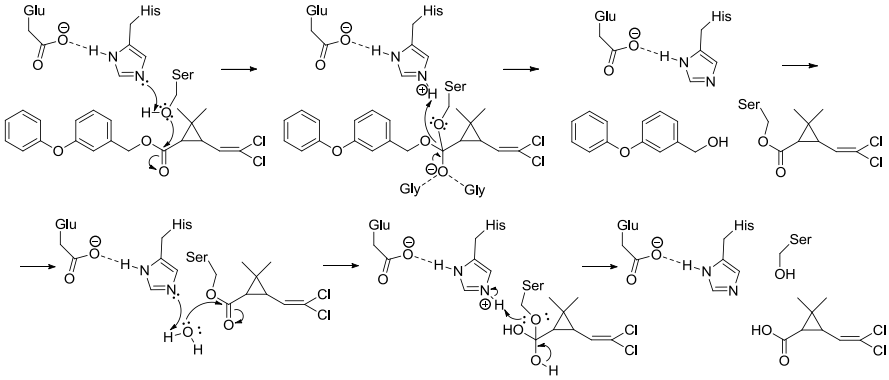


Fig. 3.25 Mechanism for the carboxylesterase-mediated hydrolysis of permethrin (Wheelock et al. 2005)

mechanisms have not yet been elucidated at the molecular level (Oakeshott et al. 2010; Farnsworth et al. 2010). Cytochrome P450-mediated synthetic pyrethroid resistance is generally attributed to over-expression via up-regulation of CYP genes (Wheelock and Scott 1992; Zhang and Scott 1996; Feyereisen 2005; Muller et al. 2008), while GSTs are involved in sequestration of synthetic pyrethroids and/or detoxification of lipid peroxidation products induced by synthetic pyrethroids, rather than direct metabolism of them (Li et al. 2007). Insects need to generate new, or enhance existing xenobiotic detoxification mechanisms, so that they can degrade insecticides efficiently at generally low but physiologically significant concentrations (Russel et al. 2011).

According to Wheelock et al., the general catalytic mechanism of mammalian carboxylesterases (Fig. 3.25) involves a catalytic triad of residues, consisting of a Serine (Ser), Histidine (His) and either Glutamic Acid (Glu) or Aspartic Acid (Asp). A proton is transferred to the His from Ser, increasing the nucleophilicity of the Ser terminal hydroxyl group, after that the histidine is stabilized by a hydrogen bond formed between it and the Glu (or Asp). The Ser nucleophile attacks the electron-deficient carbonyl moiety in the ester substrate, forming a tetrahedral intermediate, which is stabilized by two Glycine residues in the oxyanion hole. This intermediate collapses to form the acyl-enzyme complex, releasing the Ser and the alcohol portion of the substrate in the process. A His-activated water molecule then attacks the acyl-enzyme complex, repeating the above steps and releasing the acid portion of the substrate (Wheelock et al. 2005).

According to Mikata et al., the metabolism of bifenthrin, allethrin, resmethrin, β-cyfluthrin, cypermethrin, *cis*-permethrin, and *trans*-permethrin was examined in rat and human hepatic microsomes (Scollon et al. 2009). The intrinsic hepatic clearance of the pyrethroids was 5–15-fold greater to rat than to human microsomes, except for *trans*-permethrin, which showed approximately 45 % greater clearance in human microsomes. The metabolism of bifenthrin, allethrin and *cis*-permethrin in rat and in human hepatic microsomes was solely the result of oxidative processes,

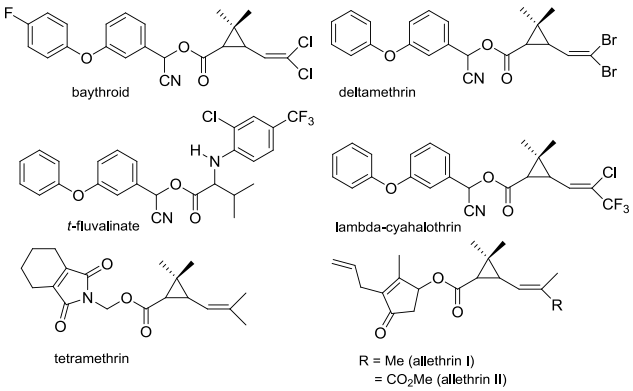


Fig. 3.26 Chemical structures of pyrethroid pesticides

while the metabolism of resmethrin and cypermethrin in human hepatic microsomes was solely the result of hydrolytic processes. Resmethrin and cypermethrin in rat hepatic microsomes and β-cyfluthrin and *trans*-permethrin in microsomes from both species were metabolized by both oxidative and hydrolytic pathways (Mikata et al. 2012).

The expression of carboxylesterases is ubiquitous in mammals. The highest hydrolase activity is present in the liver, but CbEs are also detected in small intestine, kidney and lung tissues (Satoh and Hosokawa 2006). Pure human CbEs (hCE1 and hCE2) were used to study the hydrolytic metabolism of the following pyrethroids; the (*RS*)-*trans*-resmethrin, (*RS*)-*trans*-permethrin, and (*RR*)-*cis*-permethrin. The human enzymes hCE1 and hCE2 hydrolyzed *trans*-permethrin more efficiently than *cis*-permethrin, whereas, hydrolysis of resmethrin was catalyzed efficiently by hCE1, but not by hCE2 (Ross et al. 2006). Structure-selective hydrolysis of several pyrethroids by human liver microsomes and recombinant hCE1 and hCE2 has been reported. Type I pyrethroids were generally hydrolyzed at high rates by liver microsomes. On the other hand, the majority of the pyrethroids hydrolyzed at lower rates were Type II compounds. The hCE1 preferentially hydrolyzed allethrin, resmethrin, deltamethrin, esfenvalerate and λ-cyhalothrin. However, hCE2 showed higher or similar hydrolysis activity toward baythroid, bifenthrin, *cis*-permethrin, *trans*-fluvalinate and tetramethrin than hCE1 (Yang et al. 2009; Mikata et al. 2012) (Fig. 3.26).

3.4 Conclusions

In this chapter, the most important biological methods described in the literature for the biodegradation of organophosphate and pyrethroid pesticides have been presented. Although these methods are very promising, it is not easy to eliminate the released metabolites by them into the environment. There are still serious problems concerning the contamination of soil, water and even foods. Pesticides, despite

the great benefits they bring to agricultural productivity, they also cause serious problems of contamination and increasingly need to be studied, especially in the search for compounds that are less harmful to the environment. Knowledge of the biodegradation route of pesticides and the development of new techniques that allow the improvement of these degradation pathways are essential. However, it is noteworthy that variations on results of in vivo experiments can be explained by the significant difference from in vitro experiments.

Fortunately, genetic engineering and biochemical techniques advances continue to be made in the field of pesticide biodegradation, which may enable a decontamination process that is friendly to the environment.

Another important aspect of biodegradation of organophosphate and pyrethroid pesticides by biological processes is high capacity of enzymatic system in degrading the recalcitrant metabolites, such as phenolic and carboxylic acids, formed after the hydrolysis reactions. Several metabolites are highly toxic and must be completely degraded, or eliminated by conjugation reactions catalyzed by oxidative enzymes. Therefore, there is no other method as efficient in degrading the pesticides and its metabolites as biological degradation, particularly by fungi and bacteria.

Finally, the micro-organisms are distributed throughout the environment, on the surface of soil, deeper regions, ocean, lakes and mangroves. Therefore, the microorganisms are responsible for recycling a large amount of organopollutants that are dumped in agricultural spraying, eliminating the xenobiotics and metabolites that cause serious problems for humans and the environment.

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Chapter 4
Non-target UHPLC/MS Analysis of Emerging Contaminants in Water

Fabio Gosetti, Eleonora Mazzucco, Maria Carla Gennaro, and Emilio Marengo

Contents

Abstract Contamination of water resources is one of the major problems to be faced for environment preservation and sustainability. Although anti-pollution strategies taken in the last half-century have consistently reduced in surface water the amount and the presence of many recognised contaminants, other potentially hazardous chemicals are being released into the environment, together with new substances that are continuously synthesized and whose dangerous properties are not well known. Water-pollutant monitoring makes typically use of methods developed for target analysis, focused on priority pollutants. The monitoring of target-

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compounds based on mass spectrometry (MS) and selected reaction monitoring (SRM) mode is often insufficient to definitely assess the quality of surface water, just because the presence of only a limited number of potential pollutants is considered. Also potentially harmful non-target pollutants simultaneously present must be taken into account.

In the determination of semi-polar and polar pollutants, liquid chromatography coupled with tandem MS is generally the technique of choice. To obtain a complete information on water composition, full-spectrum acquisition techniques, better if with the possibility to obtain information about MS/MS spectra, are required. For this purpose, hybrid mass spectrometers like triple quadrupole/linear ion trap (QqLIT), hybrid quadrupole/time-of-flight (QqTOF) MS and linear ion trap/orbitrap analyzer must be used. The last two instruments are the masters of the non-target approach, because their can offer the advantage of high resolution MS, that combined with a good MS accuracy allows to identify the unknown species. In addition, the hybrid nature of such instruments allows to acquire during a single chromatographic run also information about high resolution MS/MS spectra, that are fundamental in order to attribute to each unknown species a probable chemical structure. An interesting advantage associated with high resolution MS based methodologies consists in the possibility of performing retrospective analysis, since it allows the identification of organic contaminants included in the first screening: this investigation can be done at any time, without the need of new analysis or new sample injection.

In addition, the elevated acquisition speed of TOF makes it compatible with ultra high performance liquid chromatography (UHPLC). UHPLC and high resolution MS provide potent analysis rich in information on sample composition. During a non-target screening analysis, all the compounds eluted from the analytical column can be detected without any kind of selection, a part the obvious limitations derived from chromatography and ionization process in the LC-MS interface. A genuine non-target analysis involves the automated component detection from the total ion current and the use of deconvolution software to detect the presence of multiple species and to produce pure spectra for each individual component. An important limitation to this approach is the lack of availability of large compound libraries similar to those used in gas chromatography-mass spectrometry (GC-MS), making the identification of unknown species very complex and time-consuming. At the moment, spectral libraries for LC-MS are home-made and quite limited. This review presents an overview of published UHPLC-MS methods developed for post-target and non-target screening analysis of water emerging contaminants, such as pesticides and their degradation products, pharmaceuticals and drug side-reaction products, surfactants and illicit drugs. The different aspects of the current MS instrumentations, using tandem, hybrid and high resolution MS systems, are compared and discussed.

Keywords Data mining • Deconvolution software • Degradation products • Emerging contaminants • High resolution mass spectrometry system • Hybrid mass spectrometry system • Metabolites • Non-target analysis • Screening analysis • Transformation products • Surface water • UHPLC • Unknown compounds

List of Abbreviations

APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
CAD	collision activated dissociation
CE	collision energy
CID	collision induced dissociation
DDA	data dependent acquisition
DRE	dynamic range enhancement
EMS	enhanced mass scan
EPI	enhanced product ion
ESI	electrospray ionization
FFT	fast Fourier Transforms
FT-ICR	Fourier transform ion cyclotron resonance
FT-MS	Fourier transform mass spectrometry
FWHM	full width at half maximum
GC-MS	gas chromatography-mass spectrometry
HPLC-MS	high performance liquid chromatography-mass spectrometry
HR	high resolution
HRMS	high resolution mass spectrometry
IDA	information-dependent acquisition
IT	ion trap
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LIT	linear ion trap
LOD	limit of detection
LOQ	limit of quantification
LR	low resolution
LTQ-Orbitrap	linear ion trap/orbitrap
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NIST	National Institute for Standards and Testing
NL	neutral loss
NMR	nuclear magnetic resonance
nw-XIC	narrow-window extracted ion chromatogram
PI	precursor ion
Q	quadrupole
QqLIT	hybrid triple quadrupole/linear ion trap
QqTOF	hybrid quadrupole/time-of-flight
QqQ	triple quadrupole
RDB	ring double bond
SPE	solid phase extraction
SRM	selected reaction monitoring
TDC	time-to-digital converter
TIC	total ion current

TOF	time of flight
TP	transformation product
UHPLC	ultra high performance liquid chromatography

4.1 Introduction

Typically, water-pollution monitoring makes use of gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) methods developed for target analysis, that generally concerns priority pollutants legally regulated or of public concern (Ibáñez et al. 2008). These methods rarely consider several tens of analytes, being quite unusual analytical methods considering more than 100 organic pollutants. This is because there exists a physical limit to the number of analytes that can be sought in a chromatographic time window or segment. In addition, in multi-residue methods, most of the analytes are detected in the middle section of the run (Soler and Picó 2007). The reduced dwell times (i.e. 5 ms) used to attain a large number of species screened within a defined time window may easily yield low-quality chromatographic-shape peaks, due to the reduced number of acquisition points; this not only reduces sensitivity but also hampers the unambiguous identification and, especially when concentration levels are low, easily produces false negatives.

Therefore, the target-compound monitoring based on mass spectrometry (MS) methods that use single reaction monitoring (SIM) or selected reaction monitoring (SRM) modes, is often insufficient to assess the quality of environmental water, because only a limited number of analytes are recorded (rarely more than 100 compounds), while unknown and potentially harmful micro-contaminants present might represent a threat to environment and human health.

During past decades, the use of chemicals has tremendously increased as a consequence of the increased number of industrial products used in agriculture and everyday life (Hogenboom et al. 2009). The aim of synthesise chemicals with the properties to be less persistent, less bioaccumulating (because less hydrophobic) and less toxic may produce chemicals with higher mobility in aqueous media, with the risk of polluting aquatic and almost pristine environments (Fig. 4.1). As a consequence, the drinking water companies are facing stronger demands on removal processes of hydrophilic compounds, potentially toxic and difficult to remove from water. A threat to environmental ecosystems is also represented by emerging contaminants such as pharmaceuticals, hormones, endocrine distruptors, perfluorinated compounds, flame retardant, plasticizer, personal care products, impurities from commercial formulations, surfactants, drugs of abuse, transformation products of pesticides. These compounds are structurally different and represent an heterogeneous group of chemicals not currently covered by regulations or legislation and not widely studied yet (La Farré et al. 2008). It is necessary that the general shift towards the use of more hydrophilic compounds in industrial and consumer applications requires the development of appropriate analytical methods that take into account

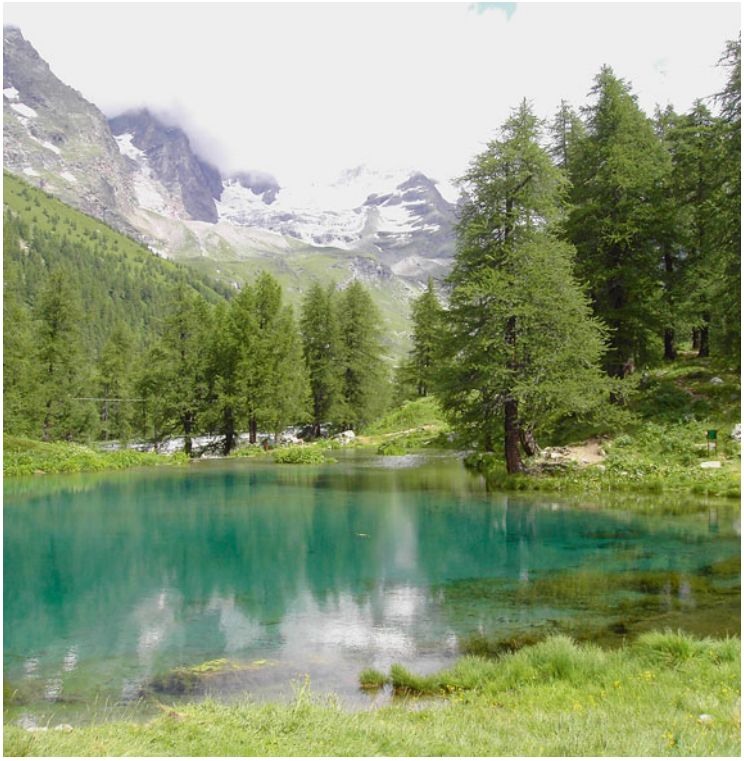


Fig. 4.1 The photo taken by Fabio Gosetti shows the wonderful Blue Lake (1980 m), Aosta Valley, Italy

the specific properties of the many different chemicals that finally end up in the environment and contaminate it. The new term "chemicalization" indicates the increased use of chemicals and the potential environmental contamination (Nurmi et al. 2012). The identification and determination of the emerging contaminants is always gaining increasing interest in the field of environmental research (Pedrouzo et al. 2009; Nödler et al. 2010; Bisceglia et al. 2010; Al-Odaini et al. 2010; Eggen et al. 2010). Furthermore, while increasing information is nowadays available for these new contaminants, scarce information is still accessible about their potential transformation products (Celiz et al. 2009; Kolpin et al. 2009). Transformation products and/or impurities from commercial formulations can be present at relevant concentrations (e.g. impurities in commercial formulations are typically in the range 10–15 %), and may be as toxic as the target compounds, or even more toxic. The unknown compounds and their main sources can be generally summarised as: (i) impurities present in commercial formulations, for example of pesticides, originating in the synthesis process; (ii) transformation products originating during storage or application of the commercial formulations; or, (iii) transformation products originating in environmental conditions. So for instance it was shown that organic contaminants can undergo transformations during the passage through the

wastewater treatment plants and also once they reach the aquatic environment (Andreozzi et al. 2003; Boreen et al. 2003). Hydrolysis ractions, photolysis and biotic transformations can lead to the formation of unexpected transformation products (TPs), that sometimes are even more toxic and persistent than the precursor compounds (Gómez et al. 2008; Trovó et al. 2009; Gosetti et al. 2010a, b).

These studies may take long time because are usually accomplished manually after a comprehensive visual inspection of the chromatograms (Agüera et al. 2005; Picó et al. 2007).

It must be underlined that studies that simulate natural conditions and that could provide a more realistic view of the problem are hampered by the complexity of the matrices, that makes difficult the identification of the non-target compounds, already unknown a priori.

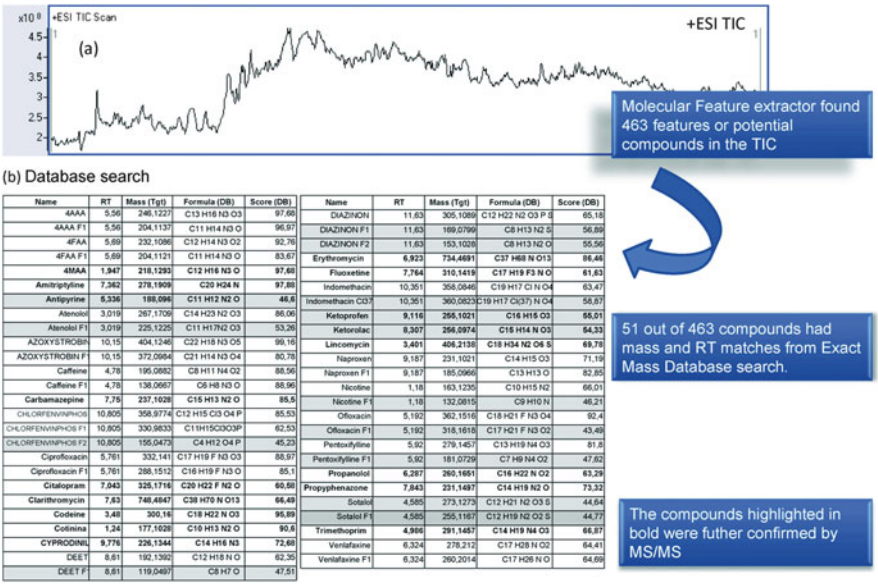
The focus of environmental analytical chemistry nowadays concerns the more polar compounds, because supported by the significant development of LC-MS techniques (Petrovic et al. 2010). Emerging contaminants, characterised by medium-to-high polarity and low volatility, are therefore properly identified and determined with these techniques, as well as their metabolites and transformation products, which are generally more polar than their precursor molecules.

The applicability of non-target analysis, i.e. full-scan screening, greatly depends on current technical developments. GC-MS methods have greatly contributed to the characterization of (semi)volatile and thermostable contaminants in water, whereas LC–MS methods have been utilized to extend the investigation of water contaminants to non-volatile, (highly) polar, and thermally labile compounds, such as for example pharmaceuticals, pesticides, endocrine disrupting compounds and personal care products (Richardson 2011).

If LC coupled with low resolution (LR) MS, in particular by using SIM or SRM mode is a great advantage in terms of sensitivity and selectivity, it is still unsuitable for not-target approach, whereas nominal full spectrum acquisition is often insufficient to obtain useful information about the unknown species, also taking into account that LC-MS libraries are few and often unreliable.

For this purpose, high resolution full spectrum acquisition techniques capable of providing accurate mass measurements for both precursor and product ions are of great help.

Highly resolved and accurate hybrid tandem mass spectrometry such as quadrupole/time-of-flight and linear ion trap/orbitrap allow for a more reliable identification in target analysis with reference standards, a screening for suspected analytes without the use of reference standards, and a screening for unknowns. A reliable identification requires both high resolving power and high mass spectral accuracy to increase selectivity against the matrix background and for a correct molecular formula assignment to unknown compounds. For the identification and structure elucidation of unknown compounds within reasonable time frame and with reasonable soundness, it is necessary the availability of advanced automated software solutions as well as of improved prediction systems for theoretical fragmentation patterns, retention times, and ionization behaviour (Krauss et al. 2010).



Name	RT	Mass (Tgt)	Formula (DB)	Score (DB)
4AAA	5,56	246,1227	C13 H16 N3 O3	97,66
4AAA F1	5,56	204,1137	C11 H14 N3 O	96,97
4FAA	5,69	232,1086	C12 H14 N3 O2	92,76
4FAA F1	5,69	204,1121	C11 H14 N3 O	83,67
4MAA	**1,947**	**218,1293**	**C12 H16 N3 O**	**97,68**
Amitriptyline	**7,362**	**278,1909**	**C20 H24 N**	**97,88**
Antipyrine	**5,336**	**188,096**	**C11 H12 N2 O**	**46,6**
Atenolol	3,019	267,1709	C14 H23 N2 O3	86,06
Atenolol F1	3,019	225,1225	C11 H17N2 O3	53,26
AZOXYSTROBIN	10,15	404,1246	C22 H18 N3 O5	99,16
AZOXYSTROBIN F1	10,15	372,0984	C21 H14 N3 O4	80,78
Caffeine	4,78	195,0882	C8 H11 N4 O2	88,56
Caffeine F1	4,78	138,0667	C6 H8 N3 O	88,96
Carbamazepine	**7,75**	**237,1028**	**C15 H13 N2 O**	**95,5**
CHLORFENVINPHOS	10,805	358,9774	C12 H15 Cl3 O4 P	85,53
CHLORFENVINPHOS F1	10,805	330,9833	C11H15Cl3O3P	62,53
CHLORFENVINPHOS F2	10,805	155,0473	C4 H12 O4 P	45,23
Ciprofloxacin	5,761	332,141	C17 H19 F N3 O3	88,97
Ciprofloxacin F1	5,761	288,1512	C16 H19 F N3 O	85,1
Citalopram	**7,043**	**325,1716**	**C20 H22 F N2 O**	**60,58**
Clarithromycin	**7,63**	**748,4847**	**C38 H70 N O13**	**66,49**
Codeine	3,48	300,16	C18 H22 N O3	95,89
Cotinina	1,24	177,1028	C10 H13 N2 O	90,6
CYPRODINIL	9,776	226,1344	C14 H16 N3	72,68
DEET	8,61	192,1392	C12 H18 N O	62,35
DEET F	8,61	119,0497	C8 H7 O	47,51

Name	RT	Mass (Tgt)	Formula (DB)	Score (DB)
DIAZINON	11,63	305,1089	C12 H22 N2 O3 P S	65,18
DIAZINON F1	11,63	169,0799	C8 H13 N2 S	56,89
DIAZINON F2	11,63	153,1028	C8 H13 N2 O	55,56
Erythromycin	6,923	734,4691	C37 H68 N O13	86,46
Fluoxetine	7,764	310,1419	C17 H19 F3 N O	61,63
Indomethacin	10,351	358,0846	C19 H17 Cl N O4	63,47
Indomethacin CO37	10,351	360,0823	C19 H17 Cl(37) N O4	58,87
Ketoprofen	**9,116**	**255,1021**	**C16 H15 O3**	**55,01**
Ketorolac	**8,307**	**256,0974**	**C15 H14 N O3**	**54,33**
Lincomycin	**3,401**	**406,2138**	**C18 H34 N2 O6 S**	**69,78**
Naproxen	9,187	231,1021	C14 H15 O3	71,19
Naproxen F1	9,187	185,0966	C13 H13 O	82,85
Nicotine	1,18	163,1235	C10 H15 N2	66,01
Nicotine F1	1,18	132,0815	C9 H10 N	46,21
Ofloxacin	5,192	362,1516	C18 H21 F N3 O4	92,4
Ofloxacin F1	5,192	318,1618	C17 H21 F N3 O2	43,49
Pentoxifylline	5,92	279,1457	C13 H19 N4 O3	81,8
Pentoxifylline F1	5,92	181,0729	C7 H9 N4 O2	47,62
Propanolol	**6,287**	**260,1651**	**C16 H22 N O2**	**63,29**
Propyphenazone	7,843	231,1497	C14 H19 N2 O	73,32
Sotalol	4,585	273,1273	C12 H21 N2 O3 S	44,64
Sotalol F1	4,585	255,1167	C12 H19 N2 O2 S	44,77
Trimethoprim	4,906	291,1457	C14 H19 N4 O3	66,87
Venlafaxine	6,324	278,212	C17 H28 N O2	64,41
Venlafaxine F1	6,324	260,2014	C17 H26 N O	64,69

Fig. 4.2 Screening of a wastewater effluent sample by LC-QqTOF MS using the automatic screening method with the user-created database: (**a**) total ion chromatogram (TIC) acquired in electrospray positive ion mode (+ESI); (**b**) database search results. The detected compounds with a score below 60 are marked in *gray* and the compounds which have no in-source fragments or characteristic isotope profile are highlighted in *bold* (Gómez et al. 2010, Fig. 4.1, with permission)

Previous works described the use of "fragmentation–degradation" relationships as a useful approach to identify and structurally elucidate pesticide-transformation products (García-Reyes et al. 2007a). They state that, from a given precursor specie, the fragmentation pattern occurred in-source (by collision induced dissociation, CID) can be used as reference to predict possible degradation products, suggesting that compounds are often transformed into their degradation products in the same fashion in which they are fragmented in the instrument. This hypothesis opens up a new dimension in the use of exact-mass databases not only for the screening of target compounds but also for unknown transformation products when the characteristic fragment ions are also included. The proposed strategy is based on: (i) the assumption that some transformation products maintain a similar structure to that of the precursor compound and therefore originate common product ions, and (ii) the use of advanced LC–MS data processing software, combined with an user-created accurate-mass database of target compounds, which includes information about the accurate mass of the target compounds and about their more significant and characteristic fragments (del Mar Gómez-Ramos et al. 2011). A representative example of this workflow is presented in Fig. 4.2 as more detailed in the text (paragraph 4).

In non-target analysis, no a priori information is available, as for instance retention time or SRM transitions. The possibility of obtaining commercially available

standards of suspected compounds for confirmation purposes is plausible in non-target analysis, but very often the standards are not available and the analysis becomes very complex.

For the developing of screening and identification methods, LC-TOF MS instruments offer unsurpassed capabilities, when compared with MS^2 instruments, that can be advantageously used in scanning modes but that in turn offer poor sensitivities. Product-ion or precursor-ion scan and constant neutral loss modes require previous information (as for example common fragment ions, or diagnostic ions, from a family of compounds), to detect possible non-target compounds. For this reason, the procedure cannot really be considered non-target analysis. Advanced MS techniques are therefore required, able to combine high-performance (high sensitivity and selectivity) target analysis with the ability to identify not only non-target compounds (which could be later included as target ones in the monitoring programs) but also possible unknown unexpected products (García-Reyes et al. 2007b).

4.2 UHPLC/MS in Non-target Analysis

GC-MS can allow the identification of some non-target compounds when the technique is suitable for the analyte (Almeida et al. 2007; Hancock et al. 2007). In this case forward-search methods enable library searching, for instance using the large library of the National Institute for Standards and Testing (NIST). But unfortunately GC-MS is only suitable for non-polar, volatile and semi-volatile compounds where for the other analytes a previous derivatisation reaction is required. For semi-polar and polar compounds, liquid chromatography combined with tandem MS (LC-MS/MS) is normally the technique of choice. Within the last 15 years LC-MS technologies have opened the analytical window to thermolabile, polar compounds and in particular polar organic micropollutants. A large number of studies showed that many polar micropollutants such as pesticides, pharmaceuticals, and industrial chemicals are present in the environment, where they can form many transformation products, not all yet identified (Celiz et al. 2009).

Further improvements have been obtained by the use of ultra high performance liquid chromatography (UHPLC) technique, that, due to the column packing particles lower than 2 μm and pressures up to 1200 bar, allows to rapidly and efficiently separate a lot of substances. According to van Deemter equation, as the particle size decreases not only a significant gain in efficiency is gained, but also the efficiency does not diminish at increased flow rates. But, on the other hand, reducing the particle size results in an increase in column back pressure and a special designed chromatographic instrument is required. The high resolution provided by the UHPLC system gives therefore greater information and reduces the risk to not detect potentially important co-eluting analytes.

With respect to HPLC, UHPLC provides fast, high-resolution separation, which increases LC-MS sensitivity and minimizes matrix interference arising from

minimal or reduced sample preparation. The minimization of the component coelution renders high mass spectra purity, improving the screening process.

Fused core or core shell HPLC technology has been utilised for similar high-throughput bioanalytical applications as an alternative to sub 2 μm UHPLC technology, without the disadvantage of so high column back pressures (Cunliffe and Maloney 2007). These phases are designed to obtain fast separations arising from both the small particle size (2.7 μm) and from the particle technology that creates a thin porous shell (0.5 μm) of stationary phase fused to a solid core particle. Fused core columns show a more separating power per unit time than columns of the same length but packed with conventional phases. This means that shorter columns operated at higher flow rates can be used to achieve remarkably fast high resolution separations, without the back pressure observed with sub 2 μm UHPLC columns. This system presents the advantage of utilising conventional HPLC instrumentation and of not requiring a dedicated UHPLC instrument with high pressure capability.

Unluckily, the absence for LC-MS of mass spectra libraries and the characteristics of CID make the identification of unknown compounds complex, time consuming, and not always successful (Bobeldijk et al. 2001, 2002; Ibáñez et al. 2005). The success greatly depends on the availability of compound databases or libraries for performing the search of an elucidated elemental composition (Bobeldijk et al. 2001, 2002; Ibáñez et al. 2005; Grange et al. 2006). Further restrictions come from the need to preselect relevant ions, which is typically based on ion abundance rather than, for example, compound toxicity. Some authors have performed genotoxicity tests in individual LC fractions to focus the research on only those fractions that cause potential harm to living organisms (Bobeldijk et al. 2001). However, other limitations come from the intrinsic characteristics and requirements of LC-MS, in relation to chromatography, ionization and fragmentation. Failure to identify the hazardous compounds within the fraction of interest is therefore still possible (Nielen et al. 2004).

To meet the challenges posed when analyzing a mixture of many known and unknown compounds present at low concentrations in complex matrices, a range of different LC-MS technologies have been put forward in recent years. In particular, the coupling of LC high resolution mass spectrometry (HRMS) with high mass accuracy emerged as powerful tool.

Generally, LC-MS techniques are considered more suitable in the identification of emerging contaminants than GC-MS techniques, because in LC-MS less fragments can be formed. Using in-source CID, where covalent bonds within the ionized molecules are broken between the ion source and the analyser by increasing the voltage in final skimmer cone. This feature can be used as a complementary tool in the identification of different analytes (Abrankó et al. 2001; Liu et al. 2010). However, in-source CID does not provide real tandem MS analysis because the precursor ion cannot be selected and thus the origin of the product ion cannot indisputably be confirmed especially in complex sample matrix, where coeluting components are usually present. In addition, the widespread applicability of the in-source CID is limited because of the low reproducibility of the spectra acquired

with instruments from different manufacturers. To obtain an unbiased dataset and compensate the lack of fragment information, tandem MS/MS or still better accurate full-scan acquisition have to be used.

4.3 Mass Spectrometry Instrumentation for Non-target Analysis

Recent developments in mass spectrometers have created a situation where many different mass spectrometers are suitable for non-target analysis, each with their specific strengths and drawbacks.

The use of analyzers like quadrupole (Q), time of flight (TOF) and ion trap (IT) evolved towards combinations of them to achieve tandem instruments, as triple quadrupole (QqQ), hybrid quadrupole linear ion trap (QqLIT) and quadrupole time of flight (QqTOF). In the last few years due to the improved characteristics of sensitivity and selectivity and to the need to collect a great amount of information for identification and elucidation purposes, we assisted to a rapid evolution of different analyzer designs.

In particular, to elucidate the structures of unknown compounds (not-target analysis), two sets of instruments are suitable. The first set concerns low resolution instruments such as QqQ or IT or QqLIT, from which information about the product ion MS/MS spectra can be obtained. The ability to generate detailed fragment ion data is a desired instrumental feature for structure determination of unknowns. The product ion scan (MS^2 scan) with a QqQ mass spectrometer (Weiss et al. 2009) and the multiple-stage MS scan (MS^n scan) (Ikatajamaa et al. 2006; Smith et al. 2006) with a tridimensional IT (3D IT) mass spectrometer are two of the most common techniques used in LC/MS analysis for generating fragment ions from small molecules.

The second set of instruments concerns the high resolution (HR) instruments such as TOF, orbitrap, Fourier transform ion cyclotron resonance (FT-ICR), from which accurate full-scan MS data can be obtained. Accurate mass data are essential for the identification of the appropriate elemental composition and the elucidation of unknown chemical structures can be obtained by using powerful software to mine the recorded chromatogram.

The optimal condition is to use a HR instrument able to give an accurate MS/MS product ion spectrum in order to obtain the elemental composition of both precursor and product ions. In this section these analyzers will be taken into account, highlighting the strengths and weaknesses. However, due to the use of different atmospheric pressure ionization sources and consequently different optimal conditions, the comparison between distinct LC-MS systems could give a non realistic comparison between mass analyzers (Soler et al. 2005, 2006, 2007). Among the possible ionization techniques, electrospray (ESI) is by far the most widely used as compared with atmospheric-pressure chemical ionization (APCI) or the more recent atmospheric-pressure photoionization (APPI) (Krauss et al. 2010).

4.3.1 LR MS: Triple Quadrupole (QqQ), Tridimensional Ion Trap (3D IT), Linear Ion Trap (LIT) and Hybrid Triple Quadrupole/Ion Trap (QqLIT)

QqQ instrument, when working in SRM, are the best choice for quantitative analysis, thank to their high sensitivity and selectivity. However, the well-known usefulness of full scan spectrum acquisition in a non-target approach limits the use of QqQ because of its rather poor detection sensitivity in this mode, when compared to other types of MS instruments. In addition to product ion scanning, also other full scan data acquisition modes, i.e. neutral loss (NL) and/or precursor ion (PI) scanning functions, have been used for screening purpose. These structure-specific acquisitions result in true positives only if the analyzed species undergo similar MS/MS fragmentation behaviour as the precursor. Therefore many unexpected metabolites or transformation products may be missed, as well as metabolites with more than one transformation site at opposite ends of the molecule. In conclusion, since QqQ instruments generally do not operate in full scan mode because of the lack of sensitivity, their utility in a true non-target analysis is rather limited.

Unlike QqQ, the 3D IT is characterized by high duty cycles because the time to fill an ion trap and generate a mass spectrum is short. Therefore, ion trap instruments can be also used for non-target analysis by virtue of their MS^n capability that allows the sequential and multistage isolation, in the same space and as a function of time, of precursor ions, fragmentation, trapping, and mass scanning.

The full scan sensitivity still increases with the coming of linear ion-trap (LIT).

With the advent of the hybrid QqLIT instrument called QTRAP™ (Hager 2002; Hager and le Blanc 2002, 2003; Petrovic and Barceló 2006a), both the traditional quadrupole and the LIT scans could be performed in a single LC run. In the past, a sample had to be run on two different instruments, namely QqQ and 3D IT, to obtain similar information. With the new QqLIT technology, Collision Activated Dissociation (CAD) occurs in a quadrupole collision cell, q2, and fragment ions are trapped and analyzed in Q3 operated in LIT mode. Furthermore, detailed fragmentation pathways were elucidated by further dissociation of each of the fragment ions in the enhanced product ion (EPI) spectrum using MS^3 mode in the same run. The MS^3 scan was performed by incorporating CAD in q2, and trapping, cooling, isolation, and resonance excitation in Q3 when operating in LIT mode. This approach allowed unambiguous assignment of all the fragment ions quickly and with fewer experiments and easier interpretation than the previous approach. It means that the QqLIT analyzer can provide an improved sensitivity in MS^2 studies. The overall sensitivity for obtaining complete fragment ion data was significantly improved for QqLIT as compared with that of QqQ and 3D IT mass spectrometers. This is beneficial for structure determination of unknown trace components.

The possibility to use the analyzer in information-dependent acquisition (IDA) mode, permits within a single run to obtain product ion spectra of precursor ions that are unknown beforehand. For screening purposes the QqLIT offers both structure-specific and data-dependent acquisition modes, of which SRM triggered

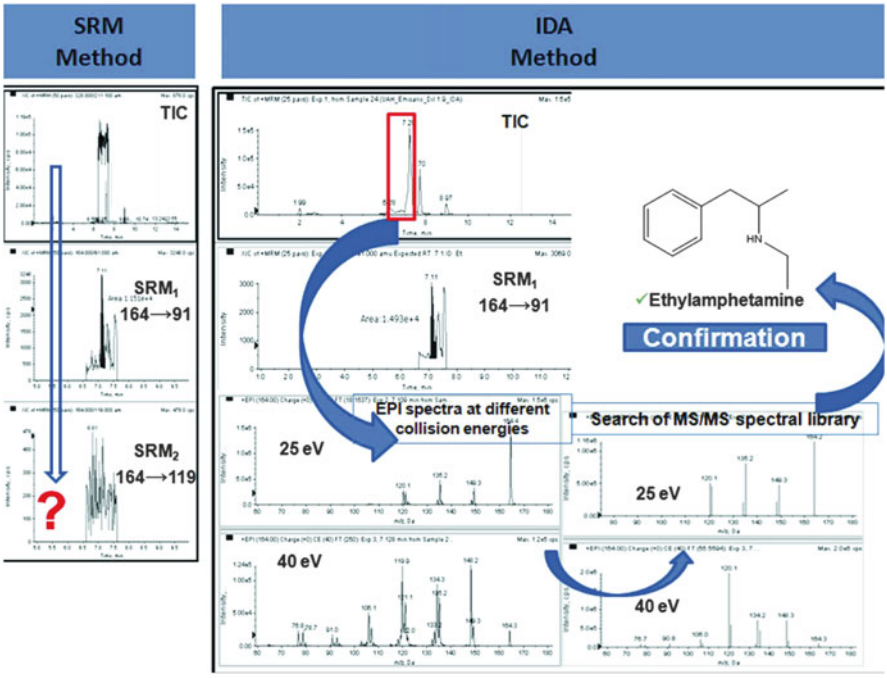


Fig. 4.3 Identification of ethylamphetamine in river water, extracting the m/z signal from Total Ion Current (TIC). The identification of the analytes was not possible in the Selected Reaction Monitoring (SRM) method because of the lack of a second transition (164 → 119). With Information Dependent Acquisition (IDA) method, the analytes was conformed through the comparison of an in-house Enhanced Product Ion (EPI) spectra library at two different Collision Energies (ECs). (Pérez-Parada et al. 2012, Fig. 4.5, with permission)

EPI MS/MS scanning is most widely used for metabolite screening (Li et al. 2005, 2007; Yao et al. 2009). In addition, approaches utilizing a wide-range scan with the ion trap as a survey scan (enhanced mass scan, EMS), followed by EPI MS/MS (EMS/EPI), have been described (Xia et al. 2003). Figure 4.3 shows, as an example, the ethylamphetamine confirmation process in a river water sample using SRM and IDA methods by direct injection. As seen in the SRM method, it was not possible to get suitable identification of ethylamphetamine because of the lack of a second transition (164 → 119). However, the IDA-based method allowed its confirmation through the comparison of an in-house EPI spectra library at two different collision energies, CEs (25 and 40 eV), avoiding the report of a false negative by using the same instrument. Anyway, *a priori* setting such as the minimum and maximum number of counts and background ions (or co-eluting ions with higher abundance than the species being studied) might become a drawback.

4.3.2 Time of Flight (TOF)

TOF is an attractive instrument due to its potentially unlimited m/z range and high-speed acquisition capabilities (Bristow 2006). The TOF MS analyzer provides the selectivity and the sensitivity required for efficient, wide-range screening, as it combines high, full-spectral sensitivity with high mass resolution that make it able to measure accurately the mass of any ionizable component in the sample. Modern TOF MS instruments provide high mass accuracy (typically below 5 ppm, according to manufacturer) and mass resolution (>10,000 full width at half-maximum height) combined with high full-spectrum sensitivity and speed. With the latest TOF mass spectrometers, mass resolving power as high as 40,000 (full width at half maximum (FWHM), m/z 922) and accuracy lower than 1 ppm are possible (Ow et al. 2010). In addition, the price of TOF MS is currently affordable, increasing its use in research.

Since TOF MS can provide a notable amount of chemical information in a single experiment, the technique is very attractive for performing non-target analysis or for searching for analytes in post-target way (when analytes are selected and searched after MS acquisition) (Hernández et al. 2005; Sancho et al. 2006). The LC-TOF MS approach enables the screening for several hundreds of compounds with high sensitivity within one run. The selectivity is based on accurate mass measurements with mass traces defined within 0.005 Da over a dynamic range of about three orders of magnitude (Decker et al. 2006). In addition, the accurate mass identification offered by LC-TOF MS system permits the determination of small organic molecules (<500 mass units) present in complex samples (e.g., environmental or food matrices). In particular, the huge amount of information provided by TOF MS, together with the measurement of accurate mass facilitates a confident identification of non-target compounds in samples. Although quantitative applications of LC-TOF MS have been reported, the quantification does not seem to be the most attractive feature of these analysers. TOF MS sensitivity is about 1–2 orders of magnitude lower than those of QqQ (Petrovic and Barceló 2006b). One of the most interesting applications of TOF MS deals with the wide-scope screening of a large number of contaminants and residues in different types of samples.

Non-selective sample treatments and chromatographic separations are required to broaden the system applicability to as many compounds as possible. Another TOF advantage is represented by the elevated acquisition speed, that makes it compatible with UHPLC.

In the past few years, some advances in mass accuracy have made LC-TOF MS a useful tool for molecular identification of unknown compounds. One of the improvements consists in the design of the double sprayer with reference solutions, which corrects the instrument drift by continuous calibration of the mass axis, with an improved mass accuracy to less than 3 ppm. In conclusion, the advantages with respect to LC-MS^2 (SRM) instruments are: (a) a large number of targets can be screened at the same time without loss of sensitivity; (b) unknown peaks can be identified on the basis of accurate mass and isotopic profile evaluation; and, (c) data

can be reprocessed *a posteriori* (retrospective analysis) for additional compounds which had not been investigated yet.

LC-TOF MS offers full-scan high sensitivity, high mass accuracy close to that provided by high-resolution Fourier-transform mass spectrometry (FT-MS) instruments (orbitrap and FT-ICR-MS).

4.3.3 Hybrid Quadrupole/Time of Flight (QqTOF)

Based on TOF technology, the development of the hybrid QqTOF MS has also revolutionized the application of TOF to accurate mass measurement with the possibility to perform accurate tandem mass experiments (Wolff et al. 2001; Ibáñez et al. 2005). The use of hybrid QqTOF, instead of single TOF, offers more possibilities in screening and identification, so it is feasible performing MS^2 experiments that offers to the researcher additional relevant information on product ion accurate mass spectra, which are very useful for structure elucidation. Some QqTOF systems have also the possibility to simultaneously acquire MS/MS spectra at low and high collision energies or in a range of these in order to provide useful information on the (de)protonated molecules and on the main product ions, respectively. Some examples of the use of this technique are the screening of antibiotics and drugs of abuse in water, screening of pharmaceutical metabolites in urban wastewater, or application to public health laboratories (Ibáñez et al. 2009; Hernández et al. 2011a, b; Diaz et al. 2012; Ibáñez et al. 2012).

However, the accuracy from accurate mass of product ions might decrease and, despite some manufacturer opinion, QqTOF does not enable mass accuracy <3 ppm from MS^2 spectrum, mainly because of the energy differences between the ions coming from the second quadrupole (García-Reyes et al. 2007a). It is quite difficult to focus the kinetic energy of all the ions before each pulse in the TOF; also, there are no calibration ions for continuous, on-line, accurate mass-measurement corrections. It is not difficult to find ±20 mDa bias in QqTOF MS/MS mode, which hampers elucidation of elemental compositions and structures of unknown compounds. This is not common in LC-TOF MS using accurate mass "dynamic calibration systems", yielding mass accuracies for fragment ions better than 3 ppm. The good results obtained with post-acquisition calibration and the possibility of performing it only when necessary (when mass drift is observed) make it the best option to obtain greater accuracy and precision (as good as that obtained with the common internal calibration). The use of reference sprayers, such as lock-spray, improves robustness in mass accuracy measurement along time. The drift in the mass measurements is mainly due to environmental factors, which lead to instability of the calibration and the necessity to daily recalibrate the mass spectrometer (Wu and Mc Allister 2003). In the older generation TOF and QqTOF instruments respectively in-source and CID fragmentation can be used, but generally the mass errors for the produced fragments are quite high (up to 30 ppm) (Gerssen et al. 2008). However, as instrumentation evolves extremely fast, last-generation TOFs utilize

new generations of detectors and thermal expansion-corrected analyzer tubes leading to better routine mass measurement accuracy. One of the most interesting advances has been extending the dynamic linear range in TOF analyzers that use time-to-digital converter (TDC) detectors thanks to the so-called dynamic range enhancement (DRE) (Grimalt et al. 2010).

Also QqTOF instruments are totally compatible with modern UHPLC, thanks to their very high data acquisition rate. The detection of all expected and unexpected analytes in a single run, without the need for pre-adjustment of detection for certain predicted species and with the possibility of various post-acquisition data filtering and processing options, makes straightforward the screening of unknowns (Tiller et al. 2008; Mortishire-Smith et al. 2005; Cuyckens et al. 2009). Because of the recent development of both technologies, few applications using UHPLC-QqTOF MS have been reported in the environmental field (Petrovic et al. 2006; Richardson 2011; Farré et al. 2008; Ibáñez et al. 2009; Gomez et al. 2010; Díaz et al. 2011; del Mar Gómez-Ramos et al. 2011; González-Mariño et al. 2011; Hernández et al. 2011a, b; Díaz et al. 2012; Pérez-Parada et al. 2012; Wang et al. 2012).

Thank to QqTOF speed, it is possible in a single run to perform dependent experiments offering high resolution and high mass accuracy for both precursor and product ions. Data acquisition is so fast that multiple precursors from co-eluting peaks can be monitored simultaneously. However, when using dependent acquisition like IDA mode (see paragraph 3.1), the same drawbacks above reported have to be taken into account.

4.3.4 Fourier Transform Ion Cyclotron Resonance (FT-ICR)

FT-ICR presents an unsurpassed high accuracy and resolving power. However, the large size, complexity, maintenance, and cost of this mass analyzer restrict the laboratory setting where it can be used. In many cases, their applications have involved extremely complex samples, such as raw seawater, which contains natural organic matter at a very low concentration. Other complex analytical problems would be the analysis of diesel fuel (Hughey et al. 2001) and of samples without pretreatment such as wine (Cooper and Marshall 2001), as well as the application in areas such as metabolomics and proteomics (Witt et al. 2001). FT-ICR mass analyzers have been rarely used in polar organic trace analytics due to their high costs.

4.3.5 Orbitrap

Orbitrap, which was introduced into the market in 2005, is a new type of mass analyser, operating by radially trapping ions about a central spindle electrode. An outer barrel-like electrode is coaxial with the inner spindle-like electrode and mass/charge values are measured from the frequency of harmonic ion oscillations, along

the axis of the electric field, undergone by the orbitally trapped ions. This axial frequency is independent on the energy and spatial spread of the ions. Ion frequencies are measured non-destructively by acquisition of time-domain image current transients, with subsequent fast Fourier Transforms (FFTs) being used to obtain the mass spectra (Hu et al. 2005).

Orbitrap technology is increasingly applied due to the combination of a very HR (up to 100,000) without the need of a superconducting magnet (Ham 2008; Perry et al. 2008), high mass accuracy (<2 ppm), and a sensitivity down to the femtogram range. Other features include intensity-independent mass accuracy and no internal calibration required.

Therefore, some applications restricted until now to FT-ICR (TOF analyzers have not been used, due to lack of resolving power) can be transferred to orbitrap analyzers, such as proteomics metabolomics, and other omics approaches. The main advantage is that orbitrap does not need such an expensive and delicate maintenance as FT-ICR does.

4.3.6 Hybrid Ion Trap/Orbitrap (LTQ-Orbitrap)

Combination of two different mass spectrometer types in so-called hybrid instruments such as linear ion trap/orbitrap (LTQ-Orbitrap) has shown excellent detection and identification capabilities for low molecular weight compounds in various matrices, based on high resolution accurate mass measurement of precursor and product ions (Hernández et al. 2005; Petrovic and Barceló 2006b; Kosjek et al. 2007; Kellmann et al. 2009).

In a LTQ-Orbitrap MS, where the high-resolution mass analyzer is usually combined with a linear ion trap, accurate mass measurements are combined with the high trapping capacity and MS^n scan function of the linear ion trap. Therefore, the LTQ-Orbitrap is capable of MS/MS and produces very high accuracy mass data (within 2 ppm using internal standard and within 5 ppm with external calibration (Makarov et al. 2006)) which makes it a useful instrument in identification of transformations or transformation products (Hu et al. 2005; Lim et al. 2007; Ruan et al. 2008).

The LTQ-Orbitrap MS when compared to QqQ MS offers the following advantages in the screening and identification of unknown compounds: (i) the sensitivity in full-scan is higher, (ii) accurate mass is used to calculate the most favorable elemental composition and (iii) the accurate mass of the product ions (in MS^n) can be determined. The advantage of the LTQ-Orbitrap MS over the QqTOF MS is the higher resolving power, which leads to better mass accuracy. The mass spectrometer can also operated, like modern QqTOF systems, in a data-dependent-acquisition (DDA) mode in which both MS and MS^n spectra can be acquired without the need to specify precursor masses. In this mode, the acquisition software probes the MS spectra in real-time on a scan-by-scan basis to select the most intense precursor ions for MS^n analysis. This technique is capable of finding true unknowns since the

method does not require any preselection of masses. The instrument is initially set to operate in full-scan ('survey') mode until an ion exceeds a preset threshold, at which the instrument switches into the product-ion mode (MS^n). However, the total cycle time depends upon the resolution: increasing resolution a reduced scan speed is obtained (at a resolution of 100,000 the scan time is 1 s) (Gerssen et al. 2011). This makes the instrument less suitable to detect the sharp peaks (<5 s) that are generated under UHPLC conditions: the relatively slow data acquisition rate of the LTQ-Orbitrap makes it often incompatible with UHPLC, at least if the aim is to analyze several types of data within a single LC/MS run, which is essential for fast high-throughput metabolite screening, like using QqTOF.

Anyway, the dilemma of what instrument between QqTOF and LTQ-Orbitrap is the more suitable for non-target screening is not so easy to be answered. As above reported, each equipment has advantages and disadvantage, strengths and weaknesses, and a comprehensive comparison is not easy at all. In fact both hybrid analyzers appear nowadays as the best option in this field.

4.4 Non-target Method and Data Mining (Software and Algorithms Solutions for Data Interpretation)

When screening a sample for unexpected compounds, it becomes difficult to pick out individual ions, especially when the matrix is complicated or when the concentration of the analyte is low. Under these circumstances, it is necessary to use powerful softwares with chromatographic peak-deconvolution capabilities to identify the presence of multiple components and to produce pure spectra for each individual component. Generally, the deconvoluted accurate mass spectrum for every component was compared with a library of spectra, giving a score based on the matching of spectral peaks and their relative intensities. The formula from the library hit is submitted to elemental composition and the two most intense ions are confirmed or rejected by accurate-mass criteria. Figure 4.2 shows an example of total ion current (TIC) and database search results. As can be observed, 26 compounds have been detected, including five pesticides, 16 pharmaceuticals and five of their major metabolites or degradation products. Fifteen of the compounds provide additional information of fragment ions from in-source CID fragmentation and indomethacin gives information about the chlorine isotopic profile, very useful for identification purposes.

This non-target approach has the advantages of identifying unexpected components present in the sample and of allowing proposal of their elemental compositions. The deconvoluted mass spectrum can retain all the exact-mass information provided by TOF, orbitrap or hydrid HR instruments, for both the molecular ion and fragment ions present in the spectrum, and it can be used to propose potential elemental compositions for the component. In this paragraph a detailed explanation is given about the development of non-target methods, screening of unknowns, their identification and elucidation of their structures by using powerful softwares.

4.4.1 Screening Methods

Screening methods are normally developed for rapidly determining the presence of contaminants in a sample. Different criteria can be found in literature about the concepts of target and non-target screening (Ibáñez et al. 2008; Cortés-Francisco et al. 2011; Nurmi et al. 2012). From one point of view (Hernández et al. 2005; Kaufmann et al. 2011; Hernández et al. 2012), three alternatives might be considered for LC-MS screening methods, depending on the objective of the analysis and, especially, on the instrumentation available:

(i) pre-target screening methods, where the analytes are pre-selected before MS-data acquisition. In this case other positives cannot be revealed. The pre-target method is the most common approach, in which only certain compounds previously chosen are determined and the method is validated solely for those compounds. These analyses are predominantly carried out with MS/MS techniques, due to their high selectivity and sensitivity. The disadvantage of MS/MS techniques is that the characteristic MS^2 ions must be known before analysis. This procedure can lead to biased information on samples, because only the user-defined data are saved and the compounds in the sample that are not beforehand specified remain unknown.

(ii) post-target screening or suspect screening methods, where all the compounds eluted from the chromatographic column are measured by MS and the m/z of target analytes are extracted afterwards from the TIC chromatogram (Hernández et al. 2005; Ibáñez et al. 2008; Nurmi and Pellinen 2011; Chitescu et al. 2012; Díaz et al. 2012). All compounds with a mass-to-charge ratio (m/z) within the defined mass range eluting from the column and ionized in the ionization chamber are measured. The analytes are examined after the analysis by plotting a narrow-window extracted ion chromatogram (nw-XIC) of 20–50 mDa. In the post-target approach, the nw-XICs of the masses corresponding to the analytes of interest are extracted from the complete dataset. It is unnecessary to totally deconvolute all the components present in the samples because these mainly belong to matrix compounds. Furthermore, processing and reviewing steps become easier as fewer compounds are searched for and consequently detected (Díaz et al. 2012). This comprehensive dataset also enables retrospective reanalysis of the sample also years after the sample was firstly analysed.

(iii) non-target screening methods, where all the compounds eluted from the analytical column and ionized in the ionization source can be detected and identified without any kind of selection (with the obvious limitations derived from chromatographic and ionization processes in the LC-MS interfaces used). In non-target analysis, previously unknown components in the sample chromatogram are extracted from TIC, using special deconvolution software that detects the ions arising from the same component without any previous information about the compound. Then, the elemental composition of the compound is deduced from the accurate mass of the ions. It is evident that post-target and

non-target approaches are incapable of revealing all the compounds in the sample, so causing possible false negative results. This is due to the inherent nature of LC-MS analysis, since the chromatography and the ionization process always exclude some of the compounds. So far, only a few applications of non-target analysis have been reported, and only few critical assessments of the screening techniques for large numbers of analytes, using TOF MS, can be found in literature (Ferrer et al. 2006; Ibáñez et al. 2008, 2012; Gómez et al. 2010; Diaz et al. 2012; Hernández et al. 2012).

In addition, in the case of non-target analysis, a further slight differentiation has been done between the suspect screening without reference standards and a genuine non-target screening of unknowns (Krauss et al. 2010).

4.4.1.1 Post-target Screening or Suspect Screening

Reference standards are currently not available for a large number of potential environmental contaminants, in particular transformation products. However, compound-specific information for suspects is available, such as molecular formula and structure, which can be efficiently used in the identification and confirmation process (Krauss et al. 2010). First, the molecular formula allows for the calculation of an exact m/z of the expected ion, which is in turn extracted from the high resolution full-scan chromatogram. The large number of suspects requires an efficient filtering approach, comprising rather straightforward and obvious criteria such as their absence in analytical blanks and the match of the observed isotope pattern with the theoretically predicted ones for the molecular formula of the suspect. As an example, Fig. 4.4 shows the TOF MS spectra of the secondary amines of alachlor and acetochlor ethan sulfonic acid. The correct elemental composition for both analytes ranks at the second score position. Furthermore, physico-chemical properties and in turn the chromatographic retention times were predicted from the molecular structure (Ferrer and Thurman 2003). An interesting example of a post-target approach is described here (Nurmi et al. 2012). In post-target process, the software extracts the given nw-XIC and lists all the components (peaks) in the chromatogram with different retention times corresponding to the desired mass. All components with Δm lower than the cut-off Δm value are labelled as positive identifications, of which only one can naturally be the correct one. A four-stage process has been developed, in which the number of possible candidates is diminished. Also log K_{ow} as a measure of the hydrophobicity can be used to determine whether the retention time (t_R) of a candidate compound is similar to that of compounds with similar log K_{ow} values (Nurmi et al. 2012). Wang et al. investigate twelve estrogen metabolites and other three progestogens in water samples by a post-target approach (Wang et al. 2012). In the TOF MS mode, using theoretical molecular mass of the selected compounds, chromatograms of extracted precursor ions in a narrow mass window (0.02 Da) were gained from total ion chromatograms. Peaks with an area greater than 50 a.u. (arbitrary units) were selected to evaluate the experimental masses of

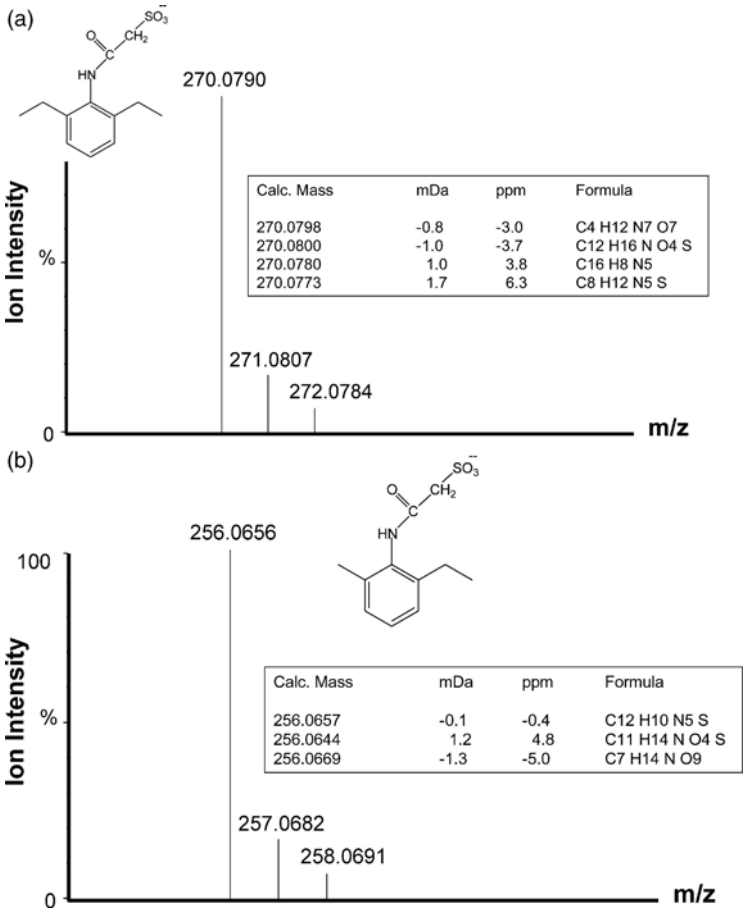


Fig. 4.4 LC-TOF MS spectra and accurate measurements for the secondary amides of alachlor (**a**) and acetochlor (**b**) sulfonic acid in a groundwater sample. The correct elemental composition for both analytes ranks at the second score position. The physico-chemical properties and the chromatographic retention times were predicted from the molecular structure (Ferrer and Thurman 2003, Fig. 4.3, with permission)

these compounds. The mass error between experimental and theoretical molecular mass was less than 3 mDa, which implied that the selected compounds may occur in the samples. Further confirmation should be performed by accurate mass measurement of characteristic fragment ions of suspected compounds in QqTOF MS/MS mode. Finally, Kern et al. tentatively identified 19 transformation products which all could be later confirmed by using reference standards (Kern et al. 2009). This indicates that under the criteria employed the probability of false positive findings is low. A less straightforward problem to assess is the possible occurrence of false negatives, as it is inherent in the post-target screening approach (as well as in the unknown screening approach, as below mentioned). Without an analytical

standard it is not possible to prove from the outset whether a compound present in a sample will be identified in the chromatogram because, as above mentioned, it could get lost during any step of the analytical procedure or not be ionized. Thus, a careful validation of the whole procedure using a range of reference standards and a comparative assessment based on the (estimated) physicochemical properties of the suspects is a prerequisite to minimize the occurrence of false negatives. Another reason for false negatives is an insufficient mass resolution, as unresolved isobaric ions will yield an inaccurate "mixed" mass (Nielen et al. 2007).

4.4.1.2 Non-target Screening of Unknowns

In contrast to suspects screening, non-target (unknown) screening in a strict sense starts without any *a priori* information on the compounds to be detected. Many studies in literature thus fall in between these two categories, as in systems with well-controlled boundary conditions such as transformation experiments (Thurman 2006; Ibáñez et al. 2006; Harir et al. 2007). According to the authors, the number of chemically meaningful structures which can be assigned to an unknown peak detected is limited to structures showing a close relationship with the precursor compound and an adequate control sample or time series is available. For this type of experiment, an identification by HRMS(/MS) alone can often be considered as definitive. A tentative identification of non-target analytes in environmental samples with unconstrained boundary conditions is more challenging, and a structure proposition for a peak detected by high resolution MS and MS/MS spectra involves several work-intensive data and expert processing steps. Although the described non-target workflows are often focused on one specific evaluation step, the following key features have emerged: (i) an automated peak detection by exact mass filtering from the chromatographic run; (ii) an assignment of an elemental formula to the exact mass of interest; and (iii) a database search of plausible structures for the determined elemental formula.

LC-HRMS is an accepted technology for generating meaningful structure suggestions of suspects and unknowns present at low concentrations in environmental samples. It must be underlined that, however, an unequivocal identification of trace-level compounds in environmental systems is in most cases not possible by HRMS alone without the application of additional knowledge, complementary techniques, or an authentic reference standard. As a fast evaluation tool for possible candidates, HRMS is ideal when it is subsequently combined with a powerful structure elucidation technique like nuclear magnetic resonance (NMR) (Godejohann et al. 2009), although this requires sufficiently high concentrations and often the isolation of the unknown compound. In fact, in natural product chemistry these two spectroscopic techniques, along with further information on functional groups and elemental composition, are typically required for the true identification of a new compound.

LC-MS non-target analysis has been scarcely applied until now, as it presents important limitations mainly due to the absence of wide commercial reproducible libraries (Ibáñez et al. 2005, 2008; Thurman et al. 2006; Hernández et al. 2008).

The main difficulty to perform a true non-target screening is due to the deconvolution process that depends to a great extent on the intensity of the chromatographic peak (Díaz et al. 2012). By using this criterion, information about other ions different from those preselect as 'relevant' ions (e.g. ions with area greater than a chosen threshold area), which could correspond to other relevant contaminants, is missing. It can be observed that the choice of threshold-area for selecting the significant ions is wrong in principle: due to the different response of analytes in an LC/MS system, higher areas do not necessary imply higher concentrations. However, a compromise has to be reached between appropriate preselection filtering and the time and effort involved in research. An additional criterion based on toxicological information appears to be one of the most interesting approaches (Ibáñez et al. 2005). Moreover, the ESI interface restricts the application to those compounds easily ionizable (from medium polar to ionic). Thus, the use of other interfaces, such as APCI or APPI, or the use of a GC/TOF system with electron ionization, would be an ideal complementary tool for example to widen the scope of the determination of pollutants in water samples towards the less polar ones. The success of a non-target procedure depends in some way on the availability of compound databases where the search can be performed. However, extending these databases to include a larger number of potential structures of a particular elemental composition could result in some cases in even more ambiguity in the interpretation of the data, because without standards one cannot, for instance, straightforwardly discriminate between various isomeric compounds from the MS/MS spectrum. One of the major limitations comes from the limited understanding of the fragmentation rules in MS/MS of (de) protonated molecules (Ibáñez et al. 2005).

As above mentioned, an efficient approach to overcome the component detection limitations is the use of "post-target" methodology, i.e. the selection of the analytes to be searched is done after MS acquisition (Díaz et al. 2012).

4.4.2 Interpretation of the Acquired Data

Since the data obtained by low resolution instruments, like QqQ, are enough simple to interpret if compared with the huge amount of data obtained by a HR instrument, interpretation of a product ion spectrum generated by the QqQ is often not straightforward, because of the complex dissociation mechanisms for formation of fragment ions. However, a structure elucidation software, such as ACD/MS Fragmenter or Mass Frontier, developed to predict the fragment ions formed by CID, has been used to assist the spectral interpretation of unknown structures.

Instead, based on the high amount of data produced by HR MS from a single run (around 200 Mb, depending on runtime, scan speed and resolution), it is laborious to mine unknown species from the TIC data, but fortunately very good software exists to ease the process. A freeware software program called metAlign has been used to reduce of about 200-fold the orbitrap MS data files (Gerssen et al. 2011).

Depending on which type of mass spectrometer has been used for metabolite screening, a number of different software programs exists for prediction and post-acquisition processing of data (Anari et al. 2004; Mortishire-Smith et al. 2005; Cuyckens et al. 2009; Zhang et al. 2009; Pelander et al. 2009; Leclercq et al. 2009). In most cases, and to guarantee the results, there is a need for manual expert evaluation and confirmation of the software-produced results, and therefore the whole process cannot be totally automated, especially with regard to the unexpected metabolites. With this approach the total workload, after finding suitable LC/MS conditions, is typically about 30 min of instrument time and 1–4 h of data processing per compound/species, depending on the number of metabolites and on the complicity of the interpretation of the fragment ion data. However, some of the recently described softwares may also take care of the fragment ion data interpretation, especially if a QqTOF instrument is used (Leclercq et al. 2009).

For post-acquisition data processing, the comparison of sample and control chromatograms with these software programs is typically straightforward, revealing the differences in sample and its negative control. The software designed to ease the screening procedure and to increase the throughput must be subjected to careful setup, as with dozens of different parameters and filters the possibility of false negatives is significantly increased (Rousu et al. 2010).

When searching for a specific compound with TOF analyzers, it is simple to plot a nw-XIC at its theoretical exact mass (i.e. post-target screening). Non-target analysis would require visual inspection of the TIC chromatogram to find potential components present in the sample. Low-abundance compounds might not be apparent by visual inspection. On the contrary, intense peaks would be easily observed in the chromatogram, but they might not be necessarily associated with a single component, as even techniques with high peak capacities (e.g., comprehensive GC-MS) would lead to partially co-eluting peaks for complex mixtures (Kind and Fiehn 2007). However, if many contaminants have to be investigated, it becomes time consuming to target them all individually.

For an automated compound detection several software packages using different peak detection algorithms are available and usually offered by the MS manufacturer (Hogenboom et al. 2009). The capabilities and limitations of the elemental composition prediction from accurate mass measurements by using heuristic rules (Kind and Fiehn 2006, 2007) or profile MS data after peak shape calibration (Erve et al. 2009) have been comprehensively investigated.

The strategy for selecting the non-target peaks is carried out in automated mode by software, after setting of some filters or constrains: for example, a chromatographic peak to be defined as such must have a peak-width or an area grater than a set threshold.

In general, the software integrates nominal mass ion chromatograms over the mass range defined to find the location of the chromatographic peaks. Then, each ion of the peak is examined to determine whether it maximizes at that retention time. Those ions not maximizing are excluded from the spectrum, and the user-defined number of maximizing ions in decreasing order of intensity are included in the final nominal mass spectrum of the component. If the mass resolution of data is

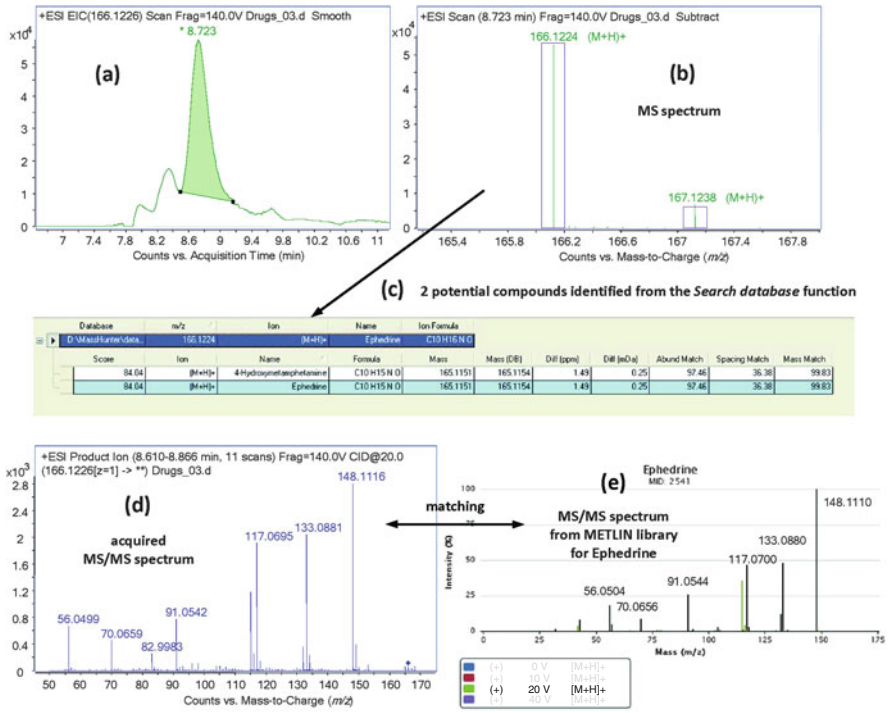


Fig. 4.5 Identification workflow of ephedrine: (**a**) peak was detected in electrospray positive ion mode (+ESI) extracting the m/z signal of ephedrine; (**b**) MS spectrum was compared to database; (**c**) database match with 4-hydroxymetamphetamine and ephedrine that actually having the same empirical formula. Once candidates were detected, sample was re-injected and MS/MS spectra acquired at several collision energies; (**d**) MS/MS spectrum acquired were compared with (**e**) METLIN library MS/MS and the ephedrine was confirmed (González-Mariño et al. 2012, Fig. 4.3, with permission)

high enough, the elemental composition of the ion can then unequivocally be calculated from the measured accurate mass of the ion. In opinion of some authors, the resolving power and mass accuracy provided by TOF MS is not sufficient for a genuine non-target screening (Nurmi et al. 2012). The spectrum formed can be compared with a library to get a preliminary identification. Finally, the accurate mass of the molecular ion measured is compared with the exact mass of the compound proposed to further improve the reliability of the identification (Nurmi et al. 2012). Figure 4.5 shows the identification workflow for ephedrine. First, the extracted ion chromatogram was automatically generated by the software (Fig. 4.5a) and its MS spectrum (Fig. 4.5b) compared to the theoretical one. In this particular instance, there were two potential positive matches with the database (Fig. 4.5c): 4-hydroxymetamphetamine and ephedrine, actually having the same empirical formula. Once candidates were detected, sample was reinjected and MS/MS spectra acquired at several collision energies. Then, in this case, the MS/MS spectra (Fig. 4.5d) were compared to those available at the METLIN public library

(Fig. 4.5e) so that the compound could be confirmed as ephedrine (González-Mariño et al. 2012; Scripps Center for Metabolomics).

Searching in large compound databases (e.g., Pubchem, Chemspider, Chembook) for possible structures of an elemental formula normally results in numerous hits which need to be ranked further by MS/MS data. The search for unknowns in MS/MS or in-source fragment ion libraries is limited to the recorded spectra of reference standards (Liao et al. 2008), which is not sufficient for a real unknown screening and suffers from limited comparability among instruments. Therefore an *in silico* strategy for determining unknown chemical structures by matching measured with computational fragmentation spectra of compounds queried from chemical databases seems to be a valuable tool (Hill et al. 2008). However, a prediction of product mass spectra yields a large number of possible fragments, of which a rather small number is actually observed. For other unknown substances containing C, H, N, O, F, and P only, which show subtle and low intensity isotope differences, both resolving power and spectral accuracy of the MS will determine the performance of the elemental formula fit (Kind and Fiehn 2006). Currently, TOF instruments seem to have a somewhat better spectral accuracy (>1 %) than orbitrap instruments, exhibiting a spectral error of 3 % and 10 % at a resolving power of 7,500 and 100,000, respectively (Erve et al. 2009). Furthermore the MS/MS prediction alone is not sufficient for a successful assignment of a suitable structure to a molecular formula; additional computational approaches are required in this workflow step. The estimation of retention times from estimated physicochemical properties as shown for the suspect screening by Kern et al. or the use of fragment libraries with accurate mass information is likely to improve the match between measured data and candidate structures (Kern et al. 2009).

Depending on the mass accuracy (mDa or ppm) obtained, there will be a higher or lower number of potential elemental formulae for a compound. Thus, the degree of accuracy required for the m/z of the ion measured has to be taken into account. The Journal of the American Society for Mass Spectrometry advice that setting fixed acceptable error limits for exact molecular mass measurement is not recommended (Cortés-Francisco et al. 2011). It should be highlighted that it has been always given more importance to accuracy rather than to precision. Precision has received too limited coverage in the literature, but both accuracy and precision should be considered when talking about uncertainty in the measurement and evaluation of mass spectrometers (Cortés-Francisco et al. 2011). In general, there is less accuracy in mass measurements for higher masses (around 500 Da) than for lower ones (Cortés-Francisco et al. 2011). Regarding precision, the same behaviour as for accuracy has been detected; precision of the measurements is worse at higher m/z values. It can be concluded that the uncertainty in the measurements is in all cases worse for higher m/z values. In contrast, a better mass measurement, in accuracy and precision terms, is needed for higher masses in order to have less candidates and the correct molecular formula (Webb et al. 2004). The aim of an accurate mass measurement is to confirm the molecular formula of a compound. The capabilities of the software when looking for the correct formulae have been evaluated (Webb et al. 2004; Kind and Fiehn 2007). Ultrahigh-resolving power and high accuracy are

powerful tools to confirm an identity, but they are insufficient for obtaining a unique structure of an unknown yet (Cortés-Francisco et al. 2011). Structural information is needed, that can be complemented with tandem mass (MS/MS) experiments and/or NMR, if possible. In order to obtain a limited list of possible candidate formulae from a mass measurement, heuristic criteria based on uncertainty in mass measurement (accuracy and precision), the number of ring plus double bond (RDB) equivalents, the charge, the adducts formed, the isotopic pattern in the mass spectra, and the elements in use can be applied. As far as possible, it is necessary to know the uncertainty associated to an accurate mass measurement: only in this way the list of theoretical candidates that should be taken into account can be known. A software parameter called mass tolerance defines how close a measured mass must be to the theoretical mass to be considered the same mass. For example, for orbitrap system with external calibration, a mass tolerance lower than 1.5 mDa can be fixed because the uncertainty expected from this mass spectrometer is better than that, and the theoretical mass of a possible candidate which differs more than 1.5 mDa from the mass measured should be ruled out as not-possible candidates.

As the number of elements (C, H, O, N, S, F…) increases, less uncertainty in the measure should be permitted because, if not, the number of candidates rises exponentially (Herniman et al. 2005).

Different isotopic filters can be applied to minimize the number of possible elemental compositions for a certain mass window. As a result of the advances in HRMS technologies discussed above, several accurate-mass based data mining tools, including isotope pattern filter (Park et al. 2011), background subtraction and noise reduction (Zhang and Zhang 2008; Zhu et al. 2009) and mass defect filter (Zhang et al. 2003, 2009) were developed to facilitate data analysis.

The algorithm isotopic pattern filter is based on the accurate m/z of ions, where the m/z differences of selected isotopic ion-pairs (e.g. M + 1:M and M + 2:M, where M is the monoisotopic peak and M + 1 and M + 2 are the first and the second isotopic peak) must fall into the pre-assigned accurate mass tolerance window (e.g. 5 ppm), so satisfying the predefined relative abundance criteria. These filters are based on the isotopic pattern deviation between the empirically measured spectrum and the theoretical spectrum. The application of carbon, chlorine, bromine or sulfur filters also allows reduction of the number of proposed elemental compositions that would fit for a certain mass-accuracy window, as their presence in the molecule produces a characteristic isotopic distribution. The additional information obtained from the isotopic signature reduces dramatically the number of proposed calculated empirical formulae to a number typically below 5. The reduced number of elemental compositions can be searched in databases using elemental composition as the search criterion (e.g. Merck Index, ChemIndex, catalogues of standards manufacturer, and Google).

Another algorithm very often used is the background subtraction and noise reduction algorithm. To apply background subtraction, LC-MS analysis is performed on a sample of interest (analyte file) and on a control sample (blank). The software surveys each ion in the analyte file against that in the control file within the mass tolerance and retention-time-shift window. If such an ion is detected in the

control file, its maximal ion intensity is then multiplied by a predefined scaling factor and subtracted from the intensity of the ion in the analyte file. Zhu et al. further improved the software by adding a noise-reduction algorithm to help further clean up the residual ion noise after background subtraction by removing ion signals that are not consistent across many adjacent scans (Zhu et al. 2009). This tool was demonstrated to be very effective in detecting metabolites and transformation products without a priori knowledge of the structure, molecular weight or mass defect in complex matrices.

As concerns mass defect filter, it represent the difference between the exact mass and the nominal mass. With high mass accuracy, analyte ions can be discriminated from background matrix ions, since the mass defects of matrix ions are different than those of the analyte ions due to difference in their elemental composition.

Notwithstanding the power of these software, the results obtained should be considered tentative as long as the identification has not been verified with an authentic standard compound.

4.4.3 Use of Library or Database

As mentioned, unlike GC-MS, LC-MS still does not have commercially available or standardized spectral libraries, essentially due to the absence of a normalized interface. The ionisation differences between the existing interfaces, together with variability in results, depending on composition of the mobile phase or the cone voltage applied, make it difficult to use standardized libraries. This means that it is necessary to build home-made libraries to facilitate searching, focusing on particularly relevant compounds.

Obviously, the higher the number of compounds included in the library, the wider the possibilities for detecting as many contaminants as possible in samples.

For an accurate mass that could not be found in our accurate mass database, an elemental composition was proposed. The elemental composition could be used to search electronic databases (other than spectral databases) to find out whether the unknown was ever patented, studied or commercialized.

MS^n measurements were performed to obtain information of fragment ions generated in the linear ion trap (nominal mass of product ions) within the same analysis. In addition, the accurate masses of these product ions could be obtained in a second analysis. The masses of these fragment ions were linked with precursor compound masses and were ordered in a so-called “fragmentation tree”. Confirmation of the identity was done by comparing the retention time and fragmentation pattern of the compound to that of a synthesized reference standard (Hogenboom et al. 2009).

Theoretical mass spectra libraries, based on the molecular formula database, can be built which facilitate increasing the number of compounds that can be searched. These libraries use accurate mass measurements and isotopic pattern information for identification (Díaz et al. 2012). Home-made empirical libraries can also be used, but these normally include much fewer compounds due to the need to inject

standards. These experimental libraries offer fragmentation and retention time information as well, providing more confidence in the compound identification process (Díaz et al. 2011). However, the possibility of detecting and identifying the sample contaminants, using both mass spectra libraries in non-target analysis, depends on the success of the deconvolution process, i.e. the capability of the software to find the component peaks and to obtain mass spectra as free as possible from sample interferents. Obviously, the more complex the matrix, the more difficult the deconvolution will be.

Recently, Thurman et al. created a home-made Microsoft Access database of 350 pesticides amenable to positive-ion electrospray (Ferrer and Thurman 2007). To identify pesticides in food and water, another research group used a semi-automated "molecular feature", and database searching for the exact monoisotopic mass of 100 compounds, one exact mass in-source fragment for each compound and retention time (Ferretti et al. 2007). In spite of the progress made, there are still some limitations using this approach, as it was not feasible to automate the matching of isotope distribution.

Ibáñez et al. built a database including 104 compounds for pesticide searching. Insecticides, herbicides and fungicides commonly used were included in this list, as were some degradation products. All compounds were detected in ESI (positive ion mode), except for six analytes, which were ionized exclusively in negative mode. For each compound, library entries were created to include compound name, nominal mass, exact mass, molecular formula, retention time and polarity/cone voltage information (Ibáñez et al. 2008).

Diaz et al. built a database containing approximately one thousand pollutants of different families (pesticides, antibiotics, pharmaceuticals, illicit drugs, mycotoxins, anabolic steroids, personal care products and metabolites) (Díaz et al. 2012). The compounds were chosen on the basis of the author experience on LC-MS/MS analysis of environmental and food samples and on the bibliographic data on LC-MS amenable organic pollutants. The database was created separating positive and negative ionisable compounds. It contains information on the molecular formula (required by the software), the exact mass of the neutral and of the (de)protonated molecule, as well as supplementary information on the compound type and on retention time, when available. From the molecular formula of each compound, two theoretical mass spectra libraries (for positive and negative ionisation modes) were automatically built, containing theoretical nominal mass spectra of the (de)protonated molecule and sodium adducts, as well as the theoretical isotopic pattern expected for each compound. A drawback of the theoretical library (and also of the empirical mass spectra library) is that TOF MS spectra are stored in nominal mass for NIST format compatibility, and in this step the mass accuracy information given by TOF MS is lost. In order to minimize this limitation, the mass errors between the measured masses of the compound detected and the exact masses of the candidate formulae are calculated and used in a subsequent step, to rank them and to propose the most plausible identity (accurate mass scoring). When a compound is not found in the library, its deconvoluted accurate-mass spectrum can be used to propose its elemental composition. When employing the theoretical, library-based, non-target

screening approach, the investigation of findings when reference standards were unavailable was carried out using the same two strategies discussed before for post-target screening. However, the non-target approach still has some advantages, especially when using an experimental library, as a comparison of the suspect compound versus library spectra is automatically performed achieving a highly reliable identification. In addition, other non-expected compounds that might be present in samples at relatively high concentrations might be detected without any kind of selection (pre- or post-target).

Genuine non-target analysis would require the identification of all components in a sample, independently on their environmental relevance (Ibáñez et al. 2008). However, excess of zeal in identifying unknown compounds could waste time working on costly irrelevance (Ibáñez et al. 2008). Rather than perform general screening for unknowns, it therefore seems reasonable to focus analytical efforts on relevant contaminants even if this approach can miss other unexpected compounds that are significantly toxic (Ibáñez et al. 2008). In this situation, the most intelligent, efficient option seems to be to build large libraries, which include as many contaminants as possible chosen with criteria of use and toxicity, as that would minimize the risk of "false negatives" with respect to other significant hazardous compounds (Ibáñez et al. 2008). From this point of view, it would be very useful for the analyst to have available wide-ranging lists of "relevant" pollutants to facilitate identification in screening environmental water (Ibáñez et al. 2008).

4.5 Non-target Analysis of Emerging Contaminants

At present one of the great challenges in environmental analysis is the control of the risks associated to mixtures of emerging contaminants, which are continuously changing. Therefore, one of the major trends in analytical chemistry is to develop fast and efficient procedures for the trace analysis of target and non-target organic compounds in complex matrices. The improvements achieved during the last years in terms of sensitivity are mostly due to the development of UHPLC/MS techniques, which are today methods of choice for the determination of trace organic analytes in environmental and biological samples.

Trace analysis of organic contaminants in environmental samples is always challenging due to the complexity and diversity of sample matrices. A very important factor, that must be taken into account in the LC/MS methods, is the matrix effect. Signal suppression or enhancement during the ionization processes is caused by co-elution of the analytes with endogenous compounds of the sample (Gosetti et al. 2010c). These effects are strongly compound-dependent, and are also more pronounced with ESI then with APCI and in less extent with APPI because in the latter interface the ionization of the analytes is dependent on the ionization energy of the analyte rather than on proton affinity like in the two former ones. This phenomenon can compromise the accuracy of the analytical results in trace analysis of complex environmental samples. However, while matrix effects have been studied

for LC/MS/MS applications with ESI, relatively few studies evaluated matrix effects when using APCI or, even less, APPI source.

Because matrix effects might exert a detrimental impact on important method parameters (limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, and precision), sample pre-treatments, involving isolation of analytes, purification of extracts and pre-concentration, are generally required. It must on the other hand underlined that in a genuine non-target analysis, each sample pre-treatment could result just in a lack of the potential unknowns of interest.

Alternatively to the use of sample preparation techniques, direct injection of large volumes of surface water in LC-MS (100–5,000 μL) to determine emerging contaminants was also assayed (Chiaia et al. 2008; Berset et al. 2010). However, in this approach the sample amount introduced in the ESI source would cause an increase of the matrix effect with introduction of dirt into the instrument, with more maintenance of the equipment and loss of result reliability. Modern highly sensitive QqLIT instruments push for a significant reduction of the volume injected into the system (5–20 μL) without significant evidence in reducing sensitivity. Application of this instrument is still rare due to the unaffordable cost for most environmental laboratories but its exceptional features have allowed even sample dilution in order to reduce the matrix load into the system (Pérez-Parada et al. 2012). Several strategies are usually required to reduce the matrix effect such as the use of matrix-matched calibration, isotopically labeled surrogate compounds, or internal standard addition. Direct injection or "dilute and shoot" concept offers several advantages, such as reduced sample handling and increased reproducibility, which are attractive features for high sample throughput analysis (Pérez-Parada et al. 2012). Recovery studies and use of organic solvent are avoided, which is in the same line with environmentally friendly trends of current analytical chemistry (Farré et al. 2010).

However, in spite of its deduced advantages, direct injection analysis was found to have a major drawback associated to the detection of those less concentrated or weakly sensitive compounds (Pérez-Parada et al. 2012).

The data of the published non-target UHPLC/MS methods for the identification and determination of unknowns (when the standards are available) in water sample are collected in Table 4.1. For each manuscript the species or the classes of compounds considered, the kind of matrix analysed, the pretreatment process, the chromatographic and detection conditions used, LOD and LOQ values, when available, are reported.

Müller et al. developed a comparative approach to data evaluation in the non-target screening of organic substance in water analysis (Müller et al. 2011). The crucial difference between this and other approaches is the comparison of samples based on compounds determined by their full scan data. The sample is not regarded as an isolated specimen, but rather it is evaluated in relation to a set of other samples based on consideration of their temporal, spatial, or process-related connections. All the detected compounds of the different samples are used for the following data evaluation. By using set operators to compare all the compounds among a set of samples, it was possible to quickly and effectively recognize the compounds relevant to a given problem independent of their intensities. Considering only the mass

Table 4.1 List of the UHPLC/MS methods for the identification and determination of unknowns (when the standards are available) in water sample

Analytes	Analytical method	Experimental conditions	LOD, LOQ	Matrix	Sample pretreatment	References
Organic contaminants and/or residues including pharmaceuticals and drugs of abuse	UHPLC-QqTOF	Stationary phase: Acquity BEH C18 (150×2.1 mm, 1.7 µm). Mobile phase: 0.01 % formic acid water solution and 0.01 % formic acid methanol solution. Gradient elution. Flow rate: 0.3 mL/min	/	Wastewater	SPE with Oasis HLB cartridge.	Diaz et al. (2012)
Pesticides, antibiotics, pharmaceutical, drugs of abuse, mycotoxins together with metabolites, degraded and transformation products	UHPLC-QqTOF MS	Stationary phase: Acquity BEH C18 (50×2.1 mm, 1.8 µm) and Acquity BEH C18 (150×2.1 mm, 1.8 µm). Mobile phase: 0.01 % formic acid water solution and 0.01 % formic acid methanol solution. Gradient elution. Flow rate: 0.3 mL/min	/	Wastewater	SPE with Oasis HLB cartridge.	Diaz et al. (2011)
Pesticides, pharmaceutical compounds and metabolites	UHPLC-QqTOF MS/MS	Stationary phase: reversed phase XDB C18 (50×4.6 mm, 1.8 µm). Mobile phase: mixture of 0.1 % formic acid in water/ acetonitrile 5/95 (v/v) solution and 0.1 % formic acid in water solution. Gradient elution. Flow rate: 0.6 mL/min	LODs < 100 ng/L	Wastewater and river water	SPE with Oasis HLB cartridge	Gomez et al. (2010)

(continued)

Table 4.1 (continued)

Analytes	Analytical method	Experimental conditions	LOD, LOQ	Matrix	Sample pretreatment	References
Pesticides, therapeutic drugs, drugs of abuse and metabolites	UHPLC-QqTOF MS/MS	Stationary phase: reversed phase XDB C18 (50×4.6 mm, 1.8 µm).	/	Wastewater	SPE with Oasis HLB cartridge	del Mar Gómez-Ramos et al. (2011)
		Mobile phase: mixture of 0.1 % formic acid in water/ acetonitrile 5/95 (v/v) and 0.1 % formic acid in water.				
		Gradient elution.				
		Flow rate: 0.5 mL/min				
Insecticides, fungicides, herbicides and degradation products.	UHPLC-TOF MS	/	/	Surface, influent and effluent wastewater	SPE with Oasis HLB cartridge.	Ibáñez et al. (2008)
TPs of bromacil	UHPLC-QqTOF MS/MS	Stationary phase: Acquity BEH C18 (100×2.1 mm, 1.7 µm).	/	Ground water spiked with bromacil (20 µm/mL) and undergone to chlorination with 1 % sodium hypochlorite	/	Ibáñez et al. (2011)
		Mobile phase: mixture of 0.01 % formic acid in water and 0.01 % formic acid in methanol.				
		Gradient elution.				
		Flow rate: 0.3 mL/min				

Antibiotic	UHPLC-QqTOF MS/MS	Stationary phase: Acquity BEH C18 (100×2.1 mm, 1.7 µm). Mobile phase: mixture of 0.01 % formic acid in water and 0.01 % formic acid in methanol. Gradient elution. Flow rate: 0.3 mL/min	/	Water sample used for animal feeding	SPE with Bond Elut Plexa cartridge. Dried over anhydrous sodium sulfate. Concentrated to dryness and reconstituted with a 90:10 mixture of cyclohexane and ethyl acetate	Ibáñez et al. (2012)
Organic trace substances	UHPLC-QqTOF MS	Stationary phase: Zorbax Eclipse XDB-C18 (50×4.6 mm, 1.8 µm). Mobile phase: mixture of formic acid 0.1 % (v/v) water solution and formic acid 0.1 % (v/v) methanol solution in positive mode ionization (PI). Mixture of 5 mM ammonium acetate water solution and 5 mM ammonium acetate methanol solution in negative mode ionization (NI). Gradient elution. Flow rate: 0.6 mL/min	/	Drinking water	SPE with Isolute ENV+ cartridges	Müller et al. (2011)

(continued)

Table 4.1 (continued)

Analytes	Analytical method	Experimental conditions	LOD, LOQ	Matrix	Sample pretreatment	References
Organic contaminants	UHPLC-QqTOF MS	Stationary phase: Acquity HSS T3 (100×2.1 mm, 1.8 µm).	/	Treated and raw leachate water samples	Raw leachate samples were diluted 50-fold with ultrapure water. All samples were centrifuged.	Pitarc et al. (2010)
		Mobile phase: mixture of 0.1 mM ammonium acetate water solution and 0.1 mM ammonium acetate methanol solution.				
		Gradient elution.				
		Flow rate: 0.3 mL/min				
Pharmaceuticals and drugs of abuse	UHPLC-QqTOF MS and UHPLC-QqTOF MS/MS	Stationary phase: reversed phase XDB C18 (50×4.6 mm, 1.8 µm).	/	River water	Direct injection and SPE with Oasis HLB cartridge.	Pérez-Parada et al. (2012)
		Mobile phase: mixture of 0.1 % formic acid water/ acetonitrile 5/95 (v/v) solution and 0.1 % formic acid water solution.				
		Gradient elution.				
		Flow rate: 0.5 mL/min				

of the neutral molecule and the retention time enables to combine data sets of the positive and negative ESI modes for example. In the example presented, from a total of over 1700 compounds (only ESI+) present in a landfill leachate, ten waterworks and three drinking water-relevant compounds have been identified. Among these, the structures for crotamiton, carbamazepine, and 1-adamantylamine were unequivocally verified. The use of this new approach to data evaluation in non-target screening analyses opens the possibilities of various other applications, for example in open- and groundwater monitoring, in the remediation of contaminated sites, or for monitoring natural attenuation as well as for evaluating the process steps during drinking water treatment (e.g., flocculation and ozonation), and for following the impact of extended waste water treatment (e.g., activated charcoal filtration and ozonation).

Del Mar Gómez-Ramos et al. proposes a systematic approach to assist and simplify the identification of transformation products (TPs) of organic contaminants (del Mar Gómez-Ramos et al. 2011). This approach is based on the use of characteristic fragmentation undergone by organic contaminants during MS/MS fragmentation events, and the relationship and consistency with the transformations experimented by these chemicals in the environment or during water treatment processes. With this in mind, a database containing accurate-mass information of 147 compounds and their main fragments generated by CID MS/MS fragmentation experiments was created using an LC-QqTOF MS/MS system. The developed database was applied to the identification of tentative TPs and related unexpected compounds in eight wastewater effluent samples. The approach comprises basically three stages: automatic screening, identification of possible TPs and confirmation by MS/MS analysis.

In this first step, the software examines the entire chromatogram in order to search and group all the ions that can be logically associated with a real chromatographic peak and may represent a “feature” of a molecule. Search parameters must be adjusted according to the application. Complex matrices like wastewater effluents require the application of some filters to diminish the number of total extracted compounds that may result irrelevant to the analysis. For this application a peak filter ≥100 counts for the ion extraction (only including peaks whose height is greater than the value entered) and a compound filter with an absolute abundance higher or equal to the height of 100,000 counts have been selected (the height of the compound must be at least the value entered). To increase the speed of the analysis and simplify the identification step, it is important to apply a mass filter. In this work a mass filter with a tolerance of 5 ppm has been selected. With this option, the algorithm searches and extracts from the raw data only those compounds whose accurate masses match with the masses included in the database. The resulting compound list of molecular features is then matched with the CSV Excel file created as a database, to identify compounds and/or fragments included in it. The defined search criteria were: accurate mass, with a tolerance of 5 ppm and retention time with a tolerance window of ±0.3 min (Bloomfield 2002). The automatic screening described above generates a report with a list of compounds. Some of them are coincident with the database in both accurate mass and retention time. These com-

pounds correspond to target analytes and their fragments present in the samples. Eight degradation products, from the pharmaceuticals acetaminophen, amoxicillin, carbamazepine, erythromycin and azithromycin and from the pesticide diazinon, were identified with a high grade of accuracy. Three of them were also confirmed by analysis of the corresponding analytical standards.

4.6 Conclusions

With regard to environmental monitoring programs, a major advantage of LC-HRMS is the possibility of retrospective analysis of full scan data, which enables laboratories to search for "new" contaminants even years after data recording. This allows investigation of the presence of organic contaminants that were included in the first screening. This can be done at any time, without the need of new analysis or new sample injections. LC-HRMS opens the possibility to identify polar target, suspect, and non-target compounds with improved reliability and robustness. Accordingly, within the 2002/657/EC guideline (European Commission 2002/657/EC of 2002) HRMS precursor and product ions (resolution>20,000) earn 2 and 2.5 identification points, respectively, instead of 1 and 1.5 points for low resolution MS precursor and product ions. Therefore it seems likely that despite the higher investment costs as compared with LC-QqQ instruments, LC-HRMS instruments will find their way from research into routine analysis. Intelligent strategies allow for combining target analysis and suspects and non-target screening into the same analytical run, including the recording of product ion spectra for target and suspected compounds and intense unknown peaks by data-dependent MS/MS analysis.

Post-target analysis with current instruments is practically feasible and can be used to find new compounds in environmental samples. Post-target analysis can quickly be used to indicate the presence of a compound in a sample, even before the corresponding standard compound is available. However, there is a risk of occasional false-negative and -positive identifications and the results must be examined with care. The identification should be treated as tentative, until it has been confirmed using a standard compound.

Emerging LC-MS ionization techniques such as APPI could possibly extend the analytical window towards less polar compounds in the future. However, the application of APPI for the screening of suspects and unknowns is complicated by the fact that from one compound a range of different ions might be formed (molecule ion, (de)protonated molecule ion, adducts ions), which depends strongly on ionization conditions and is not uniform for compounds of different properties (Kauppila et al. 2002). For reliable formula assignment, resolution >60,000 is recommended and the accuracy of the isotope intensities must be excellent to allow that also elemental formula fits for substances without highly characteristic isotope pattern (consisting solely of C, H, O, N, F, P). To increase the success rate of this step, existing approaches have to be optimized like the ability to model MS/MS spectra including product ion intensities. Furthermore, quantitative structure–property rela-

tionships could be used for an improved estimation of chromatographic retention times and ionization efficiencies to support a tentative identification.

The study by Chalcraft et al. suggests furthermore that with a refined computational prediction of ionization efficiencies from chemical structure or available physicochemical parameters some progress could be made towards a (semi)quantitative analysis of unknowns and suspects without reference standards (Chalcraft et al. 2009). To enable a non-target screening of environmental samples with several thousand peaks within a reasonable time frame, advanced software solutions are needed with capabilities for automated batch processing and fast database queries. At the moment, integration within handy workflows of software packages is mainly available in the field of metabolomics, which is only partly applicable to environmental samples.

However, the non-targeted screening presents important drawbacks at compound low concentrations, especially in more complex-matrix samples, due to the difficulties in the components detection step. Identification of non-target contaminants is greatly facilitated when the compound detected is included in the home-made libraries, otherwise the elucidation of the compound becomes an analytical challenge where the possibilities of success are rare.

Further effort should be made to speed the development of more intelligent software for screening unknown compounds. So far, results of the content of a sample can be gained with the thorough manual inspection, but larger scale automated data treatment is still hindered by the lack of advanced features in the software.

Most probably, the impact of LC-HRMS in environmental analysis will increase within the coming years and this technology will make the environment more transparent with regard to the occurrence and fate of polar micropollutants.

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Chapter 5
PAHs Pollution Monitoring by Bivalves

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Contents

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are broad environmental contaminants which due to their lipophilic profile tend to be absorbed on particles and finally accumulate in marine environments. Analysis of PAHs is conducted in various commodities, such as water, sediment and in organisms, with bivalves possessing a predominant role due to their cumulative for organic contaminants and PAHs profile. Substantial research has been performed on the effects of PAHs pollution on the bioindicator itself, the marine bivalve, in matters of subcellular, cellular, tissue or organ alterations. Taking into account the persistence of these compounds, their ubiquity and the variety of health effects they may elicit to invertebrates, it is imperative to focus on biomarkers assessment after acute or subchronic exposure to PAHs. Nowadays, a tiered approach in Mussel Watch Programs is followed with chemical analysis of PAHs in mussel tissues being accompanied by detection of effects at subcellular, cellular, tissue, organ or organism level. This combined methodology serves on a number of levels-it measures with precision current PAHs pollution status and it links this pollution to possible health effects on bivalve populations

This review aspires to consolidate knowledge on PAHs analysis and the biological effects that they elicit in bivalves. In this context, it is provided an overview on PAHs pollution as monitored with the use of bivalves. Specific focus is given on: (1) PAHs analytical methodologies and their performance (2) levels of marine PAHs as measured through bivalves within the last decade; current pollution status and (3) health effects of PAHs on bivalves as shown from field studies and laboratory experiments.

Keywords PAHs • Bivalves • GC-MS • GC-MS/MS • HPLC-FLD • Pollution • Biomarkers • *In vivo* experiment • Field deployment • Biological effects

List of Abbreviations

MWP	mussel watch program
NOAA	National Oceanic and Atmospheric Administration
SD	standard deviation
APPI	atmospheric pressure photoionization
CRM	Certified Reference Material
SPE	solid phase extraction
DCM	dichloromethane
QA	quality assurance
IDL	Instrument Detection Limit
IAEA	International Atomic Energy Agency
SPME	solid phase microextraction
IS	internal standard
LC-MS/MS	liquid chromatography tandem mass spectrometry
HMW	high molecular weight
RSD	*relative standard deviation*

SIM	selected ion monitoring
MSPD	matrix solid-phase dispersion
SRMs	Standard Reference Materials, National Institute of Standards & Technology, USA
MAE	microwave assisted extraction
QC	quality control
GC-MS/MS	gas chromatography tandem mass spectrometry
LMW	low molecular weight
d-PAHs	deuterated PAHs
ASE	accelerated solvent extraction
GPC	g*el permeation chromatography*
GC-MS	gas chromatography mass spectrometry

5.1 Introduction

5.1.1 PAHs Chemical Profile and Origins

Polycyclic aromatic hydrocarbons PAHs are compounds composed of two or more fused and thermodynamically stable aromatic rings (for some examples see Fig. 5.1). The resulting structure is a molecule where all carbon and hydrogen atoms lie in one plane. The environmentally significant PAHs are those compounds which contain two (eg. naphthalene) to seven benzene rings. In this range, there is a large number of PAHs which differ in number of aromatic rings, position at which aromatic rings are bonded to one another, and number, chemistry, and position of substituents that they bear on the basic ring system.

PAHs are found in creosote, soot, petroleum, coal, and tar; and are the only organic contaminants that have natural sources (eg. forest fires) in addition to anthropogenic sources (eg. automobiles emissions, home heating, local releases of oil).

Generally the dominant source of PAHs in the environment is pyrolytic deriving from incomplete combustion at high temperatures of current and fossil matter. Petrogenic characteristics are actually attributed to slow maturation of organic mat-

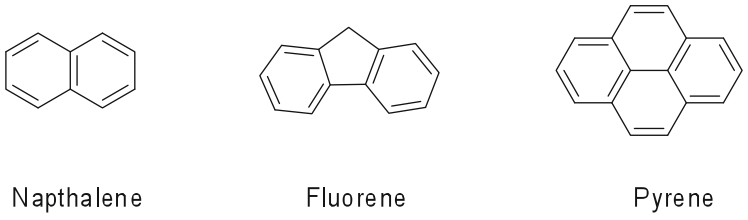

Fig. 5.1 Chemical structures of selected PAHs

ter under the geochemical conditions that preponderate and only after oil spills is a strong petrogenic profile observed. Diagenetic transformation of non-hydrocarbon natural products, chronic leakage of marine pipelines, domestic and industrial wastes have also been classified as PAHs sources. Study of PAHs distribution and PAHs concentration ratios can be used to discriminate PAHs of petrogenic and pyrolytic origin. Alkylated 2- and 3- ring molecules are derived mainly from petrogenic sources whereas 4- to 6- ring compounds are derived from pyrolytic sources. In general higher proportion of HMW PAHs suggests pyrolytic input, while a high proportion of LMW PAHs indicates petrogenic sources. This approach should be treated with caution when studying mussels and deducting conclusions because various PAHs have different uptake and depuration rates which will change their profile.

In this frame the monitoring programs such as the MWP encompass various hazardous compounds with common features such as (1) high chemical stability and persistency (2) hydrophobic behavior which indicates the tendency to accumulate in lipids and fat tissues and (3) acute toxicity.

Particularly and due to their hydrophobic nature and low aqueous solubility, PAHs tend to associate with particulate material. Subsequent particle deposition in rivers and coastal waters can lead to buildup of PAHs in sediments and filtering entities such as marine bivalves. Publications of early 2000s and afterwards showed that concentrations of PAHs in mussels' tissues from urban areas were in a range reported to exert biological responses (O'Connor 2002; Horii et al. 2009; Serpe et al. 2010a; Vinas et al. 2012). A review by Roose and Brinkman classified PAHs as some of the most hazardous and persistent organic pollutants (Roose and Brinkman 2005). PAHs levels in various compartments were efficiently reviewed by Srogi in 2007 with emphasis on bioaccumulation and biomarker responses in air, soil, water and food (Srogi 2007). PAHs concentrations in biota depend upon their proximity to the sources of pollution, their bioavailability, and species ability to metabolize PAHs.

It is widely regarded that the mussel of the genus Mytilus is one of the species most extensively used as a sentinel organism because of its broad distribution in both hemispheres. Mussels, oysters, clams and other filtrating organisms are complex matrixes with limited metabolic ability. PAHs accumulate at the fat tissue of these organisms rendering them as ideal bio-indicators for pollution. However extraction of analytes is sometimes troublesome and research teams try to circumvent this problem by appropriate extraction protocols. Thus extensive cleanup is required so as analysis results to be devoid of matrix interferences and subsequent misleading results. For fat elimination and clean-up, an initial step with chromatographic purification with silica and/or alumina is usually applied or size exclusion HPLC, followed by GPC (Sloan et al. 2004). Furthermore SPE is used extensively with various sorbents (silica, alumina, magnesium silicate Florisil, cyanopropyl). However samples prior to the introduction of SPE need to be pre-cleaned in most cases.

5.1.2 PAHs Extraction and Analysis

The classical approach to extract PAHs from lipophilic compounds which are abundant in marine matrices is by means of saponification in basic (NaOH or KOH) alcoholic solution [indicatively see (Webster et al. 2006; Rank 2009)] or the Soxhlet extraction with organic solvents mainly acetone, *n*-hexane and DCM [indicatively see (Vorkamp et al. 2010; Yoshimine et al. 2012)]. Both methods are widespread, but time consuming. For this reason more fast methods which reduce time, labour and solvent use were pursued by researchers. These include Sonication Assisted Extraction (Maioli et al. 2010; Dsikowitzky et al. 2011), Microwave Assisted Liquid Extraction (MAE) (Cortazar et al. 2008; Zuloaga et al. 2009), and Accelerated Solvent Extraction or Pressurized Liquid Extraction (ASE) usually conducted with the Dionex system (Sloan et al. 2004). Finally the matrix solid-phase dispersion (MSPD) has also been applied to extract PAHs from mussels even in miniaturized scale (Campins-Falco et al. 2008).

Mass spectrometry is a powerful analytical tool which provides unambiguous advantages in the field of identification of chemical structures. In combination with gas or liquid chromatography, it provides the ability to separate compounds with subsequent quantitative and qualitative information. The requirement of volatile or semi volatile compounds in gas chromatography is easily addressed for PAHs since these compounds usually share a volatile or semi-volatile profile.

Consequently, analysis of PAHs is mainly accomplished by GC-MS in simple or tandem mode (GC-MS/MS) and HPLC coupled with fluorescence detector (HPLC-FLD) since most of the PAHs are strong fluorophores. Multiple reaction monitoring (MRM) in a triple quadrupole GC-MS/MS is inherently more selective and sensitive than either scan or selected ion monitoring (SIM) as many matrix interferences are minimized or even removed. For this reason various groups have used tandem MS (Matozzo et al. 2010; Xia et al. 2012), however sufficient detection was accomplished also with GC-MS-SIM (Webster et al. 2006). HPLC-UV has also been used with less sensitivity as expressed by LOD values, although in one work LOD values with UV detection are reported quite low (Cravo et al. 2012). PAHs as in almost all UV or FLD methods were identified on the basis of retention time and quantification on an external standard method.

The use of LC-MS/MS has similarly been reported for PAHs analysis in other commodities -not bivalves-, with good results only when APPI was used. This is expected since ESI and APCI fail to ionize non polar compounds, such as PAHs, efficiently. The advantage of LC-APPI-MS/MS is that it can simultaneously analyze polar and nonpolar analytes with the potential to lower detection limits. Although not applied in bivalves, in this overview these limited reports will be addressed since they set new perspectives on LC-MS/MS analyses of PAHs which can be adapted to bivalves' analysis as well.

5.2 Overview of Last Decade's Respective Mass Spectrometry Analytical Methods

5.2.1 Gas Chromatography Mass Spectrometry Methodologies

The majority of research endeavors presented in this overview separate and detect PAHs, utilizing GC-MS either in full scan or in SIM mode, demonstrating satisfactory LODs by both ways e.g. (Gaspare et al. 2009; Choi et al. 2010). Separation is performed on HP5-MS or DB5-MS columns, however there are reports also on less widespread columns such as the methyl silicone column or the DB-EUPAH CF column (Uhler et al. 2005; Xia et al. 2012).

To manage a sufficient detection it is essential to extract almost quantitatively PAHs from complex matrices –such as bivalves- and minimize interferences. Simplified procedures for the analysis of PAHs in mussels have been reported by various groups whose primary aim was the optimization of GC-MS methods (Martinez et al. 2004). Indicatively Navarro et al. assessed different clean-up procedures used in the determination of PAHs in biota samples such as oysters and mussels (Navarro et al. 2006). Briefly the extraction of PAHs was pursued by (a) Microwave assisted saponification (MAS) (b) Microwave assisted extraction (MAE) and their clean up by SPE and GPC.

For the SPE, Florisil cartridges proved facile for the clean-up of MAE extracts after comparison of recoveries with the NIST 2977 SRM superseding in terms of performance the MAS approach. Finally by GPC cleaner extracts were obtained, leading to the conclusion that GPC in combination with MAE was the most sufficient approach for the extraction and purification of PAHs from biota matrices.

5.2.2 Liquid Chromatography Mass Spectrometry Approaches

An important work on the LC-APPI-MS/MS field was developed for the analysis of 12 PAHs in sediment samples (Moriwaki et al. 2004). In this context APPI was performed in positive mode aiming to overcome the low sensitivity of poor fluorescence PAHs. Extraction of PAHs from sediments was conducted with ultrasonic agitation and the mixture was purified on a silica cartridge. PAHs were eluted with a mixture of hexane: toluene (9:1) and separated on a reversed phase ODS column with a runtime of 20 min. From mass spectrometry point of view the positive molecular ions were observed as the main peaks for all PAHs. Limits of determination ranged from 6 to 91 ng/g thus they were comparable with LODs presented in HPLC-FLD analyses. The method had also acceptable recovery values (90–116%).

In the second work the utility of UPLC-APPI-MS/MS for high-sensitivity and high throughput analysis of US EPA 16 priority PAHs was demonstrated (Cai

et al. 2009). All PAHs were analyzed on column in approximately 3.5 min improving the runtime compared to previous work (Moriwaki et al. 2004) while achieving low picogram detection limits (DLs range from 1.7 to 158 pg for dibenzo[*a,h*] anthracene and acenapthylene respectively). The advantage of this method with existing U.S. EPA methods is that it improves sample throughput by at least tenfold.

Smoker's et al. work -presented in 2010- was stimulated by the accident of Deepwater Horizon rig in the Gulf of Mexico and the subsequent burden of pollutants to marine organisms that has aroused. Shrimp was the matrix organism and analysis of PAHs was achieved by LC-APPI-MS/MS in positive mode using toluene as a charged dopant to ionize analytes. The experimental part was based on QuEChERS procedure. Analytical method was robust, with good linearity and acceptable recoveries for all analytes with better results obtained when primary-secondary amine (PSA) was used. LODs ranged from 20 to 510 ng/g (Smoker et al. 2010). In regards to the LOD of 20 ng/g which corresponds to benzo[a]pyrene a report by Johnson states that GC-MS/MS is more efficient in terms of standard detection with an LOD value < 1 ng/g (Johnson 2012).

Hutzler et al. in 2011 presented an improved method for PAHs detection in the picogram scale with an LC-APPI-MS/MS method (Hutzler et al. 2011). Sample preparation consisted of Soxhlet extraction or refluxing in toluene. After evaporation of solvent, the mixture was forwarded to silica SPE cartridges and PAHs were eluted with a 98:2 mixture of n-hexane: DCM. LC separation was accomplished on an Envirosep PP column,. LODs of pure compounds dissolved in solvents were determined in the ranges of 1.5–10.9 pg, while for matrix samples (extracted) in the range of 2.3–72 pg. The application of this method to the analysis of certified SRMs proved that data of both sides were in full agreement.

5.3 HPLC Analytical Methods

HPLC fluorescence (HPLC-FLD) methods are quite extensive and are depicted in Table 5.2. In general they are efficacious and can satisfactory address the need for substantial detection and quantification of PAHs. An issue which might pose some concern over these methods is that they usually –and if targeting a large number of PAHs- require prolonged runtimes, which in some cases reach 60 min (Nieto et al. 2006).

In a recent paper the utility of HPLC-UV in PAHs determination in clams of an important ecosystem (Ria Formosa lagoon in Portugal) was demonstrated. The clam *Ruditapes decussates* has a great economic value and has been widely used as a biomonitor. Clams were sampled between July 2007 and December 2008. The detection limit was in accordance with previous work by the same group (0.01–0.04 ng/g dry weight). TPAHs mean concentration ranged for all sampling seasons from 126 to 342 ng/g dw (Cravo et al. 2012).

5.4 Levels of PAHs Marine Pollution Through Bivalve Monitoring: Trends Within the Last Decade and Current Pollution Status

In this review article as stated above PAHs pollution status is presented in works or surveys in which sampling was performed after 2000. PAHs levels in this overview are presented in Tables 5.1 (for GC-MS and GC-MS/MS) and 5.2 (HPLC-FLD) and are in ng/g scale either in wet or dry weight, or in lipid.

5.4.1 NOAA-MWP

Since 1986 the NOAA MWP has monitored concentration of trace chemicals in the Coastal United States by sampling mussels, oysters and sediment. In 2006 a follow-up on the chemicals concentration in mussels and oysters collected along the US coast (O'Connor and Lauenstein 2006) was published as a continuation to the previous works published by O'Connor in 1998 and 2002 related to MWP results on various contaminants including PAHs (O'Connor 1998, 2002). The median annual concentrations of total PAHs for 2000/2001 and 2002/2003 were 140 and 220 ng/g dw, respectively. The resulting data showed that concentration of lindane and high molecular weight PAHs were decreasing at that time on national level when compared with data from previous years.

NOAA established threshold values to compare categorize and group contamination for PAHs. These were divided in three contamination categories: (1) low from 47 to 828 ng/g, (2) medium 829–2,511 and (3) high > 2,512 ng/g.

Baumard et al. have efficiently approached the contamination levels of PAHs and their relation with sediments respective pollution (Baumard et al. 1998a, b, c, d, 1999b). Thus a baseline level for the mussel PAHs residue appears to be in the range of 300–500 ng/g for the bivalves exposed to the least contaminated sites (sediment PAHs concentration < 3,000 ng/g). This level is the outcome of the balance among uptake and depuration of xenobiotics. When the contamination is higher tissues cannot depurate contaminants at the same rate that they accumulate them (Baumard et al. 1998a, 1999a). Thus this can be considered as a reference value and can be used to categorize pollution according to NOAA threshold values.

In the following paragraphs results on PAHs pollution of bivalves will be presented according to geographical origin:

5.4.2 South America

Contamination higher than the mentioned baseline values has been observed in various works. In one of them high contamination has been reported in Ushuaia coastal zone in Argentina when it reached in one sampling point the maximum

Table 5.1 GC-MS, GC-MS/MS analytical methodologies for PAHs determination in bivalves

Sampling period	Reference	Location	Species	Extraction-sample preparation	Limit of detection/ quantification	Results/remarks validation	PAHs measured
2001–2007	Choi et al. (2010)	Korean Coast	*M. edulis, M. coruscus, Crassostrea gigas*	Digestion KOH, LLE *n*-hexane, activated silica gel column-hexane-DCM elution	1 ng/g dw	Mean .Concentration 83–387 ng/g dw	GC-MS
						Recovery 64–108%	
						No trend on PAHs distribution	
2005–2006	(Ramdine et al. 2012)	Guadeloupe, French West Indies	*Crassostrea rhizophorae*	IS addition d-PAHs,	No ref	TPAHs 66–961 ng/g dw	GS-MS-SIM
				MAE			HP5-MS
2005	Gaspare et al. (2009)	Tanzania	*Saccostrea cucculata*	Na_2SO_4 homogenization, DCM extraction, clean up silica gel-deactivated Al_2O_3, elution DCM: hexane (1:1)	<1 ng/g dw	ΣPAHs 174–647 ng/g dw Recovery 56–88%	23 PAHs
							GS-MS-SIM
							HP5-MS
May 2010 to August 2011	Xia et al. (2012)	USA, Gulf of Mexico (Mississippi)	Blue crabs (*Callinectes sapidus*),	ASE with DCM/ acetone	0.5 ppb (ng/g) ww, as IDL	Average PAHs concentration	25 PAHs
			Oysters (*Crassostrea virginica*)	Silica gel/alumina purification, elution petroleum ether-DCM, d-PAHs addition		Oyster 34 ng/g	GC-MS/MS
						Carb 42 ng/g	DB-EUPAH CF column
						Recovery 60–130%	
						LMW PAHs 40–60%	

(continued)

Table 5.1 (continued)

Sampling period	Reference	Location	Species	Extraction-sample preparation	Limit of detection/ quantification	Results/remarks validation	PAHs measured
Jan. April 2008	Pereira et al. (2011)	Estuary NW Spain	*Cerastoderma edule, M. galloprovinciallis*	Baumard et al. (1999b)	No value for LOQ	TPAHs 97–1,056 ng/g dw	19 PAHs GC-MS/MS
2006, 2008–2009	Brooks et al. (2011)	Ekofisk platform area	*M. edulis*	Homogenization, IS addition, Saponification extraction, cyclohexane, size exclusion chromatography	No reference	ΣPAHs lower in 2009 than 2006 and 2008 samplings	GC-MS-SIM
August 2006 to April 2007	Vorkamp et al. (2010)	Faroe Islands, Greenland, Ghana, South Africa, Australia, Solomon Islands, New Zealand, Chile, US Virgin Islands, Boston, Newfoundland and Shetland Islands	*M. edulis, Mytilus sp., Chlamys opercularis, Chlamys Islandicas, Saccostrea sp., Saccostrea glomerata, Perna Perna, Genoina coaxans, Isognoman alatus, Tapes pullastra*	Soxhlet-toluene, dialysis, clean up Silica column, IS addition	DLs, 0.1–1 ng/g ww.	ΣPAHs 177–5,966 ng/g dw	GS-MS HP5-MS

May to June 2011	Yoshimine et al. (2012)	SE Brazilian Coastal Zone	*Perna Perna* Linnaeus 1758	Lyophilization, Soxhlet-DCM, initial clean-up neutral alumina, GPC and adsorption chromatography with neutral alumina-silica gel-Na_2SO_4, copper	DL, 0.2 ng/g dw	Mean TPAHs concentration	38 PAHs
					QA on NIST 2974a 100% recovery	33–761 ng/g dw	GC-MS/MS
July 2002 to June 2004	Quiniou et al. (2007)	French Atlantic Coast, Arachon	*Crassostrea gigas*	Protocol by Baumard 1997	Detection threshold 0.001 ng	ΣPAHs for two sites	GC-MS
						Entrance harbor 566 ng/g dw. Inner part 384 ng/g	
2004–2006	Galgani et al. (2011)	Western Mediterranean (Spain, Italy, France North Tunisia, Algeria, Morocco) **Mytilos Project**	*M. galloprovincialis*	Lyophilization, ASE with hexane:acetone, purification silica gel cartridge	1 ng/g dw	TPAHs, 22–106 ng/g dw	16 PAHs
						Classification of stations according to hydrocarbon origin, mostly pyrolitic origin	GC-MS
							DB5-MS column
	Liguori et al. (2006)	–	*M. edulis*	Freeze-dried mussel, ASE step with Al_2O_3 and silica gel	0.11–0.88 pg/g dry mass	Validation on SRM 2977	24 PAHs
					The lowest in literature	No real samples analyzed	GC-MS-SIM
						Recovery 81–116%	HP-5MS column

(continued)

Table 5.1 (continued)

Sampling period	Reference	Location	Species	Extraction-sample preparation	Limit of detection/ quantification	Results/remarks validation	PAHs measured
November 2002 to March 2004	Bartolome et al. (2010)	Bilbao estuary, Spain	*Not specifying species*	Freeze-dried samples, dPAHs addition, MAE in acetone, SPE Florisil, elution *n*-hexane:toluene	0.5–11 ng/g dw	QA on SRM 2977	16 PAHs
						TPAHs, 5,100–18,300 ng/g dw	GC-MS-SIM
							HP5-MS column
January February 2006	Zuloaga et al. (2009)	India, Sunderban Mangrove Wetland	*Meretrix meretrix, Macoma birmanica, Sanguilonaria acuminate*	Dry biota, MAE acetone, Florisil SPE clean up, d-PAHs addition	0.23 o 25.5 ng/g dw	Validation on SRM 2977	16 PAHs
						ΣPAHs 122–534 ng/g dw	GC-MS
						Recoveries > 50% except for naphthalene > 25%	HP5 capillary column
June 2002 to September 2004	Cortazar et al. (2008)	Urdaibai, Bay of Biscay	*Crassostrea sp.*	Dry oyster, d-PAHs addition, MAE acetone, Florisil SPE clean up, elution *n*-hexane:toluene	0.5–11 ng	TPAHs, 300–1,400 ng/g dw	16 PAHs
							GC-MS-SIM
2006	Bustamante et al. (2010)	Urdaibai, Bay of Biscay	*Crassostrea sp.*	Homogenization, freeze-drying, MAE acetone, Florisil purification	LODs, 0.09–12.4 ng/g	ΣPAHs, 290–1,814 ng/g dw	GC-MS
						Validation On SRM 2977	
	Phillips et al. (2006)	USA, Santa Barbara Channel, 4H shell mounds	*M. galloprovincialis, Macoma nasuta*	EPA 3550A, 8270C methods	No reference	TPAHs, 78–1,880 ng/g dw	GC-MS-SIM

October 1999, 2003	Amin et al. (2011)	Ushuaia Coastal Zone, Argentina	*Mytilus edulis chilensis*	Na_2SO_4 wet tissue homogenization, Fractionation silica:alumina, organic solvent elution	No reference, for individual PAHs reported concentration < 5 ng/g lipid assumed as their LOD	TPAHs, 2,240–2,420,000 ng/g lipid	16 PAHs
						Recoveries for spiked mussels 87 ± 8.2 %	GC-MS-SIM
						Quality Control NIST SRM 1974b	DB5 column
July 2006, October 2007 and February 2008	Maioli et al. (2010)	Mundaú Lagoon, Maceió, Alagoas, Brazil	*Mytella charruana*	Lyophilized samples, Ultrasonication with DCM, Al_2O_3 clean up, fractionation with silica gel, elution with hexane-DCM	Quantification Limit 0.1 ng/g	ΣPAHs (16 PAHs)	17 PAHs (16 USEPA+ Perylene)
						17.7–144.9 ng/g ww	GC-MS-SIM
							DB5 column
–	Martinez et al. (2004)	–	–	Lyophilization, Soxhlet Utrasonic-PLE (DCM:hexane, 1:1), For Soxhlet-Ultrasonic Alumina SPE, for PLE alkaline digestion KOH	0.5–8 ng/g dry mass	Recoveries 64–121%	16 PAHs
							GC-MS-SIM
							DB5 column
From 1999 each month to 2005	Webster et al. (2006)	Scotland	*M. edulis*	Homogenization of sample, d-PAHs addition, saponification NaOH *iso*-hexane extraction, HPLC fractionation	0.05–0.2 ng/g ww	TPAHs 12.5–151.2 ng/g ww	16 PAHs
						Recoveries >80%, for 1 ng/g ww.	GC-MS-SIM
							HP5-MS column

(continued)

Table 5.1 (continued)

Sampling period	Reference	Location	Species	Extraction-sample preparation	Limit of detection/ quantification	Results/remarks validation	PAHs measured
October 2005 to September 2007	Duedahl-Olesen and Ghorbani (2008)	Denmark	*M. edulis, Ostrea edulis, Cerastoderma edule, Spisula solida*	Homogenized mussel tissue, IS addition, Dionex ASE with *n*-hexane: acetone (1:1), GPC on S-X3 and SPE silica	0.05 ng/g to 1.0 ng/g fresh weight for LVI	Recoveries ranged for two spiking levels 2 and 11 ng/g from 72 to 124 %. With LVI from 89 to 110 %, for only 5 PAHs, RSD_r 1–22 %, RSD_R 4–38%	5PAHs
					0.1–1.8 ng/g fresh weight splitless injection		GC-MS with LVI and splitless, DB-5MS
February 2003	Perez-Cadahia et al. (2004)	Galicia, NW Spain	*M. galloprovincialis*	See Baumard et al. (1999a, b)	No reference	TPAHs, 5,978–17,033 ng/g dw	GC-MS
August 2008	Li et al. (2010)	Yuandnag Lagoon, China	*Crab (Portunus pelogicus), clam (Ruditapes philippinarum)*	ASE with hexane: DCM, glass column alumina, elution with hexane: DCM, addition of IS	MDLs, 0.6–9 ng/g dw	ΣPAHs mean value	16 PAHs
						Clams 251 ng/g dw	GC-MS-SIM
						Crabs 215 ng/g dw	HP5-MS column
						RSDs < 20%	
August 1999 to April 2000	Yim et al. (2002)	Sori, Kumo, Dolsan Islands-Korea	*Mytilus sp.,*	Na_2SO_4 dried mussels, dPAHs addition, Soxhlet with DCM, Si/Al column chromatography, HPLC size exclusion	No reference	ΣPAHs 172–1,443 ng/g dw monitoring sites, 278–431 ng/g dw control, 144–664 ng/g dw transplanted,	24 PAHs
			Crassostrea sp.			Recoveries from 80 to 111%	GC-MS
							DB-5MS

Srping and Fall 2001	Salazar et al. (2005), Uhler et al. (2005)	Delaware River, USA	Clam, *Rangia cuneata*	Modified USEPA 8270 method protocol,	Approximately 1 ng/g dw	Spring TPAHs, 468–5,876 ng/g dw, Fall 600–2,999 ng/g dw	16PAHs+ alkylated PAHs
				Na_2SO_4 assisted extraction with DCM, dPAHs addition, KD evaporation, GPC-HPLC purification			GC-MS-SIM
							Methyl silicone capillary column
February and November 2003	Fernandez-Gonzalez et al. (2008)	Punta Insua, San Anton, Spain	–	Freeze dried mussels, dPAHs addition, MAE hexane: acetone (1:1), glass column alumina, hexane elution	IDL, 1–26.9 ng/L	PAHs concentration 0.23–80.4 ng/g ww	27 PAHs
					LOD method, 1.9–79 pg/g	Validation on SRM 2977	PTV-GC-MS/MS and GC-MS-SIM
						RSD% for repeatability 0.5–8%	
						Reproducibility 1.1–11%	
November 2002 to November 2003	Bellas et al. (2007)	Göta Älv Estuary, Sweden	*M. edulis*	Homogenization, drying, Soxhlet toluene,	DL, 2–10 ng/g dw	ΣPAHs, average 3,700–5,200 ng/g lipid basis	GC-MS
						Highest 7,000–14,000 ng/g lipid basis	
						In the range of 40–420 ng/g dw	

(continued)

Table 5.1 (continued)

Sampling period	Reference	Location	Species	Extraction-sample preparation	Limit of detection/ quantification	Results/remarks validation	PAHs measured
June 2007	Matozzo et al. (2010)	Lagoon of Venice, Italy	*Ruditapes Philippinarum*	Cold Soxhlet Acetone: *n*-hexane 1:1, drying of extracts, *n*-hexane recovery of PAHs, digestion, clean up Silica gel/ Florisil, elution hexane: DCM	LOD 0.1 ng/g dw	ΣPAHs,142–655 ng/g lipid weight	GC-MS/MS and GC-MS-SIM quantitation
–	Navarro et al. (2006)	Estuary of Bilbao-Urdaibari, Spain	*M. edulis, Crassostrea sp.*	MAS-MAE, SPE (Si or Florisil)-GPC with DCM		Better recoveries with MAE in conjunction with 5 g Florisil, cleaner extracts with GPC	16 PAHs GC-MS
December 2005	Arias et al. (2009)	Bahia Blanca Estuary, Argentina	*Brachidontes sp., Tagelus sp.*	Matrix digestion, *n*-hexane extraction, clean up Alumina-Si column, *n*-hexane: DCM (9:1) elution	LOD 0.1–0.2 ng/g dw	PAHs 348–1,597 ng/g dw QC on Recovery of IS range from 75 to 105%	17 PAHs GC-MS
–	Tsangaris et al. (2011)	East Mediterranean, Greece, MYTIMED PROJECT	*M. galloprovincialis*	Dried mussels, saponification, hexane elution, alumina purification	No reference	Peak concentration 72.9 and 75.3 ng/g dw Accuracy, precision based on CRM IAEA-432	GC-MS

October 2000	Skarpheoinsdottir et al. (2007)	Nordic Coastal Ares, Iceland-Norway-Sweden	*Mytilus spp.*	Homogenization, dPAHs addition, saponification, *n*-pentane extraction, GPC	As DL, 0.5 ng/g ww.	TPAHs 10–11,670 ng/g dw	32 PAHs
						Accuracy test with SRM 2977	GC-MS-SIM
August–September 2007 and January 2008	Sureda et al. (2011)	Eivissa and Formentera Islands, Spain	*M. galloprovincialis*	Planas et al. (2006)	No reference	ΣPAHs	16 PAHs
						Monitoring site 15.8 ng/g dw, control site 4.6 ng/g	GC-MS
						SRM 2977 used	DB5 column
November 2003	Rank (2009)	Lynaes Denmark	*M. edulis*	Saponification KOH, *n*-pentane extraction, silica gel- Al_2O_3 column,	LOD, 0.1–2 ng/g ww.	ΣPAHs for two sites, 85 and 636 ng/g ww, reference point 32 ng/g ww	GC-MS
April 2004	Namiesnik et al. (2008)	Mokpo Bay, Korea	*M. galloprovincialis*	Freeze dried samples, digestion extraction with cyclohexane, silica gel column, elution DCM	LOD, from 0.3 to 1.3 ng/g ww	ΣPAHs from 1 to 31 ng/g ww	GC-MS
March to December 2010	Nahrgang et al. (2012)	Norwegian Fjord, Barents Sea	*M. edulis, Chlamys islandica*	Homogenization, IS addition, saponification, pentane extraction, GPC-SPE purification	LOD, 5 ng/g ww.	Concentration up to 22 ng/g ww, *M. edulis*, 5.7–17 ng/g ww *Chlamys islandica*	GC-MS
February 2003	Labarta et al. (2005), Babarro et al. (2006)	Galician coastline	*M. galloprovincialis*	Dry sample saponification, LLE hexane: DCM,	LOD, 0.67–7.85 ng/g dw.	PAHs levels, 196–502 ng/g dw	GC-MS
						Close to industrial area 4–6 ring PAHs predominate	

(continued)

Table 5.1 (continued)

Sampling period	Reference	Location	Species	Extraction-sample preparation	Limit of detection/ quantification	Results/remarks validation	PAHs measured
2000–2003	O'Connor and Lauenstein (2006)	US Coast	*M. edulis, M. californianus, C. virginica*	Tissuemizer DCM extraction, DCM-hexane exchange, fractionation alumina-silica chromatography or HPLC or ASE	No reference	Median ΣPAHs 2000–2001, 140 ng/g dw, 2002–2003, 220 ng/g dw	GC-MS-SIM
						HMW PAHs decreasing compared to previous decade	DB-5 fused capillary
September 2008	Dsikowitzky et al. (2011)	Segara Anakan, Indonesia	*Polymesoda erosa* clam, *Saccostrea sp*. oyster, *Episesarma versicolor* crab	Soft tissue homogenization, Na_2SO_4 drying, acetone ultrasonication, SPMs	No reference	Contaminant burden	GC-MS
						P. erosa ≥ E. versicolor ≥ Saccostrea sp.	
January 2005	Lima et al. (2007)	NW coast Portugal	*M. galloprovincialis*	Homogenization, IS addition, hexane: DCM extraction, silica gel-alumina chromatography	LOD 10 ng/g dw	TPAHs 124,000–549,000 ng/g dw	GC-MS
Feb. June and Nov. 2003	Soriano et al. (2006)	Galician coast	*M. galloprovincialis*	Soxhlet DCM,	LOD 0.68–3.4 ng/g dw	See HPLC respective ref.	GC-MS

Sep.–Oct. 2001	Fung et al. (2004)	China East Coast	*Perna viridis, M. edulis*	Homogenization, freeze drying, IS addition, tissumizer, DCM extraction, fractionation silica gel, hexane: DCM elution	DL, 1–2 ng/g dw	TPAHs 457–3,496 ng/g dw	24 PAHs
						Recoveries 84–88% spiked samples	GC-MS-SIM
						SRM 2974, 84–97%	
1999 to Dec. 2004	Francioni et al. (2007)	Guanabara Bay, Brazil	*Perna perna*	Freeze drying, homogenization or chemical drying sodium sulfate, Soxhlet MeOH, hydrolysis KOH, column Al_2O_3, DCM:hexane elution, adsorption chromatography, elution DCM:hexane, dPAHs IS addition	DL, 0.5 ng/g dw	Σ 35PAHs from 60 to 6,271 ng/g dw, Σ 16PAHs from 9 to 273 ng/g dw	35 PAHs including 16 USEPA
						Alkylated homologues predominate	GC-MS
						Recoveries > 80%	DB-XLBITD column
Nov. 2006 to Jan. 2008	Fernandez-Tajes et al. (2011)	Muros-Noia estuary, Galicia	*M. galloprovincialis*	See Baumard et al. (1999a, b)	No reference	TPAHs lower than 800 ng/g	35 PAHs
							GC-MS
Aug. 2003 to June 2004	Laffon et al. (2006)	Lira-Ancoradoiro beaches, Galicia	*M. galloprovincialis*	See Baumard et al. (1999a, b)	No reference	TPAHs, 500–3,500 ng/g	35 PAHs
							GC-MS

Table 5.2 *HPLC-FLD, HPLC-UV analytical methodologies* for PAHs determination in bivalves

Sampling period	Reference	Location	Species	Extraction-sample preparation	Limit of detection	Results/remarks	PAHs measured
November 2007	Trisciani et al. (2012)	NW Adriatic Sea	*M. galloprovincialis*	ASE Dionex	LOD, 0.01–0.5 ng/g dw	ΣPAHs 14–56 ng/g dw	16 PAHs
						QC with CRM	HPLC-FLD and HPLC-PDA for acenapthylene
						Precision-reproducibility RSD from 4.3 to 18.5 %	
January 2008 to March 2009	Serpe et al. (2010b)	Campania, Italy	*M. galloprovincialis*	Homogenization, Saponification, cyclohexane extraction, Purification on SEP-PAK cartridges	LOD, 0.2–0.8 ng/g ww	ΣPAHs 0.2–16 ng/g ww	11 PAHs
						Recoveries 51–72% acceptable	HPLC-FLD
From Nov. 1998 to Nov. 2008	Vinas et al. (2012)	Ria de Vigo, NW Spain	*M. galloprovincialis*	Freeze dried samples, Soxhlet hexane: Acetone 1:3, Alumina column purification, hexane elution	LOD, 0.1–0.4 ng/g dw	ΣPAHs 24–480 ng/g dw	13 PAHs
						Reproducibility better than 70–90%	HPLC-FLD
July 2006 and 2007	Bouzas et al. (2011)	Eastern Mediterranean Spanish Coast, Communidad Valenciana	*M. galloprovincialis, Donax trunculus* (clam)	Lyophilization,	LOD 0.05–35 ng/g dw	ΣPAHs 1.1–44.5 ng/g dw (mussels), 2.9–67.3 ng/g dw (clams)	8 PAHs, HPLC-FLD
				In tube SPME			
–	Yusa et al. (2005)	–	*M. edulis*	Homogenization, ASE DCM:acetone,	LOQ 0.1–0.25 ng/g dw	Validation on SRM 2977	12 PAHs, HPLC-FLD
						ASE with DCM:acetone better than methanolic saponification	

2004/2005	Curtosi et al. (2009)	Antarctica, Potter Pove	*Laternulla elliptica*	Sonication DCM, Hexane reconstitution, SPE, elution hexane: DCM 9:1	LOD 0.1–0.3 ng/g dw	ΣPAHs 105 ± 40 ng/g dw gland, 124 ± 40 ng/g dw gonad	25 PAHs
						Accuracy SRM 2977	HPLC-FLD
						Recovery 80–96%	
2004	Perugini et al. (2007)	Central Adriatic Sea	*M. galloprovincialis,*	Homogenization, KOH saponification, cyclohexane extraction, Florisil clean-up	LODs 0.05–0.25 ng/g ww	Mean PAHs concentration 0.15–15.64 ng/g dw	13 PAHs
							HPLC-FLD
–	Bihari et al. (2007)	Gulf of Rijeka, Adriatic Sea, Croatia	*M. galloprovincialis*	KOH saponification, cyclohexane extraction, addition of Al_2O_3	As DL 0.1 ng/g ww	ΣPAHs 49–134 ng/g ww	10 PAHs
						Preference for LMW PAHs	HPLC-FLD
–	Campins-Falco et al. (2008)	Comunidad Valenciana	*Mussels, tellins*	Miniaturized MSPD-samples C18 phase-Florisil, in tube SPME	LOD 0.05–0.6 ng/g dw	Optimization of method	HPLC-FLD
						SRM 1974b NIST	
						Overall recoveries 10–28%	
–	Rey-Salgueiro et al. (2009)	Galicia-Rias Gallegas, Spain	*M. galloprovincialis, clams (Venerupis pullastra), cockles (Cerastoderma edule)*	Lyophilization, Ultrasound *n*-hexane,clean up SEP-PAK and C18 cartridges	LOD 0.02–1.4 ng/g ww	ΣPAHs 0.48–5.5 ng/g ww for clams, 5.7–13 ng/g ww for cockles, and 7.4–47 ng/g ww for mussels	11 PAHs
						Recoveries by spiking mussels 68–95%	HPLC-FLD

(continued)

Table 5.2 (continued)

Sampling period	Reference	Location	Species	Extraction-sample preparation	Limit of detection	Results/remarks	PAHs measured
From Nov. 2002 to Feb. 2003	Nieto et al. (2006)	Galician Coast, Spain	*M. galloprovincialis*	Lyophilization, Soxhlet acetone:hexane, SPE-Alumina	No reference	ΣPAHs 2,500–5,900 ng/g dw – immediately after spill, to 130 ng/g dw late season	16 PAHs HPLC-FLD
May 1999 and 2001	Pikkarainen (2004)	Gulf of Finland to Southern Baltic Proper	*Macoma bathica* *Astarte borealis*	Saponification, IS addition, n-hexane extraction, alumina-silica column	No reference	ΣPAHs 44–298 ng/g dw Relative high bias value of 26%	12 PAHs HPLC-FLD 62 min
Summer 2001	Vassura et al. (2005)	Pialasa Baiona (Ramsar site), Italy	*Tapes phillippinarum*	Saponification, n-pentane extraction, fractionation over alumina, elution with DCM:n-pentane, column chromatography silica	No reference	ΣPAHs for two sites 100–130 ng/g dw	HPLC-FLD
Feb. June and Nov. 2003	Soriano et al. (2006)	Galician coast	*M. galloprovincialis*	Soxhlet acetone:hexane (1:3), clean-up column chromatography deactivated alumina	LOD 0.1–0.4 ng/g	ΣPAHs High concentration 1,000–778 ng/g dw, Low 54–65 ng/g dw	13 PAHs HPLC-FLD
March 2008	Langston et al. (2012)	Milford Haven Waterway, UK	*M. edulis*	Freeze dried samples extracted ACN:tetrahydrofuran with sonication	LOD 0.1–0.7 ng/g dw	ΣPAHs 110–262 ng/g dw	17 PAHs HPLC-FLD

–	Valavanidis et al. (2008)	Saronikos Gulf, Greece	*M. galloprovincialis*	Lyophilized samples, Sonicaton hexane: DCM, Alumina SPE	LOD 0.5–4.5 ng/g dw	ΣPAHs 430–2,454 ng/g dw gills	PAHs
						ΣPAHs 380–1,800 ng/g dw	HPLC-FLD
						Recovery on SRM 2977	
–	Bolognesi et al. (2004)	Ligurian Coast, Italy	*M. galloprovincialis*	Saponification, pentane extraction, Silica column purification	No reference	High ΣPAHs 190–2,110 ng/g dw, industrialized Genoa site	9 PAHs
							HPLC-FLD
Feb. 2001 to Aug. 2002	Ruiz et al. (2011)	Ria of Vigo Estuary, Galicia, Spain	*Mytilus spp.*	Lyophilized samples, Soxhlet hexane:acetone 3:1, clean-up deactivated alumina.	DL, 0.1 ng/g dw	ΣPAHs 59.6–739 ng/g dw	HPLC-FLD

concentration of ΣPAHs at 2,420,000 ng/g lipid (Amin et al. 2011). This was attributed to the nearby presence of an oil jetty used to discharge to shore storage tanks, indicating that proximity to oil sources can lead to increased concentration of PAHs.

Less pollution was measured in the South East Brazilian Coast, and it was reported in 2012, with sampling conducted from May to June 2011 on the species *Perna Perna* (Yoshimine et al. 2012). The mean ΣPAHs concentration varied from 33 to 761 ng/g dw with areas adjacent to urban settlements prone to PAHs pollution. In a lagoon in Brazil *Mytella charruana* species were monitored for PAHs pollution (Maioli et al. 2010). Results showed low contamination reaching a maximum of 145 ng/g ww.

5.4.3 Atlantic Europe

A second research report with high concentration in bivalves has been published with sampling in 2005 at the NW coast of Portugal (Lima et al. 2007). The ΣPAHs varied from 124,000 to 549,000 ng/g. Further investigation of the effects of petrochemical contamination in *M. galloprovincialis* was reported in the same study.

Bartolome et al. reported elevated levels of PAHs in the Bilbao estuary in Spain, one of the most industrialized and populated areas of the Bay of Biscay (Bartolome et al. 2010). Bivalve samples were collected between 2002 and 2004 and the pollution ranged from 5,100 to 18,300 ng/g dw with the highest concentration observed in January 2003. Regarding the distribution LMW PAHs were the most abundant with one exception in one sampling period in which HMW PAHs were the predominant. According to the concentration ranges of PAHs the authors classified this part of estuary as moderate to highly polluted area.

This research was complementary to other works performed in the same region by other groups e.g. (Orbea et al. 2006). In these cases, the concentrations of (Amin et al. 2011) micro-contaminants were at the same order of magnitude, showing once again a moderate to high level of contamination in all the compartments of this estuary, taking into consideration that sampling was performed before 2000.

5.4.4 North Western Spain, Galicia

The NW coast of Spain (Galicia) has a characteristic hydrography, defined by a continuous suite of estuarine systems called “Rias Gallegas”. Some of these estuaries support important industrial and urban centers while others are preserved from human influence.

On November 13th 2002, the oil tanker ‘Prestige’ which carried 77,000 t of heavy fuel-oil, began to leak oil ending up polluting more than 1,000 km of coastline, especially affecting Galicia.

Nieto et al. presented work on PAHs pollution effect on bivalves immediately after the "Prestige Oil Spill" (Nieto et al. 2006). ΣPAHs ranged from 2,500 to 5,900 ng/g dw, which clearly indicated that the oil spill affected the pollution status of bivalves in this region, with concentration being nearly one order of magnitude higher than the concentration range of least contaminated sites. Similar conclusions were deducted by another group who found even higher concentration of PAHs with a maximum of 7,780 ng/g dw established in samples of Costa de Morte 2–3 months after the Prestige Oil Spill (Soriano et al. 2006).

Laffon et al. reported the same year PAHs pollution on M. galloprovincialis collected in Galician beaches from Aug. 2003 to June 2004 (Laffon et al. 2006). ΣPAHs ranged from 500 to 3,500 ng/g. In a 5 year follow-up study the same group noted that the average ΣPAHs were below 800 ng/g, lower than previously reported (Fernandez-Tajes et al. 2011).

Labarta and Babarro have also studied PAHs burden on *M. galloprovincialis* in the Galician coastline (Labarta et al. 2005; Babarro et al. 2006). Concentration were comparable to other works (Vinas et al. 2012) however much lower than the high concentration obtained in sampling points near the "Prestige Oil Spill".

Perez-Cadahia et al. sampled mussels form Galicia in Spain, at Betanzos estuary not affected by the oil spill of the tanker "Prestige". Their rationale was to expose these mussels in crude oil which was collected from the Muros–Noia estuary (West coast of Galicia, especially affected by the oil spill). In this work although sampling was not performed in "real-time samples" it was exhibited that mussels accumulated high concentration of PAHs in their tissues, above the baseline threshold values (Perez-Cadahia et al. 2004).

Cortazar et al., published work on PAHs burden in oysters (*Crassostrea sp*.) from the Urdaibai estuary an UNESCO Reserve unique for the species that it hosts. This estuary can be considered a low-contaminated estuary, although in some of its sites the maximum ΣPAHs belong to the moderate level of pollution. Bartolome's group published research carried out also in the same estuary (Bustamante et al. 2010). Sampling was performed 2 years later and the levels were in the same range, showing stability in the PAHs load of this estuary.

Another estuary in NW Spain was selected by Pereira et al. for monitoring PAHs pollution in bivalve species (Pereira et al. 2011). The TPAHs concentrations were comparable to those reported form moderately contaminated areas, however additional concern has been raised since in one sampling point the respective values were higher than those obtained in mussels 3 days after the Prestige shipwreck (Nieto et al. 2006).

Monitoring two sites at the French Atlantic Coast on *Crassostrea gigas* resulted in low contamination (384 and 566 ng/g dw, Quiniou et al. 2007). The entrance of the Arachon harbor was more polluted in respect to the inner part, which is somehow expected due to the presence of many ships and nearby activities which seem to affect hydrocarbon pollution status.

In Scotland a 5 year monitoring of PAHs levels was performed in farmed mussels of the reference site in Loch Etive and in Loch Leven (Webster et al. 2006). ΣPAHs

ranged from 12 to 151 ng/g dw in the reference site setting this way a benchmark concentration that can be used to evaluate contamination incidents.

5.4.5 Asia

Increased production and chemical usage which is reported during the last decades at the east coast of China can potentially affect the aquatic environment. As a result various research groups have monitored chemicals burden in bivalves originating from China. Fung et al. reported in 2004 monitoring of organic contaminants – including PAHs- for bivalves in China (Fung et al. 2004). The results showed that pollution especially in Shanghai sampling points in *M. edulis* was elevated (>3,000 ng/g dw) signifying substantial contamination of aquatic environment nearby this metropolitan region. In other sampling sites the pollution was low to moderate. Li et al. in a work on a lagoon in China monitored lower PAHs levels in crabs and clams (Li et al. 2010).

Mytilus and *Crassostrea* spp. were used in three islands in Korea to evaluate the effect of the spilling of 5,040 t of crude oil by the "Sea Prince" oil tanker on the 23rd of July 1995 (Yim et al. 2002). Sampling was performed between August 1999 and April 2000. ΣPAHs reached a maximum concentration of 1,443 ng/g dw at Kumo island in 1999 – although it was not the place of the spilling incident- and almost 1 year after the respective value was one order of magnitude less. In the same time period the respective concentration at Sori Island (near the accident) was 2.5 times higher, indicating a transient trend for the high concentration in Kumo sampling point. The comparison with other regions where spills accidents have taken place is difficult since sampling in this report was far later than the incident.

In the Korean Coast in the frames of MWP a survey has been conducted on bivalves and PAHs accumulation on them (Choi et al. 2010). Sampling was performed in a time window of 6 years thus it was quite prolonged. The mean concentration of PAHs ranged from 83 to 387 ng/g dw signifying low pollution. On the whole PAHs substances in Korean mussels met the current guideline for human health.

5.4.6 Global Survey

Vorkamp et al. in a global survey have reported elevated levels of PAHs (Vorkamp et al. 2010). Sampling in this work has been done in 2006 and 2007 thus it was quite contemporary. The ΣPAHs reached a maximum of 5,966 ng/g dw in the Boston harbor and 1,453 ng/g dw in the Sydney estuary. These levels were above the baseline value as set by (Baumard et al. 1999a, b) and in the case of Boston harbor

exceeded the NOAA threshold, indicating medium to high PAHs contamination in the specific areas.

5.4.7 Mediterranean

In Greece PAHs pollution was described recently in the Saronikos Gulf. ΣPAHs varied from 430 to 2,454 ng/g dw in gills (Valavanidis et al. 2008). Elefsis Bay and Salamis Island which are located in the coastal area of Saronikos Gulf are considered to be the most polluted, and this was confirmed by analytical results (1,482 and 2,454 ng/g dw for Elefsis Bay and Salamis Island correspondingly). Both values were substantially higher than the baseline value set by (Baumard et al. 1999a, b) and in the case of Salamis Island approximating the high level as defined by NOAA.

On the other hand a multisport analysis of PAHs in the Western Mediterranean was conducted in the frames of Mytilos Project (Galgani et al. 2011). Monitoring was conducted on caged mussels *M. galloprovincialis* at 123 stations along Spain, France, Italy, North Tunisia, Algeria and Morocco. Low contamination was reported with total PAHs levels ranging from 22 to 106 ng/g dw. The highest amounts of PAHs were found in all areas known for their contamination (large towns, industrial areas such as Marseille, Genoa golf etc.), posturing additional concern for these areas due to their proximity to large populations.

Low levels were obtained in the Valencia Community in two types of bivalves collected in July 2006 and 2007 (Bouzas et al. 2011). In this context in mussels the ΣPAHs varied from 1 to 45 ng/g dw, while in clams from 2.9 to 67.3 ng/g dw. Fluoranthene was the most abundant PAHs in mussels while benzo(b)fluoranthene in clams. In the same region, and specifically in Eivissa (Ibiza) island Sureda et al. measured the level of PAHs in *M. galloprovincialis* obtaining low levels reaching a maximum in total PAHs of 16 ng/g dw (Sureda et al. 2011).

Same maximum level has been observed in Campania, Italy when *M. galloprovincialis* were monitored (Serpe et al. 2010b). Finally a quite efficient analytical methodology revealed likewise low levels in the Punta Insua in Spain for samples collected during 2003 (Fernandez-Gonzalez et al. 2008).

5.4.8 Adriatic Sea

Various groups have published works on PAHs burden in bivalves sampled from the Adriatic Sea (mainly Italy and Croatia) at sites which have been impacted for decades by industrial discharges (Vassura et al. 2005) or still possess industrial activity or are near to harbours (Bolognesi et al. 2004; Matozzo et al. 2010; Trisciani et al. 2012). A relatively high concentration was observed in Cornigliano in Genoa reaching 2,110 ng/g dw. The lowest concentration was measured in the NW Adriatic as reported by Trisciani et al. not exceeding 60 ng/g dw.

5.4.9 Northern Europe

In northern Europe PAHs significant levels have been reported by Skarpheðinsdóttir et al. (Skarpheoinsdottir et al. 2007). Sampling was conducted during October 2000 in Nordic Coastal areas. ΣPAHs ranged from very low to the high concentration of 11,670 and 10,830 ng/g dw in the harbours of Reykjavík and Ólafsvík respectively. These sites had also the highest DNA adduct levels demonstrating that harbours are significant sources of pollution for marine organisms. Less contamination has been observed in Sweden when -during 2002 and 2003- sampling was conducted in *M. edulis*, which were in the range of 40–420 ng/g dw (Bellas et al. 2007).

Low but not negligible levels of PAHs were also detected in *Macoma Bathica* and *Astarte Borealis* species in a survey extending from the Gulf of Finland to the Southern Baltic Proper. The ΣPAHs reached a maximum of almost 300 ng/g dw in the Bornholm basin.

5.4.10 Antarctica

A work motivated by the necessity to establish the environmental status of areas near to Antarctica was published in 2009 (Curtosi et al. 2009). For this reason 25 PAHs were measured in bivalve *Laternulla elliptica* from the area of Potter Pove (the cove is characterized by a benthic fauna dominated by this bivalve). The rationale of selecting that area was on the significant traffic mainly of large ships involved in station logistics and science. Sampling was performed during the Antarctic summer 2004/2005. TPAHs in digestive gland and gonad of *Laternulla elliptica* were 105 ± 40 and 124 ± 40 ng/g dw, suggesting low contamination from PAHs.

5.4.11 Conclusions

From the above it is evident that the majority of works presented here are in the "safe side" with ΣPAHs being in the range of 47–828 ng/g. In case of monitoring sites adjacent to areas of "oil spills accidents" or heavily industrialized metropolitan areas elevated levels of PAHs were witnessed.

An interesting approach on this section is to quote on the public health levels of concern (LOC) for PAHs in bivalves (crabs and oysters) (NOAA 2006) and compare with actual levels of individual PAHs found on bivalves tissues from monitoring points (Xia et al. 2012). The LOC values vary from 132 (benzo[a]pyrene) to 2,000,000 (anthracene/phenathrene) ng/g ww.

Table 5.3 Levels of concern (LOC) for PAHs in crabs and oysters

PAHs compound	Crabs LOC[a] (ng/g ww)	Oysters LOC[a] (ng/g ww)
Napthalene	123,000	133,000
Fluorene	246,000	247,000
Anthracene/Phenathrene	1,846,000	2,000,000
Pyrene	185,000	200,000
Fluoranthene	246,000	267,000
Chrysene	132,000	143,000
Benzo[k]fluoranthene	13,200	14,300
Benzo[b]fluoranthene	1,320	1,430
Benzo[a]anthracene	1,320	1,430
Indeno[1,2,3-cd]pyrene	1,320	1,430
Dibenzo[a,h]anthracene	132	143
Benzo[a]pyrene	132	143

[a]Data from FDAs July 29th 2010 document: "Protocol for interpretation and use of sensory testing and analytical chemistry results for re-opening oil-impacted areas closed to seafood harvesting due to the deepwater Horizon oil spill" (NOAA 2006)

Indicatively in some of the works of this review the ΣPAHs value is given in ng/g ww. Thus it is convenient to establish if in some cases PAHs individual concentration levels exceed the LOC value. For instance in the work by Fernandez-Gonzalez et al. the ΣPAHs value range was far below the lowest LOC which is reported for dibenzo[a,h]anthracene and benzo[a]pyrene (Fernandez-Gonzalez et al. 2008). Similar conclusions, indicatively, were derived from works in Korea and the Barents Sea where ΣPAHs reached a maximum 31 and 22 ng/g ww respectively (Namiesnik et al. 2008; Nahrgang et al. 2012) (Table 5.3).

5.5 Comparison of Analytical Performance in PAHs Detection in Bivalves

The validation of the methods should typically follow some guidelines as proposed by the NOAA Quality Assurance Plan for Analyses of Environmental Samples (NOAA 2006). This technical memorandum includes PAHs and their metabolites and sets minimum analytical quality assurance criteria depending on the analytical method and instrumentation used. The analytical performance of the methods is evaluated on certain criteria which have to be addressed so a method to ensure its credibility.

Laboratory precision and accuracy were evaluated using laboratory replicates of field samples and SRMs when available. In a substantial number of reports presented here SRMs mainly SRM 2977 and SRM 1974b were used by the authors for the QA

part. Precision is usually expressed as the RSD for repeated measurements. Recoveries were checked not only in spiked samples but also in internal standards (usually dPAHs) and considered acceptable if they were between 60 and 130%. In one study the acceptable matrix spike recovery criterion for tissue analysis was that the average recoveries for PAHs should fall between 40 and 120% (Yim et al. 2002).

5.5.1 LOD-Sensitivity

Sensitivity reflects methods' ability to determine analytes at low concentration levels. Limits of Detection and Quantification LOD and LOQ are usually calculated/ defined as the concentration of the analyte that produces a signal to noise ratio of 3 and 10 respectively (usually the LODs are calculated by spiking lyophilized blank biota and are the concentrations that resulted to S/N ratio 3). This approach was followed by the majority of works included in this overview.

The LOD values range in most reports of GC-MS methods from 0.01 to 27 ng/g. Two reports had LOD values at pg/g scale using GC-MS-SIM and advanced purification (Liguori et al. 2006) and GC-MS/MS with PTV injection system (Fernandez-Gonzalez et al. 2008) which set new perspectives in sensitivity as they manage to refer to extremely low concentration An additional work sets the detection threshold at 0.001 ng however authors do not provide further details on how they determined that so actual comparison with other LODs, IDL or DLs of presented methods is rather difficult (Quiniou et al. 2007). The above results demonstrate that GC-MS is a powerful tool to identify PAHs and quantify them owing to its proven sensitivity.

The LOD values vary in most reports of HPLC-FLD methods from 0.01 to 35 ng/g. The latter proves that HPLC-FLD is "antagonizing" GC-MS methods since the LOD values are comparable. Studies with HPLC-FLD demonstrating LODs in the pg/g scale do not exist, which implies that GC-MS and GC-MS/MS are superior to HPLC-FLD. However if the goal of the lab is only the analysis of PAHs and not the inclusion of other contaminants HPLC-FLD is a reliable tool. However if the analyses intends to be expanded to other contaminants (such as pesticides) GC-MS or LC-MS are the instrumentation of choice.

As regards HPLC-UV reported LOD values vary from 0.01 to 0.04 ng/g dw (Cravo et al. 2012). The LODs reported in this study are of the lowest reported in literature even after compared with theoretically more sensitive detectors such as the fluorescence or mass spectrometer. However to our knowledge it is the only work on PAHs with UV detection that its LOD reached that order of magnitude.

5.5.2 Linearity

Good to excellent linearity was also obtained for all PAHs studied ($r^2 > 0.99$) with a linear range in the scale of ng/g ww or dw.

5.6 *In Vivo* (and *In Vitro*) Exposures of Marine Bivalves to Individual PAHs and PAHs Mixtures-Biochemical Responses

Taking into account the ubiquity of PAHs in marine environments, the controlled exposure of mussels under laboratory conditions to PAHs, as to elicit biochemical responses suggestive of reaction in the field, may be proven very useful. Since bivalves accumulate these pollutants to high degree, it is probable that health consequences are possible for them. For these reasons, a variety of exposure regimes as regarding duration, design, husbandry conditions and PAHs candidate substance have been tried *in vivo* with marine bivalves. Different endpoints have been recorded. The research in this field which started almost three decades ago has been intensified in the last decade with novel biomarkers being explored along already established ones (see Table 5.4 for a number of *in vitro* and *in vivo* experiments).

Starting with bivalve cellular systems, *in vitro* exposure of *M galloprovincialis* haemocytes to the model PAHs benzo[a]pyrene (BaP) elicited some remarkable responses (Gomez-Mendikute et al. 2002). It caused a dose-independent increase in the production of the reactive oxygen species (ROS) superoxide anion, disruption of the actin distribution pattern and compromised the endocytic ability. A subsequent experiment by the same group (Gomez-Mendikute and Cajaravllle 2003) on *M galloprovincialis* haemocytes verified the above-mentioned results. Oxidative stress is a probable way of action of PAHs in bivalves since it has earlier been proven in mussels that BaP metabolism gives rise to mainly three classes of products: dihydrodiols, quinones and phenols. Out of these, quinones may produce Reactive Oxygen Species (ROS) via continuous redox cycling (Michel and Narbonne 1996).

Regarding *in vivo* approaches, ROS-relevant damage caused by PAHs is also corroborated by the work of Machella et al 2005 which focused on the detection of the highly relevant DNA oxidative lesion of 7,8-dihydro-8-oxodeoxyguanosine (8oxodG) in marine organisms exposed to BaP. There was a significant increase in immunofluorescence staining of BaP in relation to control with no clear dose trend. Differences in antioxidant enzyme activities have been noted after *in vivo* exposure of bivalves to PAHs (Luna-Acosta et al. 2011). Higher statistical correlation showed that enzyme activities after exposure were generally inhibited in the gills and plasma while they were generally activated in mantle and haemocytes. It was also found that enzyme activities in digestive gland, mantle and haemocytes were generally positively correlated with PAHs body burden, whereas the opposite was true for gills and plasma. Superoxide anion detection, lipid peroxidation levels (due to ROS attack to lipids) and other relevant biomarkers were chosen for quantifying the ability of anthracene and phenanthrene to compromise mussel (*M galloprovincialis*) health (Giannapas et al. 2012). The results were highly indicative of ROS-mediated effects in PAHs-exposed mussels.

Detrimental effects of PAHs on mussel DNA have also been found; a simulation of bivalve exposure to oil spill was performed by Pérez-Cadahía et al. in an experiment utilizing *M galloprovincialis* and crude oil from an oil spill (Perez-Cadahia

Table 5.4 *In vitro/in vivo* experiments utilizing mussel biomarkers after PAHs exposure

Year	Reference	Species	Experimental set-up	Biomarker tested	Results	PAHs measured
2012	Croxton et al. (2012)	*C. virginica*	Exposure to naphthalene, pyrene, BaP: 5, 125, 625, 1,000 μg/L (contaminated feed)	Total and differential haemocyte counts	Generally granular haemocyte counts and superoxide production positively correlated with PAHs burden	GC/MS
			Exposure to BaP: 100, 1000 μg/L (contaminated feed) for 7 days	Haemocyte aggregation		
				Superoxide production (haemolymph)		
				Phagocytosis (haemolymph)		
2011	Giannapas et al. (2012)	*M. edulis*	Exposure to 0.1 mg anthracene/L or 0.1 mg phenanthrene,/L mixture of the two 0.2 mg /L	Mortality on air (stress on stress)	Stress on stress: enhanced in all exposed groups	–
				Haemocyte viability	Superoxide anion production enhanced in all exposure groups -all other markers negatively affected by exposure	
				Acid phosphatase activity, LPO, MN, superoxide production (haemolymph)		
2011	Luna-Acosta et al. (2011)	*C. virginica*	Exposure to different regimes of 67 mg/L BAL110 for 2 days	GPX, CAT, Catecholasephenoloxidase, laccasephenoloxidase, lysozyme activity (gill mantle, digestive gland, plasma, haemocytes)	Activities generally inhibited in gill and plasma	GC/MS
					Activities generally activated in mantle and haemocytes	
					Chemical dispersants modulated these activities	
2011	Okay et al. (2011)	*M. edulis*	Exposure to 800 μg phenanthrene/L with or without oil removing sorbent 9 days	NR (haemolymph)	NR,FR: negatively affected in all exposed groups	Fluorescent spectrophotometry
				FR		

2011	Pan et al. (2011)	*R. phillipinarum*	Exposure to 0, 0.01, 0.2 ppb for 10 days	CYP4 sequencing	Increase in mRNA CYP4 in digestive gland at high dose group	–
				mRNA CYP4 quantification (digestive gland, gill, mantle, adductor muscle)		
				Quantification of RpCYP4 mRNA in gill, dig gland, adductor muscle and mantle		
2011	Sundt et al. (2011)	*M. edulis*	Exposure to 0.125, 0.25, 0.5% PW from oil field for 29 days	NR, MN (haemolymph)	MN elevated only at the highest dose group	GC/MS
					No differences in NR	
2011	Yakan et al. (2011)	*M. galloprovincialis*	Exposure to 3, 6, 9 µg BaA /L for 29 days (total experiment duration)	NR (haemolymph)	FR was not significantly affected	GC/MS
				FR	NR retention time negatively correlated with BaA burden	
2011	Zhang et al. (2011)	*R philippinarum*	Exposure to 0.02, 0.2 µM BaP for 3 days	NMR based identification of metabolites (gill)	Metabolite shift characteristic of disturbances in osmotic regulation	–
					Metabolite shift characteristic of increased anaerobic metabolism	
2010	Hannam et al. (2010)	*P. maximus*	Exposure to 50, 100 and 200 µg phenanthrene/L for 7 days	Total haemocyte count	Most markers negatively affected by exposure	GC/MS (water samples)
				Plasma protein levels, LPO, GT levels, membrane stability, phagocytosis (haemolymph)	GR depletion	
					Increase in circulating haemocytes	
2010	Xu et al. (2010)	*V. phillippinarum*	Exposure to 0.01, 0.2 µg BaP/L for 10 days (total experiment duration)	GST-pi sequencing	Increase in transcription levels at high dose group	–
				GST mRNA levels (gill, digestive gland)		

(continued)

Table 5.4 (continued)

Year	Reference	Species	Experimental set-up	Biomarker tested	Results	PAHs measured
2009	Lewis and Galloway (2009)	*M. edulis*	Exposure to 0.01, 0.1 and 1.0 mg BaP/L for 3 days	SSB (sperm, haemolymph)	Increase in SSB in sperm in parents	–
				% abnormal embryos	Increase in % abnormal embryos	
					Correlation between sperm and hemocyte SSB in parents	
2008	Einsporn and Koehler (2008)	*M. edulis*	Exposure to 150 μg phenanthrene /L for 10 days	Pathological changes (digestive gland)	Accumulation of phenanthrene in lysososomal system of digestive gland	–
				Subcellular localization of phenanthrene	Ultrastructural changes in lysosomes and other organelles	
2008	Okay and Karacik (2008)	*M. edulis*	Exposure to phenanthrene:100, 400 μg/L	FR	Different responses according to chemical tested, UV light presence, assay chosen	Fluorescent spectrophotometry
			Pyrene: 40, 120 μg/L	NR (haemolymph)		
			Fluoranthene:100, 250 μg/L			
			Chrysene: 1, 1.8 μg/L			
			Either in dark or under UV light for 7 days			
2007	Frouin et al. (2007)	*M. arenaria*	Exposure to coke dust (2g/day), PAHs-contaminated feed (100 ng/L/day), smelter soot-spiked sediment (2% w/w), smelter discharge-spiked sediment (2% w/w) for 50 days (total experiment duration)	Phagocytosis (haemocytes)	Phagocytosis negatively affected in all regimes in males	Autoradiography of ^{14}C
				LPO, Glycogen reserves, CAT activity (digestive gland)	Subsequent amelioration	15PAHs (HPLC)
				Reproductive cycle stage	Persistent bioaccumulation of ^{14}C in digestive gland from contaminated feed	

2006	Burlando et al. (2006)	*M. edulis*	Exposure to 500 ppb NSO, 500 ppb NSO+100 ppb alkylphenols +100 ppb PAHs for 3 weeks	Protein tyrosine phosphorylation (gill, digestive gland, mantle)	Significant increase in all exposed groups in all tissues.	–
2006	Hoarau et al. (2006)	*M. galloprovincialis*	Exposure to 100 µg BaP/L for 7 days	GST sequencing	Decrease of activity in gill	
				mRNA GST quantification (digestive gland)	Decrease of transcription in digestive gland without concomitant activity decrease	
				GST activity (gill, digestive gland)		
2006	Jonsson et al. (2006)	*M. edulis*	Exposure to 500 ppb NSO, 500 ppb NSO+ 164 ppb alkylphenols+36 ppb PAHs	CYP2 like and CYP4 like protein levels (digestive gland)	Cross reaction of CYP2 with a 57 kDa protein	–
				Total cytochrome content (digestive gland)	Cross reaction of CYP4 with a 55 kDa protein	
				Proteomic analysis of upregulated/downregulated spots	No difference in levels after treatment	
					No difference in levels of total CYP	
					No comparable findings of protein spots	
2006	Okay et al. (2006)	*M edulis*	Exposure to 20, 40 µg/L pyrene in contaminated feed for 15 days (*M edulis*),	FR	FR and NR negatively affected with recuperation	Fluorescent spectrophotometry
		M galloprovincialis				
			Exposure to 40 µg/L in contaminated feed for 15 days (*M galloprovincialis*),	NR (haemolymph)	Results most pronounced in *M galloprovincialis* under continuous illumination	
			Exposure to continuous contaminated feed and illumination for 15 days (*M galloprovincialis*)			

(continued)

Table 5.4 (continued)

Year	Reference	Species	Experimental set-up	Biomarker tested	Results	PAHs measured
2005	Machella et al. (2005)	*M galloprovincialis*	Exposure to 100 500, 1,000 ppb BaP for 1 week	8oxodG levels (digestive gland) via immunofluorescence staining	Increase with no clear dose trend	–
2004	Aarab et al. (2004)	*M. edulis*	Exposure to 500 ppb NSO, 500 ppb NSO+100 ppb alkylphenols +100 ppb PAHs for 3 weeks	Histopathological findings (mantle)	Generally unaffected mantle with normal spermatic follicles.	–
				Vitellin levels (gonads)	Some specific histopathological lesions in the exposed groups	
					Vitellin significantly elevated in NSO only.	
2004	Perez-Cadahia et al. (2004)	*M. galloprovincialis*	Exposure to seawater/ shipwreck oil mixture (1:500 or 2:500) for 12 days	SSB (gill)	Significant increase in all exposed groups	GC/MS
					Correlations of effect with levels of PAHs groups in tissue or water	
2003	Gomez-Mendikute and Cajaravllle (2003)	*M. edulis* cells	Exposure of haemolymph to 1–20 μg BaP/mL for 1 h	Superoxide anion production	All markers negatively affected	–
				Endocytic ability		
				Actin distribution patterns		
2003	Wootton et al. (2003)	*M.edulis, C.edule,*	Exposure to phenanthrene: 50, 100, 200, 400 μg/L for 14 days	Total and differential haemocyte count	*M edulis* resilient to the lethal dose of 400 μg/L	–
		E.siliqua		Enzyme cytochemistry (haemocyte)	Significant alterations in *M edulis*and *C edule* haemocytes	
				Phagocytosis (haemocyte)	*E siliqua* showed generalized stress	
				Intracellular /extracellular superoxide production (haemocytes)		

2002	Durand et al. (2002)	*M. edulis*	1 ppb BaP (7 days/subtidal, 14 days/intertidal total experiment duration)	LPO (mantle)	Increased retention of BaP in mantle and slower depuration of whole body burden in intertidal	Liquid scintillation radiography of ^{14}C
					Higher LPO in intertidal	
2002	Gomez-Mendikute et al. (2002)	*M. edulis* cells	Exposure of haemolymph to 0.5–40 µg BaP/mL	Superoxide anion production	Dose independent increase in superoxide anion production	–
				Endocytic ability	Disruption of actin distribution patterns	
				Actin distribution patterns		
2002	Guerra-Rivas et al. (2002)	*M. galloprovincialis*	Exposure to 2mM anthracene via injection for 3 days (total experiment duration)	Buffering capacity of pallial fluid	Increase in buffering capacity in all air exposed groups.	–
			water submerged or air exposed,		Highest increase in the anthracene treated group	
2002	Large et al. (2002)	*M. edulis*	Exposure to 0.5, 20 ppb BaP in contaminated feed for 14 days	SSB (digestive gland)	SSB initial increase with subsequent recuperation	GC/MS
					Continuous bioaccumulation	
2002	Micic et al. (2002)	*M. galloprovincialis*	Exposure to 5–20 µg BaP/g tissue via injection	SSB (haemolymph)	Increase in SSB	–
				Conventional and pulsed field gel electrophoresis flow cytometry (haemolymph)	No apoptotic patterns	

(continued)

Table 5.4 (continued)

Year	Reference	Species	Experimental set-up	Biomarker tested	Results	PAHs measured
2001	Snyder et al. (2001)	*M. galloprovincialis*	Exposure to 0, 0.01, 0.1, 1.0 5.0% w/w AWCO for 1 day	HSP60-like, HSP70-like and HSP90-like protein levels (digestive gland)	Cross reaction of HSP70 antibody with 67, 70, 74kDa proteins.	–
				CYP4Y1 mRNA levels (digestive gland)	Cross reaction of HSP60 antibody with a 60 kDa protein	
					Elevation of some bands at all exposed groups	
					Significant reduction in CYP4Y1 mRNA at all exposed groups	
2000	Akcha et al. (2000)	*M. galloprovincialis*	Exposure to 1.786 μg BaP/mg dw feed for 28 days (total experiment duration)	BPH, CAT, DTD (digestive gland)	Two distinct bulky DNA adducts in digestive gland	GC/MS
				GST activity (digestive gland, gill)	Induction of BPH activity	
				ACh activity (gill)		
				Bulky DNA adduct detection (digestive gland)		

8oxodG 8-oxo-deoxyguanosine, *AChe* acetyl-cholinesterase, *AWCO* artificially weathered crude oil, *BAL110* brut Arabian light crude oil, *BaA* benzo[a]anthracene, *BaP* benzo[a]pyrene, *BPH* benzo[a]pyrene hydroxylase, *CAT* catalase, *CYP* cytochrome, *DTD* NADPH DT-diaphorase, *FR* filtration rate, *GR* glutathione reductase, *GST* glutathione S-transferase, *GPX* glutathione peroxidase, *GT* total glutathione, *HSP60/70/90* heat shock proteins, *LPO* lipid peroxidation, *MN* micronucleus, *NMR* nuclear magnetic resonance, *NR* neutral red, *NSO* north sea oil, *SSB* single strand breaks

et al. 2004). DNA damage in the form of Single Strand Breaks (SSB) was detected in gills. Results showed a statistically significant increase in SSB in the oil exposed mussels. A significant correlation was found between tissue Total PAHs content and SSB. In another experiment on *M edulis*, BaP was also genotoxic; SSB increased in digestive gland after 3 and after 7 days of exposure via contaminated feed (Large et al 2002). At 14 days however, both low and high dose group returned to control levels, despite continuous BaP bioaccumulation, highlighting the adapting mechanisms existing in bivalves. *M. galloprovincialis* acute direct exposure to BaP by means of injection in the adductor muscle also caused SSB in haemocytes and gills (Micic et al. 2002)

PAHs exposure may cause DNA damage not only on the parent organism but also to its progeny. This was depicted in the experiments of Lewis and Galloway, in 2009 where *M. edulis* was exposed to up to 1 mg/L BaP. SSB were elevated in spermatozoa in a dose-related manner (Lewis and Galloway 2009). Furthermore, percentage of abnormal embryos (embryos with irregular cleavage, incomplete blastula development, necrosis or discoloration) was higher in the parental exposed populations in a dose-related manner.

Other DNA lesions after exposure to BaP have also been found, under *in vivo* exposures. One of the major metabolic pathways of PAHs (and BaP in particular) leads to the formation of a "bay region diol epoxide" which eventually creates stable miscoding DNA adducts (McCoull et al. 1999). The adducts were found in *M galloprovincialis* after BaP exposure via contaminated feed (Akcha et al. 2000). Histopathology techniques have also offered substantial input into the effects of PAHs on bivalvian health. *In vivo* exposure of *M edulis* to phenanthrene showed subcellular localization of this PAHs into the lysososomal system of digestive gland cells and relevant pathological changes there (Einsporn and Koehler 2008). Another simulation exposure of *Mytilus edulis* to an oil spill (NSO) was performed by (Aarab et al. 2004). Histopathology of gonads revealed a generally unaffected tissue with normal spermatic follicles however some individuals showed irregular ejaculating patterns. Vitellin levels were significantly increased in the NSO group. Furthermore, some individuals exposed to NSO spiked with alkylphenols accumulated haemocytes resembling vertebrate melanomacrophage centers.

The Filtration Rate (FR) biomarker which measures the impact of noxious stimuli of the bivalve on its feeding capacity and the Neutral Red (NR) biomarker which highlights the haemocyte health status have commonly been used for *in vivo* exposures to PAHs. Exposure of *M galloprovincialis* to benzo[a]anthracene (BaA) showed varying results according to the biomarker examined (Yakan et al. 2011). In brief, lysosomal stability (as measured via NR) was negatively affected by BaA while Filtration Rate of the PAHs exposed mussels remained unaffected. Using the same experimental organism (*M galloprovincialis*), Okay et al., performed an *in vivo* exposure to phenanthrene with or without simultaneous exposure to an oil removing sorbent (Okay et al. 2011). NR and FR showed that phenanthrene exposure was harmful in terms of mussel physiology and the sorbent did not manage to change the outcome. NR retention time was also used by Sundt et al. for *M edulis* exposed to different concentrations of oil platform effluent discharge (Sundt et al.

2011). The mussels responded generally well to the exposure with minimal MN formation and unaffected NR retention times.

Despite their universal nature, signaling pathways have seldom been examined in bivalves. Among signaling mechanisms, tyrosine-kinase dependent pathways play an important on/off role in signal transduction and cellular activity regulation. An *in vivo* experiment on *Mytilus edulis* exposed to different spiked mixtures of NSO revealed significant alteration in tyrosine phosphorylation in gill, digestive gland and mantle for proteins of 50–100 kDa (Burlando et al. 2006). Despite no clear attribution potential to a specific group of pollutants (PAHs, alkylphenols or other oil components), it is evident that crude oil mixtures affect signaling pathways in mussels *in vivo* whereas consequences are still unknown.

Many PAHs members have been proven to be immunotoxic in cell systems and mammalian models, regardless of their carcinogenicity profile. Thus, immunological effects of PAHs on bivalves have also been considered. Experiments on the scallop *Pecten maximus* revealed adverse effects on its immune system due to phenanthrene exposure (Hannam et al. 2010). Frouin et al 2007, also examined the immunotoxic effects of coke dust, PAHs-contaminated feed, smelter soot-spiked sediment and smelter discharge-spiked sediment on *Mya arenaria*. Phagocytosis was compromised in males in all treatments, although in a reversible way. In a related study, *M.edulis*, *C.edule* and *E.siliqua* were exposed to varying concentrations of phenanthrene (Wootton et al. 2003). A number of immunological parameters differed significantly according to organism tested. In brief, *M edulis* was considered hardier, with resilience to the lethal (for the other two species) dose of 400 μg/L. Responses to *E.siliqua* were minimal, probably due to the generalized stress laboratory caused to this organism. *C virginica* also behaved in a durable manner when exposed to naphthalene, pyrene or BaP via contaminated feed (Croxton et al. 2012). Principal Component Analysis however revealed an increase in granular haemocyte counts and ROS production with a possible inhibition of phagocytosis.

A relatively unexplored bivalvian biomarker (buffering capacity of pallial fluid) was examined by (Guerra-Rivas et al. 2002). In their experiment *M. galloprovincialis* was injected with anthracene. The buffering capacity of pallial fluid (which is the amount of base in meq needed to change pH in one unit) differed significantly between groups, with the PAHs-treated group exhibiting the highest buffering capacity. This acidic reaction of anthracene exposure may be the result of the implication of PAHs metabolism into the pentose phosphate pathway in bivalves under hypoxic conditions. A recent metabolomics study in the clam *Ruditapes philippinarum* regarding effects of BaP (Zhang et al. 2011), also noted implications of this PAHs in anaerobic metabolism pathways.

Xenobiotics as well as endogenous substances are primarily metabolized by cytochromes P450 in living organisms. As such, effects of pollution by means of their up or down-regulation exert an enormous impact on metabolism and homeostasis. Unfortunately, bivalvian cytochrome P450 identification is a relatively unexplored research area. It is certain that it bears striking differences to the vertebrate P450 system; there is an apparent absence of CYP1A-like enzymes in mollusks, as

it also happens in *C elegans*, Drosophila and other sequenced insects. The induction or suppression of bivalvian CYPs is also a matter of research since classic CYP1 and CYP2 inducers in vertebrates may or may not exert similar action on bivalvian functional analogues. Under this light, exposure to classic AhR receptors like PAHs plays two roles: it facilitates research in PAHs metabolism towards toxic products in bivalves and it elucidates the relationship between CYP bivalvian-mammalian orthologues. In the experiments of Pan et al 2011 healthy clam specimens (*Ruditapes philippinarum*) were exposed to BaP for up to 10 days with interim samplings. Exposure to BaP caused statistically significant increase of CYP4 mRNA in digestive gland at the 10 day exposure group only. On the contrary, significant reduction in CYP4Y1 mRNA after exposure to artificially weathered crude oil was noted for *M. galloprovincialis* for all concentrations tested (Snyder et al. 2001). These results not only show that a CYP4 isoform is present in bivalves but that its transcription can indeed be regulated by PAHs. Whether CYP4 take active part in PAHs metabolism and/or other important xenobiotics found in polluted waters is a matter of further research. Studies by Jonsson et al., verified the differences between vertebrates and mollusks (Jonsson et al. 2006). *M edulis* specimens were exposed to NSO spiked mixtures and digestive gland extract was matched with polyclonal antibodies designed upon conserved CYP2 and CYP4 peptide sequences. Cross reaction with some bivalvian proteins was found, but no induction/suppression due to NSO was evident and no identification of proteins was possible.

Given the still evolving field of bivalve protein identification and sequencing, it is probable that only highly conserved proteins among species may be robust predictive biomonitoring tools. The Heat Shock Proteins may well fall under this category, since they have been identified in mussels (Snyder et al. 2001). HSP70 antibodies indeed reacted with proteins in *M galloprovincialis* digestive gland. Furthermore, some of these proteins were significantly elevated after 24 h exposure to artificially weathered crude oil HSP are expressed in response to an array of stresses, including hyperthermia, oxygen radicals and heavy metals and are rather non-specific in terms of stress origin. Nevertheless their induction shows the unfavorable situation of PAHs exposure to the mussel and may become a useful biomonitoring tool.

Regarding other detoxifying enzymes, Xu et al. detected an increase in the transcription levels of GST, which was conserved for the whole experiment duration, in V. *phillipinarum* digestive gland and gill (Xu et al. 2010). On the contrary, another *in vivo* exposure to BaP led to a decrease of gene expression for pi-GST, cloned in *M galloprovincialis* digestive gland (Hoarau et al. 2006). Given the vast use of this kind of biomarkers (antioxidant enzymes activity) in biomonitoring studies, further insight in their transcriptional control will be proven useful in the future.

Special exposure circumstances may affect the toxicity exerted by PAHs; it has been shown that some PAHs may be activated by UV-light to more toxic intermediates. The effects of UV light on the toxicity of selected PAHs were examined by Okay and Karacik(Okay and Karacik 2008). *M galloprovincialis* specimens were exposed to phenanthrene, pyrene, fluoranthene or chrysene, under dark or UV light conditions. The toxicity was quantified via the methods FR and NR. Photoactivation

to more toxic substances was evident with increase of toxicity mainly in the case of chrysene, but also in the case of phenanthrene. In a previous study, both *M edulis* and *M galloprovicialis* were exposed to pyrene through contaminated feed (Okay et al. 2006). Under a continuous feeding regime of polluted algae and constant illumination, *M galloprovincialis* showed significant decrease in performance in both assays, which was time-dependent.

Mussels in intertidal conditions may also react differently to pollution than mussels which are continuously submerged. Specimens of *M edulis* exposed to a single dose of BaP depurated quicker when they were continuously submerged (Durand et al. 2002). Lipid peroxidation was also more pronounced in the mussels subjected to tidal oscillations since for the same degree of bioaccumulation of BaP, more damage was present in the intertidal mussels.

In summary, a variety of biomarkers in different bivalves have been assessed after acute or subchronic exposure to PAHs. Results are variable and dependable to numerous factors. Toxicity of PAHs to bivalves however is probable, since bioaccumulation in invertebrates soft tissues is significant.

5.6.1 In Vivo *Exposures to PAHs and PAHs-Mixtures: General Remarks and Considerations*

The universal presence of PAHs in marine environments is a good reason for their popularity in controlled, laboratory-based experiments with bivalves; investigation into the causes and effects is valuable when extrapolation to field situations is needed. Furthermore, the significant vertebrate toxicity, genotoxicity, carcinogenicity and immunotoxicity profile of members of the PAHs family render them suitable models for exploring whether similar reactions take place in invertebrates. It should be noted however that the response at subcellular, cellular, tissue, organ or organism level may be affected by a number of other factors rather than toxicity *per se*. Knowledge of the confounding contributions aids the explanation of the results.

The choice of organism is a factor of utmost importance. Despite the general resilience of bivalves towards pollution, differences in fitness between species are expected. It is therefore questionable to use a less sensitive species to detect fine, early-signaling responses to PAHs. On the other hand, generalized stress in a quite sensitive species will mask effects caused exclusively by PAHs and will ultimately be of limited value for field situations, where by virtue, only the most adapted species will be present.

Choice of tissue is also important in terms of response of the biomarker. Each tissue has evolved for well-defined physiological and biochemical activities i.e. gill are the first line of defense against environmental pollutants whereas digestive gland is responsible for metabolism and detoxification. As a result, enzyme levels and

activities, antioxidant and detoxifying defenses, rate of cell renewal and cell morphology, resistance to chemical and physiological stress, degree of inducibility of cell pathways might be tissue-specific. An ideal biomarker suite would be applicable to most tissues of the organism so that the tissues would be simultaneously compared. This strategy however may be hindered by practical limitations and it is of no value when a specific biomarker (i.e. related to immune response) is chosen. It is advisable when a biomarker is applicable to be tested to a range of types of cells since inhibition or induction is frequently tissue-specific.

The exposure regime to PAHs is also an important factor since most of these chemicals are highly lipophilic and attach to particles in water. Thus, a good simulation of field situations would be through contaminated feed or sediment rather than through direct water exposure. However, water exposure with a compatible PAHs solvent, provides good bioavailability of the pollutant. When acute, precise exposure is desirable, direct injection of the animal may be preferred. In any case, bioavailability of the pollutant should be analytically verified whenever possible. Other parameters such as light intensity, food abundance, temperature and salinity may also affect the condition of the animal. Light intensity and duration may also augment the toxicity of certain PAHs.

The relative unexplored genome of bivalves poses an attractive challenge to ecotoxicologists, but at the same time, complicates the choice of a PAHs-specific biomarker. As already mentioned PAHs are probably not metabolized by CYP1A and CYP1B, as happens it humans. As a result, alkoxyresorufin *O*-dealkylation activity levels cannot be used as a CYP-specific biomarker. In the same context, it is not known whether benzopyrene hydroxylase (BPH) is inducible by PAHs or other possible pollutants. This lack of knowledge also hinders the manufacture and use of bivalve-specific antibodies for relevant enzymes. Until substantial progress is made in bivalve genotyping, only highly conserved proteins and enzymes among species may be utilized.

Oxidative stress is probably implicated in PAHs toxicity in bivalves. This is corroborated by the PAHs metabolic pathways found in invertebrates which give rise to oxidative products. Effects and responses might be tissue, dose and pollutant-specific and necessitate further elucidation. Immunotoxic effects of PAHs on bivalves are also possible and further research in this domain should be encouraged. Finally, specific lysosomal alterations and other histopathological lesions may also be proven useful for development of a PAHs-focused biomarker.

In brief, PAHs exposure has elicited numerous alterations in bivalves at subcellular, cellular, tissue, organ and organism level, after several *in vitro* exposure regimes. No ideal biomarker that both covers a broad spectrum of effects and is simultaneously PAHs-specific exists; choice of relevant biomarkers and endpoints will depend on the function investigated and it will be affected by the exposure scheme and duration. The evolving field of invertebrate genotyping will certainly offer new biomarkers along with already established ones.

5.7 Field Experiments Utilizing Bivalves for Detecting PAHs Pollution Levels and Its Effects

PAHs pollution levels have been effectively measured through MWPs since 1970s. Biochemical and biological responses have accompanied many of these studies; nowadays this tiered strategy is recommended in environmental monitoring and biomarkers are rapidly becoming part of health assessment and management of aquatic ecosystems (Sundt et al. 2011). For the scope of the present review, only marine field studies characterized by detection of PAHs pollution are included. The review is further narrowed to the last decade (see also Table 5.5).

The blue mussel (*M. edulis*) is commonly chosen as a sentinel species for colder European climates. The biomonitoring study of Nahrgang et al., involved native populations of blue mussels and Icelandic scallops (*Chlamys islandica*) along with vertebrates, from a sub-Arctic location in Norway at autumn, winter, spring and summer (Nahrgang et al. 2012). A variety of biomarkers were examined including NR, energy reserves levels, antioxidant enzyme activity, total oxyradical scavenging capacity (TOSC), lipid peroxidation levels and electron transport system activity. In most cases, PAHs levels were low and comparable to values found in "reference" sites for biomonitoring programmes. As a result, correlations between PAHs and biomarkers were not significant. On the other hand, most of the biomarkers examined were under strong seasonal variation, a matter which merits attention.

M edulis has also been used for another study in Norway (Aarab et al. 2011). Samplings of natural populations were performed in a previous aluminum smelter and a reference site. Histopathological and histochemical markers were measured. Heamocytic aggregates were always significantly higher in polluted specimens whereas digestive gland atrophy and to a lesser extent lipofuscin (LP) and neutral lipid levels (NL) were also elevated at certain time points. A preceding experiment in the same Norwegian aluminum smelter also involved sampling of native *M edulis* (Aarab et al. 2008). Histopathological lesions, digestive gland atrophy and LP levels were detected in digestive glands at higher rates in the polluted sites, while Lysosomal Membrane Stability (LMS) was also lower there, when compared to a reference site. In Norwegian ocean waters, the efficiency of an effluent discharge treatment system installed in an oil exploitation platform was tested using *M edulis* (Brooks et al. 2011). NR retention time and MN formation were measured in haemolymph. Results were satisfactory with all sites exhibiting "healthy status" 2 years after the installation. These results were verified by analytical findings in tissues which also showed reduced PAHs values. Previous experiments at the same sites with transplanted *M edulis* had revealed a precarious situation; NR retention time of haemocytes was worse in all stations than the references site(s) and MN frequency in haemocytes was higher (Sundt et al. 2011).

Natural populations of *M edulis* in Denmark harbors were chosen for detection of common marine pollutants (Rank 2009). Most PAHs were found in the old harbor and most SSB in mussel gill were also found there However, it has to be highlighted that other pollutants of important toxicological profile (PCB, butyl-tin

Table 5.5 Field experiments utilizing mussel biomarkers for PAHs pollution monitoring

Year	Reference	Species	Location	Biomarkers utilized	Results/remarks	PAHs measured
2012	Fernández et al. (2012)	*M. galloprovincialis*	Mediterranean coast, Spain	BPH, GST, DTD, SOD, CAT, GPX (Se and nonSe-dependent) activity, LPO, MT levels (digestive gland)	BPH induced by PAHs burden	13PAHs (GC/MS)
					Two final clusters of sites characterized by different factors	
2012	Nahrgang et al. (2012)	*M. edulis*	Tromsø, Northern Norway	NR retention time (haemolymph/mussel)	Very low PAHs pollution	16PAHs
		C. islandica		CAT, GPX, GST, ETS activity, LPO, energy reserves, TOSC (digestive gland/ mussel, scallop)	Strong seasonal component	
2012	Ramdine et al. (2012)	*C. rhizophorae*	Guadaloupe, West Indies	LPO, CAT activity (gill, digestive gland)	CAT inhibited in most polluted site (gill)	25PAHs and PAHs groups (GC/MS)
					LPO induced by total and potentially carcinogenic PAHs burden	
2011	Aarab et al. (2011)	*M. edulis*	South Norway	Heamocytic aggregates (digestive gland and gonads)	Heamocytic aggregates, atrophy and LP/NL levels generally higher in polluted area	16PAHs (GC/MS)
				Tissue atrophy (digestive gland)	Temporal differences	
				LP and NL levels (digestive gland)	LP and NL significantly higher in certain time points	
					PAHs significantly higher in polluted site	

(continued)

Table 5.5 (continued)

Year	Reference	Species	Location	Biomarkers utilized	Results/remarks	PAHs measured
2011	Brooks et al. (2011)	*M. edulis*	North Sea, Norway	NR retention time (haemolymph)	Amelioration of mussel health after effluent discharge installation treatment:	16PAHs (GC/MS)
				MN formation (haemolymph)	"Healthy status" according to NR, increased MN in considerably fewer sites	
2011	Fernandez-Tajes et al. (2011)	*M. galloprovincialis*	Galicia, Northwestern Spain,	SSB (haemolymph, gill)	SSB (haemolymph, gill) significantly higher in the affected sites at all time points	16PAHs (GC/MS)
					Haemolymph SSB induced by PAHs burden in one of the sites on arrival (0 days)	
2011	Garmendia et al. (2011)	*M. galloprovincialis (PCR verified)*	Spain, Portugal	LMS, LSC, LRI (digestive gland)	LMS, LSC performed worse the first 2 years with a subsequent recovery trend	No analytical measurement
					LRI enabled classification to less and more impacted sites	High PAHs expected (oil spill)
2011	Moschino et al. (2011)	*M. galloprovincialis*	Venice lagoon, Italy	NR retention time (haemolymph)	Spatial variations not effectively reflected in biomarker response	15PAHs in sediment
		R. philippinarum		LP/NL levels and lysosomal to cytoplasm ratio (digestive gland)	Bioaccumulation in most polluted site only	
				Stress on stress (exposure to air)		
				Reburrowing test (clams)		

2011	Pereira et al. (2011)	*M. galloprovincialis* *C. edule*	Galician coast, Spain	SSB (haemolymph, gill)	SSB significantly higher in the affected sites at all time points	16PAHs
2011	Sundt et al. (2011)	*M. edulis*	North Sea, Norway	NR retention time, MN (haemolymph)	NR, MN affected by PAHs burden	24PAHs
2011	Sureda et al. (2011)	*M. galloprovincialis*	Eivissa island, Spain	PC (digestive gland)	All biomarkers significantly different at impacted sites at 1-month sampling.	16PAHs
				LPO (digestive gland)	Subsequent samplings varied with total recuperation 6 months after the spill	
				CAT, SOD, GPX, TR, GST, EROD activities (digestive gland)		
				GSH and GSSG (digestive gland)		
				mRNA MT10 and MT20 (digestive gland)		
2011	Tsangaris et al. (2011)	*M galloprovincialis*	Aegean coast, Greece	AChe (gill)	IBR slightly affected by PAHs burden	24PAHs
				GST and CAT activity MT levels (digestive gland)		
				DNA: RNA ratio (whole mussel)		
				Fusion into IBR		
2010	Fernandez et al. (2010a)	*M. galloprovincialis*	Galician coast, Spain	IBR (gill)	IBR higher at the worst affected and the intermediate affected site(s) in relation to season	13PAHs
				SFG	SFG higher in the reference site only at spring	
					Strong seasonal component	

(continued)

Table 5.5 (continued)

Year	Reference	Species	Location	Biomarkers utilized	Results/remarks	PAHs measured
2010b	Fernandez et al. (2010b)	*M. galloprovincialis*	Mediterranean coast, Spain	MT, LPO levels, GR SOD, CAT, GPX (Se and nonSe-dependent), DTD activity (gill)	CAT positively affected by PAHs burden	13PAHs
				CI		
2010	Pereira et al. (2010)	*P. perna*	Sao Paolo coast, Brazil	EROD, DB, CAT, GPX, GR, GST activity (gill)	CAT, GPX and GR activities negatively affected by PAHs burden	ΣPAHs
2009	Rank (2009)	*M. edulis*	Lynæs harbor, Denmark	SSB (gills)	SSB increased along the pollution gradient	19PAHs
2008	Aarab et al. (2008)	*M. edulis*	Høgevarde smelter, Norway	Histopathological lesions, tissue atrophy, LP levels, LMS (digestive gland)	All markers performed worse in impacted site	16PAHs
2008	Culbertson et al. (2008)	*G. demissa*	Wild harbor, US	Shell growth measurements	Native species performed worse in all parameters	No analytical measurement
				CI	Transplanted species to polluted site negatively affected	High PAHs expected (oil spill)
				FR	Transplanted species to clean site positively affected	
2008	Martinez-Gomez et al. (2008)	*M. galloprovincialis*	Mediterranean coast, Spain	NR retention time (haemolymph)	NR retention time negatively correlated with PAHs burden	13PAHs
				CI		
2008	Namiesnik et al. (2008)	*M. galloprovincialis*	Mokpo coast, South Korea	Antioxidant activity (whole mussel extract)	Antioxidant activity affected by PAHs burden	16PAHs
2007	Bihari et al. (2007)	*M. galloprovincialis*	Rijeka Gulf, Croatia	Anoxic survival (stress on stress)	Anoxic survival time inversely affected by toxicity of biological extract	10PAHs (HPLC)
				Microtox assay (mussel biological extract)		

2007	Francioni et al. (2007)	*P. perna*	Guanabara Bay, Brazil	NR retention time (haemolymph)	NR retention time negatively affected by PAHs/ and total dibenzothiophenes burden	37PAHs and PAHs groups
				CI		
2007	Thomas et al. (2007)	*M. trossulus*	Prince William Sound, USA	SSB (haemolymph)	SSB: increased in all impacted sites (mussel, clam)	39PAHs
		P. staminea			SSB positively correlated with PAHs burden (mussel)	
2007	Viarengo et al. (2007)	*M. galloprovincialis*	Ligurian coast, Italy	SSB (gill)	Most markers performed worse in intermediately impacted site	16PAHs
				Anoxic survival (stress on stress)	No differences in survival	
				LP/NL levels, lysososomal/ cytoplasm ratio, LMS, MT levels (digestive gland)	Spill impacted sites: low PAHs burden	
				MN (gill)		
2006	Babarro et al. (2006)	*M. galloprovincialis*	Galician coast, Spain	TFAA (protein and non-protein free) (whole tissue)	TFAA and derived indices not directly affected by pollution	ΣPAHs
					Endogenous factors affect spatial variability	

(continued)

Table 5.5 (continued)

Year	Reference	Species	Location	Biomarkers utilized	Results/remarks	PAHs measured
2005	De Luca-Abbott et al. (2005)	*P. viridis*	Hong Kong coast	CI	Similar CI at all sites	15PAHs
		R. philippinarum		CAT,, GST, GPx activity, GSH levels (digestive gland, gill)	GST activity in clams correlated with PAHs burden	
					Strong seasonal component	
2004	Bolognesi et al. (2004)	*M. galloprovincialis*	Ligurian coast, Italy	SSB (gill)	SSB increased in polluted sites. Effects more pronounced at caged mussels	9PAHs
				MN frequency (gill)	MN increased in polluted sites. Effects more pronounced at native mussels	
2004	Halldorsson et al. (2004)	*M. edulis*	Reykjavík harbor, Iceland	SSB (gill, haemolymph)	SSB increased in subtidal (gill) and intertidal (gill, haemolymph) mussels in polluted site	No analytical measurement
						High PAHs expected (harbor)
2003	Romeo et al. (2003)	*M. galloprovincialis*	Atlantic coast, France	MT, LPO levels, AChe, GST activity (gill, digestive gland)	Responses in relation to mussel origin (transplanted versus native ones)	ΣPAHs
2001	Porte et al. (2001)	*M. galloprovincialis*	Galician coast, Spain	Total cytochrome levels, BPH activity (digestive gland)	No increase in BPH activity, total cytochrome levels	14PAHs
				HSP70-like proteins (gill)	Increase in HSP70-like proteins (72 kDa band)	
					Increase in HSP70 positively correlated with PAHs burden	

2000	Dyrynda et al. (2000)	*M. edulis*	Milford Haven, Wales	Total and differential haemocyte count	All parameters affected by PAHs burden	18PAHs and PAHs groups
				Phagocytosis, extra and intracellular production of superoxide, lysozymal activity (haemocytes)	Extracellular production positively affected by oil-derived PAHs and intracellular production/ phagocytosis negatively correlated with combustion-derived PAHs	
2000	Fisher et al. (2000)	*C. virginica*	Tampa Bay, USA	HD, HM, RHL	Especially HM, RHL negatively correlated with individual PAHs groups burden	25PAHs and PAHs groups
				Superoxide generation, lysozyme levels (haemolymph)		

AChe acetyl-cholinesterase, *BPH* Benzo[a]pyrene hydroxylase, *CAT* catalase, *CI* condition index, *DB* dibenzofluorescein, *DTD* NADPH DT-Diaphorase, *EROD* ethoxyresorufin-O-deethylase, *ETS* electron transport system, *FR* filtration rate, *GC/MS* gas chromatography–mass spectrometry, *GR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *GST* glutathione S-transferase, *GPX* glutathione peroxidase, *HD* haemocyte density, *HM* haemocyte mobility, *HPLC* high performance liquid chromatography, *HSP70* heat shock protein, *IBR* integrated biomarker response, *LMS* lysosomal membrane stability, *LP* lipofuscin, *LPO* lipid peroxidation, *LRI* lysosomal response index, *LSC* lysosomal structural changes, *MN* micronucleus, *MT/ MT10/ MT20* metallothionein, *NL* neutral lipids, *NR* neutral red, *PAHs* polyaromatic hydrocarbons, *PC* protein carbonyls, *RHL* rate of haemocytelocomotion, *SFG* scope for growth, *SOD* superoxide dismutase, *SSB* single strand breaks, *TFAA* total free amino acids, *TOSC* total oxyradical scavenging activity

compounds) were also present in the harbors. The effects, therefore, can be attributed to each pollutant category or even to a synergistic outcome of the chemicals present.

As also mentioned for *in vivo* exposures, synergy between intertidal conditions and PAHs is possible; the effect of hypoxic oscillations on the toxicity of PAHs was investigated by Halldórssonet al, under field situations (Halldorsson et al. 2004). *M edulis* was transplanted in an Icelandic harbor and a reference site. The mussels were divided in two groups: subtidal (all time submerged) and intertidal (half time submerged). SSB were significantly elevated for both subtidal and intertidal mussels in gills but only for intertidal mussels in haemolymph. The levels of ambient PAHs in this case were unknown, however due to the nature of the site (commercial harbor) they were expected to be substantial.

The Sea Empress oil spill was a major ecological disaster in Wales in the 1990s, when more than 200 km of coastline was polluted. Right after the spill, environmental quality was monitored with the use of native *M edulis* in three oil-impacted and one reference site in UK (Dyrynda et al. 2000). Immunological responses were recorded at high frequency for the following 2 years. Significant correlations of varied strength were recorded for all the biomarkers in relation to total PAHs and to individual PAHs body burden. The most alarming finding was that high levels of PAHs, found immediately after the spill, severely impaired immune defenses such as phagocytosis and intracellular production of ROS. Nevertheless recuperation within 2 years was noted in the populations tested.

The cosmopolitan *M. galloprovincialis* species has probably diverged from *M edulis* when the Mediterranean Sea was cut off from the Atlantic during a Pleistocene ice age (Gosling 1992). *M galloprovincialis* is therefore the organism of choice for biomonitoring studies in warmer maritime environments in Europe and US. The toxic potential of *M. galloprovincialis* biological fluids was examined by Bihari et al. in the Adriatic Sea of Croatia (Bihari et al. 2007). Mussel biological extract obtained from the specimens' tissues was tested in the Microtox assay (inhibition of chemiluminescence of *V fischeri*). Good correlation ($R^2 = 0.86$) between anoxic survival time and toxicity of biological extract (1/EC50) was noted. In other words, mussel metabolic pathways rendered PAHs (and/or other pollutants found in water) toxic for marine organisms.

In France, when *M galloprovincialis* was transplanted to two intermediately polluted sites, their responses to pollution were different than the natives' ones (Romeo et al. 2003). In brief, mussels were transplanted for 4–8 months, and their digestive glands and gills were measured for MT and LPO (lipid peroxidation) levels as well as for acetylcholinesterase (Ache) and GST activity. PAHs analysis verified a pollution gradient between the reference and the two transplantation sites. PCA based on biochemical responses and metal burdens distinguished three clusters of mussels; two clusters comprising the indigenous "polluted" populations and a third one comprising the reference population together with the transplanted groups.

A large study on the Spanish coasts was undertaken (Fernandez et al. 2012), utilizing indigenous *M galloprovincialis* in 17 sites of varied suspected pollution. A

battery of biomarkers was recorded. A significant and quite robust correlation was found between BaP hydroxylase (BPH) and total PAHs burden in tissue; in other words it was probable that PAHs pollution induced metabolism of BaP (and other aromatic hydrocarbons) to genotoxic diols. Finally, multivariate analysis of all the parameters tested grouped sites to a cluster of intermediate-to-high pollution profile with mussels exhibiting medium-to-low antioxidant activities and to another cluster which was not characterized by these factors. Another study on the Spanish coasts was carried out by Pereira et al.,(Pereira et al. 2011). In this study both mussels (*M galloprovincialis*) and cockles (*C edule*) were used. SSB were the biomarker of choice. For each season, SSB were significantly elevated in the impacted sites in relation to the time relevant control. No actual correlation between PAHs body burden and DNA damage was established for neither species. A wide scale biomonitoring study was also carried out in Spain by Fernández et al. 2010b. In this study, natural populations of *M galloprovincialis* were collected from 16 sites on the Mediterranean coast. Condition Index (CI) as well as MT, LPO and various enzymes activity were measured in gill. Biomarker responses differed across sites, however only CAT activity was significantly correlated with PAHs body burden. The presence of a cocktail of pollutants consisting of PAHs, PCBs and heavy metals, as it usually happens in real estuarine situations makes direct causality to PAHs problematic. A large scale biomonitoring study along Spanish Mediterranean coasts utilized native populations of *M galloprovincialis* of 16 sites (Martinez-Gomez et al. 2008). The specimens were checked for CI and NR retention time and results were correlated with known pollution levels of PAHs in the area. NR was indicative of pollution levels since a significant ($p<0.05$ between sites) but low correlation (Spearman's, $R^2=-0.6$) was found. A previous study concentrating on the Galician coast of Spain, examined indigenous populations of *M galloprovincialis* from five sites of different anthropogenic contact. BPH and total cytochrome content in digestive gland were not elevated in the most polluted sites. In contrast, HSP70-immunopositive levels in gill were elevated in polluted sites and indicating high stress conditions. (Porte et al. 2001).

Oil spills are a major ecological disaster with detrimental effects both in short and in long term. Monitoring of long term effects is possible, with the use of relevant bivalvian species, either native or transplanted. The Prestige oil spill in 2006 polluted thousands of kilometers of coastline on the Spanish, French and Portuguese coast, creating one of the most notable environmental disasters in the Mediterranean. In this context, (Fernandez-Tajes et al. 2011) collected natural *M galloprovincialis* populations 4 years after the accident, in three spillage-affected sites and one reference site. DNA single strand breaks (SSB) were quantified on arrival as well as 7 and 14 days after laboratory depuration. Significantly higher SSB from the three affected sites in relation to the time relevant control were evident at all three timepoints Another biomonitoring study focused on effects of the Prestige oil spill, 2 years after the accident (Fernandez et al. 2010a). Natural populations from four sites affected by the spill and from one reference site were examined. Various enzyme activities and their integration in the IAR (Integrated Biomarker Response) marker were assessed in gill, whereas Scope for Growth (SFG) was assessed in

whole mussel. An interesting outcome of the study was that some differences were evident in spring but autumn differences were more subtle with identical SFG in all sites. This study, among others, highlights the effect of seasonality in bivalves' response. The severity of the spill was also shown in a large scale monitoring study which commenced 1 year after the accident (Garmendia et al. 2011). Native specimens of *M galloprovincialis* were collected from 17 sites in Spain during the first year and 22 sites in Spain and Portugal during the three following years. LMS retention time, Lysosomal Structural Changes (LSC) and their integration in Lysosomal Response Index were calculated. All markers showed compromised health of the affected population with a subsequent recovery trend Finally, the effect of the Prestige oil spill on sensitive populations (*M galloprovincialis* juveniles) was recorded in winter, 3 months after the spill, in natural specimens of the Galician coast (Babarro et al. 2006). In this case, Total Free Amino Acids (TFAA) content in soft tissues was not significantly affected by the spill, at least for the time period tested.

The aftermath of another oil spill in Spain, the Don Pedro oil spill in 2011 was closely monitored by Sureda et al. (2011). Natural populations of *M galloprovincialis* from one contaminated and one reference site were collected within 6 months since the spill. Protein carbonyls and lipid peroxidation levels, various enzyme activities, reduced and oxidized glutathione (GSH) levels and mRNA levels of metallothionein (MT10 and MT20) proteins were examined. All biomarkers were significantly different from the reference site at the 1 month sampling. Results from subsequent samplings varied with total recuperation 6 months after the accident. Similar trend was found for the PAHs body burden. The results show that the effects of the spill to a variety of biomarkers were significant but reversible. It has to be noted however that this reversibility may be due either to pollution decrease or to adaptation of the populations to the new conditions (see also Large et al. 2002).

In Italy, the HAVEN oil spill was a fatal wreckage in the Ligurian Sea in 1991. For the long-term monitoring of its effect, a 30-day transplantation of *M galloprovincialis* in two impacted and one reference site was performed, 6 years after the spill (Viarengo et al. 2007). A variety of biomarkers were been assessed including DNA SSB, MN formation, anoxic survival (stress on stress), lipofuscin levels, lysosomal/cytoplasm ratio, neutral lipid quantification, lysosomal membrane stability and MT levels. No differences in survival between sites was noted, however biomarkers such as LP, lysosomal/cytoplasm ratio, NL, lysosomal membrane stability performed worse in the intermediate pollution site, which surprisingly exhibited the lowest PAHs burden in mussel tissues. As eloquently shown by the present survey, field experiments are frequently confounded by unaccounted factors and/or concomitant pollutants which are also capable of alterations of biochemical and physiological processes in bivalves.

The various advantages and disadvantages of using native instead of transplanted mussels were examined in a study along the Ligurian coast in Italy by Bolognesi et al. (Bolognesi et al. 2004). Either transplanted or native *M galloprovincialis* were collected from sites along a suspected pollution gradient. SSB and MN lesions were detected. There was a statistically significant increase in SSB in rela-

tion to control (mussel farm) at all sites with more pronounced effects at the transplanted mussels. There was also a statistically significant increase in MN frequency in relation to control at all sites but this time the effects were more pronounced at wild mussels. A weak correlation ($R^2=0.19$ $P<0.01$) between PAHs content and SSB was also noted

The Mediterranean mussel as well as the clam *Ruditapes philippinarum* was chosen for a biomonitoring programme in the Venice lagoon (Moschino et al. 2011). Transplanted mussels and native clams were sampled from ten sites in the lagoon. NR, LP and neutral lipid levels, lysosomal to cytoplasm ratio in digestive glands and stress on stress in both species and as well as reburrowing capability of the clam were examined. Results varied with significant differences between sites. It is worth mentioning that only organisms from the most polluted site bioaccumulated PAHs.

A large scale study in Greece, utilized transplanted *M galloprovincialis* for 3 months along the Aegean Sea islands (Tsangaris et al. 2011). Biomarkers such as various enzyme activities, DNA: RNA ratio and MT levels were measured and introduced into the Integrated Biomarker Response (IBR) index. PCA verified that stress as shown by high IBR values is generally attributed to elevated levels of organic contaminants and metals

The cosmopolitan species *M galloprovincialis* has also been used in a South Korea study (Namiesnik et al. 2008). The biomarker examined was the radical scavenging activity of whole mussel extract,. Higher activity was found in the polluted site with a significant correlation ($R^2=0.96$) between activity and PAHs body burden. It is worth mentioning that other pollutants were also positively associated, however PAHs correlation was the strongest one found.

Quality of Hong Kong coastal waters has been assessed via another widely distributed mussel species (*Perna perna*) as well as via the clam *R. philippinarum* (De Luca-Abbott et al. 2005). Mussels and clams were transplanted in four anthropogenically impacted sites. CI, CAT, GST, and GPx activity and GSH levels were quantified. Despite the complex and different biochemical responses in each site, CI did not reveal compromised health status and the only significant correlation ($R^2=0.53$) was this between PAHs burden and GST activity in clam digestive gland.

The brown mussel *Pernaperna* may be also found in tropical and sub-tropical regions of the Atlantic Ocean, as a native species. Thus, it is regularly used for biomonitoring studies in Brazil and other South American countries. Transplanted *P perna* was exposed to four sites of suspected PAHs, metal and municipal waste pollution (Pereira et al. 2010). At the end of every exposure period a number of enzyme activities were quantified. Principal Component Analysis (PCA) attributed some of the variations found to individual pollutants; as far as PAHs were concerned, suppression of CAT, GPx and GR activities were linked with PAHs burden. It was apparent that other pollutants, probably of pharmaceutical origin, also contributed to the results.

A large scale study on a heavily degraded estuary involved monitoring of native populations on the brown mussel at ten sites in Brazil (Francioni et al. 2007). PAHs pollution was substantial and in some cases prohibitive for human consumption. A positive Spearman's correlation was found between MN and total PAHs levels

($R^2 = 0.62$, $p < 0.05$). A significant increase in MN frequency was evident at a concentration greater than 1,000 μg/kg bw of mussel tissue.

In US, The Exxon Valdez oil spill in 1989 has polluted Alaskan coast with dire ecological consequences. Fifteen years after the spill natural populations of *M trossulus* from four oil impacted beaches and four nearby non impacted beaches and natural populations of *P staminea* from three oil impacted beaches and three nearby non impacted beaches were chosen, in order to monitor the long term effects of the spill (Thomas et al. 2007). Two heavily polluted sites from recent oil spills were also included in the survey. DNA damage on haemolymph was significantly higher in impacted beaches in relation to corresponding reference for both kind of organism. Considerable concentrations of PAHs were measured in mussels from the oil impacted sites and a significant correlation between DNA damage and tissue PAHs in mussels ($R^2 = 0.8$) was noted.

The aftermath of a very old spill in Massachusetts was measured 12 years after, in salty marshes of the area (Culbertson et al. 2008). Native populations of *Geukensia demissa* were transplanted to a nearby clean site and vice versa, while indigenous populations were also incorporated in the study. Growth rates of three age groups, CI and FR showed that impact of the spill was still significant; indigenous populations performed worse in all endpoints while transplanted mussels to the polluted site were also negatively affected

Individual PAHs were incriminated for compromised immune activity of *Crassostrea virginica* in US (Fisher et al. 2000). Briefly, native population of the oyster were collected in winter at 16 sites and examined for haemolymph characteristics including haemocyte density, mobility, rate of locomotion, superoxide anion production and levels of lysozyme. Low but significant correlations ($R^2 = 0.54–0.65$, $p < 0.05$) were noted for mobility and rate of locomotion parameters with individual PAHs groups.

In the Caribbean, the unique ecosystem of mangroves has been effectively monitored with the use of *Crassostrea rhizophorae* (Ramdine et al. 2012). In brief, native specimens of the oyster were collected from seven stations at dry season and tested for CAT activity and LPO in gills and digestive glands. The high pollution burden of the most impacted site inhibited significantly CAT activity. Furthermore, LPO in gills was correlated with total PAHs and potentially carcinogenic PAHs burden.

5.7.1 Field Exposures: Conclusions, General Remarks and Considerations

The characteristics of marine bivalves render these organisms ideal for short or long term monitoring of organic and inorganic pollution. As such, bivalves are frequently preferred rather than vertebrate sentinel species and they have been proven along the years to provide robust, reproducible and representative results for sediment or

water column pollution. Some of the considerations on measuring effects of PAHs on mussels in the field are analyzed here.

The type of biomarker chosen should be appropriate for the exposure duration and the adaptation potential of the biomarker should always be considered. As classified by Wu et al. (2005), a biomarker will (i) show slow induction and slow adaptation; as such it will only be appropriate for monitoring environments with little fluctuation of contaminants over time, (ii) show fast induction and fast adaptation; as such it will be able for a dose-related response but unable of time-integrated estimates, (iii) show slow induction and fast recovery and will solely be relevant for long-term pollution with little changes, (iv) show slow induction and slow recovery thus it will provide a better time-integrated response but no detection of fluctuations, (v) show fast induction and fast recovery and as such it will be ideal for recent pollution monitoring only, (vi) show fast induction and slow recovery; as such it may provide both quantitative and time-integrated responses while it will be unable to detect sudden pollution fluctuations. According to the same researchers, Type 5 and especially Type 6 biomarkers are more appropriate for realistic pollution patterns. Only some of the biomarkers traditionally used for bivalves may be allocated to one of these groups. Furthermore, particularly for PAHs, use of Type 5 biomarkers requires frequent samplings in the field (Wu et al. 2005).

The adaptation capacity of populations chronically exposed to unfavorable conditions is a strong factor in biological response. This has been highlighted in a number of studies (see Romeo et al. 2003) and it has also become the main research theme in others (see Bolognesi et al. 2004). The basis of this adaptation may be either a selection towards hardier populations or merely epigenetic changes via modifications of critical functions. Knowledge of this basis would aid enormously the interpretations of findings; however given the limited genetic bank of bivalves this is not always possible. Research in indigenous populations in parallel to transplanted ones would clarify how chronic exposure affects each population separately however this is not always possible, especially in large scale field deployments. Careful consideration and application of the most responsive biomarker in relation both to exposure duration and to genetic origin of the bivalve is a necessary step.

Pollutants are rarely found alone in nature; as shown by numerous studies, anthropogenically impacted sites contain a variety of PAHs along with PCBs, organo-tin compounds, heavy metals and agrochemicals. Nowadays, emerging chemicals such as Personal Care Products, nanomaterials and medicinal compounds have also been incriminated for endocrine-related and other responses in bivalves (Gagne et al. 2006). It is therefore highly improbable that the responses noted can be ascribed as a whole to PAHs pollution unless response and PAHs burden are highly correlated. Furthermore, synergism or antagonism is frequently noted in aquatic organisms in complex ways (see Kungolos et al. 1999).

Variance among individuals as well as variance at different seasonal points has been commonly observed in responses of bivalves. Environmental and internal factors including temperature, food availability, food quality, salinity and

reproductive status are all variables thought to affect responses to a varied extent according to the biomarker tested, the species, the tissue, the age and the sex of the bivalve. This has been mentioned in numerous field studies; furthermore it has become the main research theme in a number of them (i.e. see also Nahrgang et al. 2012). Since it is rarely possible to know *a priori* the degree of effect due to each factor, all possible variables should be recorded. Repetition of deployment in distinct seasonal frequencies and extension of biomarker assays in more than one tissue is ideal, however one should always take into account financial limitations and difficulties present in large scale field research. The best compromise should always be sought. Some special circumstances such as the photoactivation of certain PAHs or the synergism between tidal oscillations and redox cycling PAHs merit particular attention.

To conclude, one should always bear in mind that a biomarker should be quick, cheap and easy to perform, robust and reproducible and subjected to the least possible experimenter's bias. Especially for PAHs the effect should be attributable to the cause (PAHs pollution). This is not straightforward as shown by the *in vivo* experiments, since no biomarker can be exclusively characteristic of PAHs exposure. Therefore it is rather preferred that a battery of biomarkers of appropriate induction/recovery combinations are used and that results are statistically correlated to PAHs levels and fluctuations. Since bivalves are a promising group of sentinel organisms, progress in the integrated field of chemical/biological marine monitoring should always be encouraged.

5.8 Conclusions

The monitoring of PAHs pollution by bivalves constitutes an important aspect of current environmental chemistry since the sources of these pollutants are diverse and ongoing and their toxicological profile is of serious concern and vigorous investigation. In this review the integration of analytical chemistry methods and important biological approaches of new millennium was presented to address the effects of PAHs. The low metabolic profile of bivalves and their cumulative character is ideal for establishment of analytical and biochemical protocols and assays to determine PAHs' impact on the aquatic organisms and monitor pollution levels. It is certain that bivalves will remain an ideal bioindicator considering their profuse presence in sea-water and the characteristics presented in this overview.

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Chapter 6
Selenium and its Role in Higher Plants

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Abstract Selenium (Se) is a naturally occurring metalloid element which occurs nearly in all environments in the universe. The common sources of Se in earth crust occurs in association with sulfide minerals as metal selenide whereas, it is rarely seen in elemental form (Se^0). Furthermore, Se is considered a finite and non-renewable resource on earth, and has been found to be an essential element for humans, animals, micro-organisms and some other eukaryotes; but as yet its essentiality to plants is in dispute. Thus, plants vary considerably in their physiological and

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biochemical response to Se. Therefore, this review focuses on of the physiological importance of Se for higher plants, especially plant growth, uptake, transport, metabolism and interaction of selenium with other minerals. Biogeochemistry of Se, its relationship with S, application of Se-containing fertilizers, Se in edible plants and finally, red elemental Se nanoparticles in higher plants will be highlighted.

Keywords Selenium • Higher plants • Stress • Elemental nano-Se • Se-containing fertilizers

List of Abbreviations

APS	Adenosine 5′-phosphosulphate
APSe	Adenosine 5′-phosphoselenate
Cys	Cysteine
Cysth	Cystathione
DMS	Dimethylsulphide
DMSP	Dimethylproprionate
DMSe	Dimethylselenide
DMDSe	Dimethyldiselenide
DMSeP	Dimethylseleniopropionate
GPX	Glutathione peroxidase
GSH	Glutathione
GSSeSG	Selenodiglutathione
HAST	High affinity sulphate transporter
LAST	Low affinity sulphate transporter
MeCys	*S*-methylcysteine
MeSeCys	*S*-methylselenocysteine
MeSeCysSeO	Methylselenocysteine seleno-oxide
Met	Methionine
S	Sulphur
Se	Selenium
SeCys	Selenocysteine
Secysth	Selenocystathione
SeGSH	Selenoglutathione
Sehocys	Selenohomocysteine
SEM	SeCys + MeSeCys
SeMeSeCys	Selenomethylselenocysteine
SeMet	Selenomethionine
SeMMet	Selenomethylmethionine
SeCys	Selenocysteine

6.1 Introduction

Selenium (Se), a metalloid mineral micronutrient, is an essential component for the adequate and healthy life of humans, animals, archaea, and some other microorganisms. The Se field is expanding at a rapid pace and has grown dramatically in the last years. All aspects of Se biology have advanced with many new approaches and insights into the genetic, biochemical, molecular, and health areas of this intriguing element (De Filippis 2010). It is well known that, the element Se was discovered in 1817 by the Swedish chemist Berzelius, Jons Jakob and named after the Greek moon goddess 'selene'. Se belongs to the Periodic Table Group 16 (previously Group VIa); the group that also contains sulphur (S) and tellurium (Te). However, Se compounds, minerals and seleniferous soils have a long history. Marco Polo reported that during his famous journey from Venice through Asia Minor to China, in 1295, his horses suffered from a typical necrotic hoof disease when the horses ate poisonous plants; the symptoms are now known to be due to Se toxicity from animals ingesting high levels of Se present in accumulator plants (Birringer et al. 2002). As early as 1842 evidence became available for the toxicity of Se to animals, but the first recorded written evidence of Se poisoning in livestock was reported in 1856 by the US Army surgeon, Madison (Whanger 2002). In 1884, a television system was developed using Se pohotocell technology in imaging (Chasteen and Bentley 2002). Therefore, Se played a fundamental role in xerography, or in other words early versions of televisions and photocopiers. The photoconductivity of Se compounds has had a profound influence on humanity, and Se compounds have found many roles in the electronic, electrical, and semiconductor industries. As well, Se is often used in agriculture, volcanisation, paint and pigment production, glass manufacturing, oil refinery, coal and electricity generation, metallurgy and lately medicine as reviewed by De Filippis (2010).

It is well documented that, certain lower plants, such as algae, need Se for normal growth and development, with species from six classes having a Se requirement such that the element is either essential for growth or strongly promotes it (Price et al. 1987). The dinoflagellate *Peridineum cinctum* can even be used as a bioassay for Se in freshwater lakes (Lindstrom 1983), while selenate can induce activity of the important antioxidant selenoenzyme glutathione peroxidase (GSH-Px) in the green alga *Chlamydomonas reinhardtii* (Lyons et al. 2009). Thus, fungi, including those used as human food, can also accumulate and metabolize Se, though apparently not requiring it for growth (Piepponen et al. 1983). Levels of about 10 μg g^{-1} dry weight are commonly recorded in mushrooms cultivated in normal compost, while those in compost enriched with sodium selenite more than 1,000 μg g^{-1} dry weight can be accumulated (László and Csába 2004), Much of the Se in mushrooms appears to be in the form of selenomethionine, with some in other organic forms (Werner and Beelman 2001). On the other hand, Se is apparently not required for growth by majority of higher plants, though there is still some doubt about this (Novoselov et al. 2002). In fact, it is toxic to many plants, which cannot grow on seleniferous

soils. However, a small number of species actually thrive on high-selenium soils and these do appear to require the element (Trelase and Trelase 1939). These unusual plants are sometimes called *primary indicator plants* because their presence indicates that selenium is a component of the soil in potentially large amounts. Some of them are *hyperaccumulators* (Baker and Brookes 1989), able to accumulate extraordinarily high levels of Se, even, in some cases, from soil that contains relatively little of the element (Brown and Shrift 1982). Certain species of the vetch *Astragalus*, for example, have been found to contain more than 3,000 mg kg^{-1} dry weight in their leaves (Broyer et al. 1972) as reviewed by Reilly (2006).

Environmental pollution of Se can have an impact on human health, agricultural productivity and the stability of natural ecosystems. Even low-level contamination if present on a large enough scale can represent large economic and logistical barriers to effective and timely treatment (Lindblom et al. 2012). In many situations, and because of the low toxicity of Se contamination the economic value placed on remediating this type of pollution is often not considered a high priority. The chemistry of Se has been reviewed extensively by a number of authors (Pilon-Smits et al. 2009). Selenium is a naturally occurring element in most soils, and can be found at very high levels in alkaline soils where Cretaceous shale or other seleniferous rocks are the soil parent material (Beath 1982). Non-seleniferous soils contain less than 1 mg Se kg^{-1}, whereas seleniferous soils can range between 2 and 100 mg Se kg^{-1} (Mikkelsen et al. 1989). Selenate is thought to be the predominant bioavailable form of Se in seleniferous soil (Zhao et al. 2005) as reviewed by Lindblom et al. (2012).

In contrast to many other organisms, Se has not been shown to be essential for higher plants (Zhang and Gladyshev 2009). Otherwise, Se does appear to be a beneficial nutrient for many plants, especially hyperaccumulators, which can reach twofold higher biomass in the presence of Se (Pilon-Smits et al. 2009). Thus, the functional significance of Se hyperaccumulation may be to offer better growth, perhaps due to better oxidative stress resistance (Hartikainen 2005). An additional benefit of Se hyperaccumulation is enhanced resistance to Se sensitive herbivores and pathogens (Quinn et al. 2010). Thus, it could be considered that, Se hyperaccumulation is a form of elemental defense (Boyd 2010). As with any plant defense, herbivores and pathogens are likely to overcome it over time. There is indeed evidence of Se tolerant herbivores and rhizosphere microbes (Wangeline et al. 2011). Se tolerant microbes can use different Se tolerance mechanisms, including Se reduction to insoluble, non-toxic elemental Se (Se^0), volatilization, or conversion to MeSe- Cys. Some plant-associated microbes have been shown to affect plant Se accumulation and volatilization (de Souza et al. 1998). The capacity of microbes to affect plant Se speciation has not been investigated (Lindblom et al. 2012).

Therefore, it could be concluded that, this review will basically cover Se physiology and biology in higher plants. Plant Se essentiality, uptake and bioavailability, metabolism, toxicity and biogeochemistry also well be reviewed. Selenium and its relationship with sulfur and effects of elemental nano-Se on higher plants will be highlighted.

6.2 Historical Background of Selenium Research

It is well documented that, the first interest in Se related to its toxicity and the early work on Se was summarized by Moxon and Rhian (1943). Se research must be said to have began in 1817, when Berzelius discovered this element. The first genuine publication describing this research was published by Berzelius in 1818, in a paper where he also named the element as *Selenium*. The history of Se research could be considered an attempt and it is made to take a "bird's-eye" view at the development of this research field since 1817 until today (Arnér 2012). The tool chosen is an analysis of the scientific literature on Se research, thereby attempting to give an unbiased assessment of this research field. Finally, as in all assessments of historic trends, we should also ask where the future of Se research might take us. By necessity, the answer to that question is uncertain. However, it could be concluded that never before has Se research been as vigorous and expanding as it is today, which also holds major promise for the future (Arnér 2012). Although as early as 1842 evidence was obtained for the toxicity of Se, apparently the first authentic written record of Se poisoning in livestock was the report by Madison in 1856, who was an Army surgeon stationed at Fort Randall which was then in the Nebraska territory. He described a fatal disease among horses grazing certain areas near the fort (Whanger 2002). Many reviews have described the development of Se research and the findings that have shaped current day's knowledge in the field, including personal recollections by some of the pioneers of Se research. It could be used simply repeat information given in previous reviews on the Se research field. Therefore, the reader is referred to other papers on the history of selenium research for discussions on specific details or topics of that research (Arnér 2012).

It could be found eight articles in the ISI Web of Science database, from the first year covered by the database (1945) using the keyword selenium. These articles covered subjects of Se toxicity (three articles), Se levels in soil, plants, or animals (two articles), or the photodynamic properties of Se, its spectral properties, or the oxidizing capacity of selenium dioxide (one article each). The subjects of those eight papers from 1945 that focused on physical, agricultural, or chemical properties of Se are in principle the very same subjects that have made "selenium" a much more studied topic in research than the more specific "selenocysteine" or "selenoprotein" topics (Arnér 2012).

It could be noticed that, with industrial usage of Se in glass, ceramics, solar cells, photocopiers, rectifiers, and more, and because of its properties as a catalyst in nonorganic chemistry, a large number of research publications on Se are not at all related to biology or biochemistry (Arnér 2012). It is rather straightforward to look back and discuss how the history of Se research has developed. What results will a similar analysis give when performed in 10 years from now, or in 50 or 200 years? Naturally, we cannot know how the future of selenium research will unfold, but it could be trusted that it shall be exciting. With this field of research at present being under rapid development, it is clear that the potential of new Se-related discoveries of major importance waits around the corner as reviewed by Arnér (2012).

6.3 Essentiality of Selenium

The symptoms of Se toxicity were probably described for the first time long before the discovery of the element. The essentiality of Se for higher plants is still unproven, but Se is considered a beneficial nutrient for many plant species (Pilon-Smits et al. 2009), perhaps because of better oxidative stress resistance (Hartikainen 2005). Plants readily take up and assimilate Se, a capacity that may be used to alleviate both Se deficiency and toxicity in animals and humans. Plants can be used to clean up excess Se from polluted areas (phytoremediation), and Se-enriched plant material may be considered fortified food (biofortification) (El Mehdawi et al. 2012). There is no doubt that, the element Se is considered a finite and non-renewable resource on earth, and has been found to be an essential element in humans, animals, microorganisms and some other eukaryotes; but as yet its essentiality to plants is in dispute. However, Se has not been shown to be an essential microelement to vascular plants. There is some evidence that Se may be required for growth and development in algae (Pilon-Smits et al. 2009). There is no doubt also that, adequate levels of Se are important to animal and human health, and some Se compounds have been found to be active against cancers. A limited number of plants growing on Se rich soils can accumulate very high levels of Se (i.e., hyperaccumulate Se), and are classified as Se tolerant, however, many more plants do not accumulate Se to any great extent, and are Se sensitive. Plants vary considerably in their physiological and biochemical response to Se, and a revision of the physiological responses of plants to Se is presented; especially growth, uptake, transport and interaction of Se with other minerals as reviewed by de Filippis (2010).

In Se accumulating plants, indications are that Se may be required for maximum growth potential, especially those endemic to seleniferous soils (Broyer et al. 1972). Even in the best studied Se accumulating plant *Astragalus pectinatus* the results of additional Se application in experiments have had differing results (Stadtman 1990). It is fair to point out that other nutrients can complex the situation such as sulphates, phosphates and however the experiments so far have not used controls where residual Se is not present at all; and indeed such experiments may be near impossible to perform (Stadtman 1996). This is simply because there will always be trace amounts of Se in plants, coming from impurities in the nutrients used or even coming from the atmosphere. An alternative approach to try to resolve essentiality was to try to detect Se incorporation into Se dependent enzymes, with an integral SeCys residue as present in animals and bacteria (Axley et al. 1991). To conclude, the evidence so far from molecular studies available is quite strong that there is no clear evidence for essential selenoproteins in higher plants, but part of the machinery for the synthesis of selenoproteins may be present in plants as reviewed by de Filippis (2010).

It is well known that, Se is a contradictory nutrient, where it has been called *the essential poison*—too much of it in the diet can be toxic; too little can result in chronic, and sometimes fatal, deficiency (Reilly 2006). Organisms that require Se for normal cellular function contain essential selenoproteins, such as glutathione peroxidase, formate dehydrogenase, and selenophosphate synthase. Interestingly,

the incorporation of selenocysteine into these selenoproteins is directed by a specific tRNA that recognizes a UGA-opal codon (Ellis and Salt 2003). Normally, the UGA codon acts to terminate translation. In combination with a selenocysteine insertion sequence (SECIS), however, the UGA codon is recognized by the selenocysteine tRNA, which directs the insertion of selenocysteine (Low and Berry 1996). There is no direct evidence for the specific incorporation of selenocysteine in vascular plants. Several selenoproteins that include a glutathione peroxidase homologue and selenocysteine tRNA have, however, been identified in the model plant system *Chlamydomonas reinhardtii* (Fu et al. 2002). Evidence for the specific insertion of selenocysteine in vascular plants is less definitive as reviewed by Ellis and Salt (2003).

Therefore, it could be concluded that, the essentiality of Se for higher plants is still unproven, but Se is considered a beneficial nutrient for many plant species. This review focuses on the biochemical responses of plants to Se, the assimilation of Se in plants and possible incorporation into proteins. Molecular approaches to understanding Se toxicity and tolerance have increased the knowledge of mechanisms of action, and the molecular biology of Se in transgenic plants is detailed; with special reference to the similarity with sulphur metabolism, S/Se transporters and important assimilation enzymes.

6.4 Physiological Importance of Selenium for Higher Plants

As mentioned before, Se has not yet been classified as an essential element for higher plants, although its role has been considered to be beneficial for plants that are is capable of accumulating large amounts of the element (Shanker 2006). The role of Se in plant depends mainly on its concentration. According to Hamilton (2004), Se has three levels of biological activity: (1) trace concentrations are required for normal growth and development; (2) moderate concentrations can be stored to maintain homeostatic functions and (3) elevated concentrations can result in toxic effects (Fig. 6.1; Hasanuzzaman et al. 2010). The Se function in plants has been investigated in many studies and there is still little evidence that Se is essential for all plants (Combs and Combs 1986; Germ and Stibilj 2007; Pilon-Smits 2015). However, according to Terry et al. (2000), Se may be required for the growth of algae, but its essentiality to higher plants is controversial and yet unresolved. Recent studies on some grasses and vegetables provide indications that at a proper Se addition, the growth rate of these plants may be enhanced (Hartikainen 2005). Some data have indicated that this element may be required for Se-accumulating plants (Moxon and Olson 1974). Several compounds of Se, mainly with cysteine and methionine, were found in such plants such as *Astragalus* species, but their metabolic functions have not been conclusively established. The Se-accumulator plants synthesize Se-methyl-cysteine, whereas nonaccumulator species produce Se-methyl-methionine. The physiological significance of this difference is not yet understood as reviewed by Kabata-Pendias (2011).

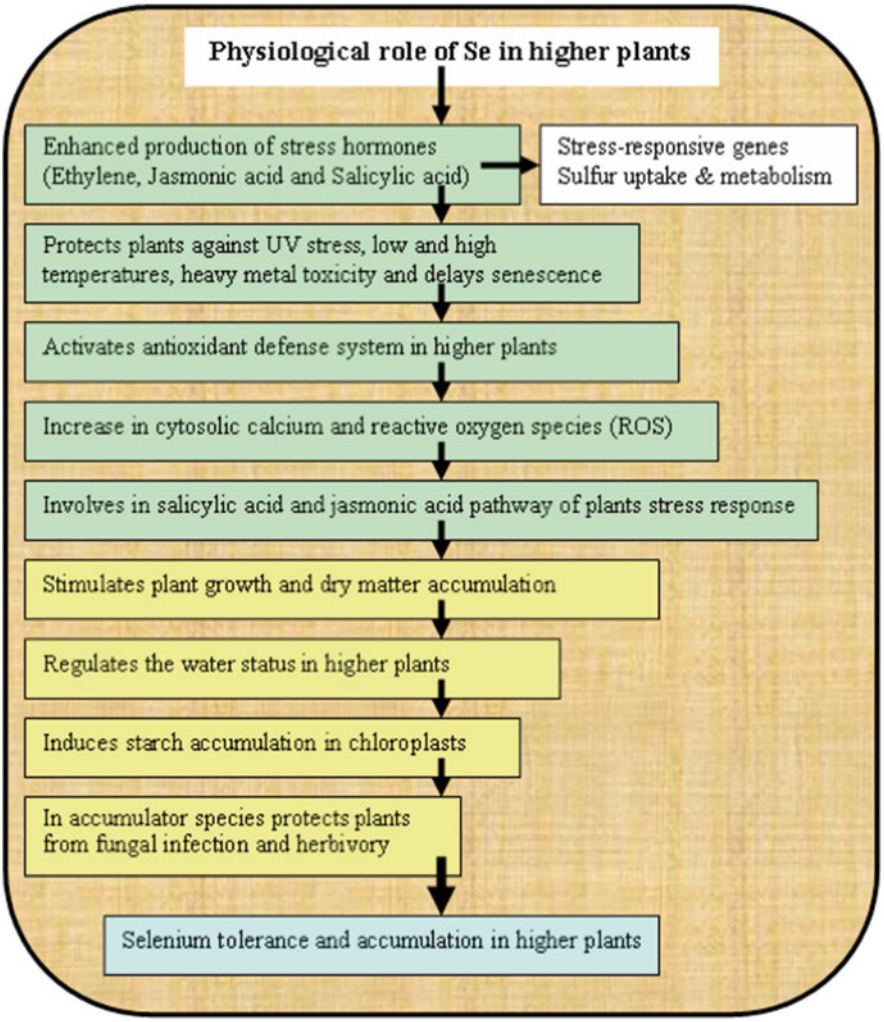

Fig. 6.1 Physiological functions or roles of Se in higher plants (From Tamaoki et al. (2008), Pilon-Smits and Quinn (2010), Hasanuzzaman et al. (2010) and Hajiboland (2012))

Although the essentiality of selenoproteins in higher plants has not been documented, syntheses of selenoproteins in some plants e.g., sugar beet were reported (Terry et al. 2000). Several selenoamino acids, SeMet (selenomethionine), SeCys (selenocysteine), and SeMSC (Se-methylselenocysteine) in association with glutathione peroxidases were found in both bacteria and higher plants (Kabata-Pendias 2011). Pyrzynska (1995) cited that there are several naturally occurring Se species: selenocysteine, methylselenocysteine, selenomethionine, selenotaurine, selenobetaine, selenoecholine, dimethylselenine, dimethyldiselenide,

and trimethylselenium. Predominated forms of Se in plants, as reported by Djujic et al. (2001), are SeMet in cereal grains and legumes seeds, and SeMSC in vegetables. In lentils, the highest proportion (up to 95 % of total Se) is present as SeMet (Thavarajah et al. 2007). In plants grown in soils with selenate addition, this Se species dominated; however, SeMet also was present at the concentration of 23 and 45 % of the total Se content, in fodder radish and Indian mustard, respectively (Simon et al. 2007). The Se taken up by plants (ryegrass and lettuce) from Se-amended soil is incorporated mainly (up to 75 %) in insoluble proteins. The activity of glutathione peroxidase increases in plants with increasing Se doses, while superoxide dismutase activity (SOD) and the concentration of vitamin E decrease. Lobanov et al. (2007) discussed the metabolism of Se-proteins in eukaryotes and concluded that aquatic organisms support the Se utilization whereas terrestrial ones reduce its uses. Species and functions of Se in plants (mainly *Cruciferae*) were broadly discussed in several publications (Finley et al. 2005). Lyi et al. (2005) reported that the key factor responsible for the SeMSC formation (the most effective anticarcinogenic Se compound) is the SMT enzyme. Lyi et al. (2007) also observed that metabolism of S and Se in broccoli is controlled by the same enzymes and apparently, this similarity occurs in other plants (Kabata-Pendias 2011).

Most probably the first positive effect of Se on plant growth was reported by Singh et al. (1980), who showed that the application of 0.5 mg kg^{-1} Se as selenite stimulated growth and dry matter yield of Indian mustard (*Brassica juncea* L.). More recently, it was revealed that Se, applied at low concentrations, enhanced growth and antioxidative capacity of both mono- and dicotyledonous plants. The growth-promoting response to Se was demonstrated in lettuce (*Lactuca sativa* L.) (Hartikainen et al. 1997) and in soybean (*Glycine max* L.) (Djanaguiraman et al. 2005). Se can also delay senescence and promote the growth of aging seedlings (Xue et al. 2001). Se has also demonstrated its effect on germination. Carvalho et al. (2003) reported that at higher supplementation level than 29 mg kg^{-1}soil, Se inhibited the growth and germination of tomato, lettuce and radish (*Raphanus sativus* L.) seeds. In contrast, priming of seeds with selenite promoted germination of bitter gourd (*Momordica charantia* L.) seeds at sub-optimal temperatures (Chen and Sung 2001). The positive effect on germination was linked to antioxidative activity as reviewed by Hasanuzzaman et al. (2010).

It is well known that, plant species with a high capacity to accumulate and tolerate Se could be used in the phytoremediation of Se-contaminated sites (Terry et al. 2000). However, most cultivated crop plants have a low tolerance to high Se levels and in general, they contain less than 25 μg Se g^{-1} DW and are considered to be non-accumulators like potato (White et al. 2004). Although non-accumulators are sensitive to high Se concentration, they can tolerate as well as accumulate even high concentrations of Se without growth reduction when grown in Se-enriched soils (Rani et al. 2005). The critical Se concentration in plant tissues, which decreased the yield in Indian mustard was 105 μg g^{-1} DW, in maize (*Zea mays* L.) 77 μg g^{-1} DW, in rice (*Oryza sativa* L.) 42 μg g^{-1} DW and in wheat 19 μg g^{-1} DW, when Se additions as selenite were 5, 5, 4, and 10 μg g^{-1} soil for Indian mustard, maize, wheat and rice, respectively (Rani et al. 2005). Se uptake and metabolism also differ

due to the plant species, growth stage and the plant organs. Broccoli (*Brassica oleracea* var. *italica*) is known for its ability to accumulate high levels of Se, with the majority of the selenoamino acids in the form of Se-Met (SeMeSeCys) (Lyi et al. 2005). The majority of plants accumulate more Se in shoot and leaf than in root tissues, but there are exceptions (Zayed et al. 1998). It is observed that, the Se concentrations in the upper leaves, roots, stolons and tubers of potato increased with increasing Se supplementation (Turakainen 2007). The highest Se concentration was reached in young upper leaves, roots and stolons, indicated that added selenate was efficiently utilized and taken up at an early stage. During the growing period the Se concentration declined in the aerial parts, roots and stolons of potato plants whereas an intensive accumulation took place in immature and mature tubers (Turakainen 2007).

Se accumulation was also affected by the methods of application, where foliar application with selenate significantly increased Se content in the tea leaves (Hu et al. 2003). Other results showed that, the Se content of pea seeds obtained from untreated and once and twice foliarly-treated plants was directly proportional to the number of sprayings (Smrkolj et al. 2006). It is clear that, from several studies, Se is taken up from the soil by plants primarily as selenate (SeO_4^{2-}) or selenite (SeO_3^{2-}) (Ellis and Salt 2003). It is suggested that, for higher toxicity of selenite compared to selenate is due to the faster incorporation of selenite than selenate (Lyons et al. 2005). In addition, the uptake of selenate into roots and its distribution in plants is much faster than that of selenite (Cartes et al. 2005). De Souza et al. (1998) reported that total Se accumulation in a plant was about tenfold higher from selenate compared to selenite as reviewed by Hasanuzzaman et al. (2010).

Therefore, it could be concluded that, Se has not yet been classified as an essential element for higher plants, although its role has been considered to be beneficial for plants that are is capable of accumulating large amounts of the element. Although the essentiality of selenoproteins in higher plants has not been documented, syntheses of selenoproteins in some plants e.g., sugar beet were reported. Se, at low concentrations, enhances growth and antioxidative capacity of both mono- and dicotyledonous plants. It could be evaluated the physiological importance of Se for higher plants within the following topics: anti-oxidative and pro-oxidative effects of Se and role of Se under abiotic stresses.

6.4.1 Anti-oxidative and Pro-oxidative Effects of Selenium

It is well known that, oxidative stress describes a condition when the generation of reactive oxygen species (ROS) in a system exceeds the system's ability to neutralize and eliminate them (Sies and Cadenas 1985). The imbalance can resulted from a lack of antioxidant capacity caused by disturbance in production, distribution, or by an overabundance of ROS from endogenous sources or environmental stressors. If not regulated properly, excess ROS can damage cellular lipids, proteins or DNA,

thus inhibiting signal transduction pathways, and, in general, normal cellular function. Recent researches have demonstrated that Se not only able to promote growth and development of plants but also increase resistance and antioxidant capacity of plants subjected to various stresses (Peng et al. 2002). The beneficial effect of Se in plants subjected to stress conditions has in most cases been attributed to increased antioxidant activity. It is reported that, the effect of Se application in the form of selenate on senescence in lettuce and soy bean, confirming that the decline in antioxidant enzyme activity was milder in plants treated with this element, which offsets oxidative damage by boosting growth in plants treated with Se (Djanaguiraman et al. 2005). It is well indicated that, the selenate form of Se is less toxic than selenite. Also, the plants treated with selenate induced higher increases in enzymes that detoxify H_2O_2, especially glutathione peroxidase (GPX) and ascorbate peroxidase (APX), as well as an increase in the foliar concentration of antioxidant compounds such as ascorbate (AsA) and glutathione (GSH). These data indicate that an application of selenate at low rates can be used to promote the induction in plants of the antioxidant system, thereby improving stress resistance (Hasanuzzaman et al. 2010).

It is also reported that, a significant change in the activities of oxido-reductase enzymes in response to added Se in wheat plants. The nature of the changes can not be clearly determined, however, they were found to depend on both the concentration of Se and the enzyme (Nowak et al. 2004). Ryegrass was cultivated at Se addition levels ranging from low to very high to study the possible antioxidative function of Se (Hartikainen et al. 2000). After 5 years, Hartikainen followed these impacts of Se additions on harvested ryegrass, where the growth-promoting response agreed with the enhanced antioxidative capacity manifested in decreasing lipid peroxidation. This response coincided with a marked increase in glutathione peroxidase (GSH-Px) activity and a peak concentration of tocopherols (vitamin E), scavengers of lipid peroxide radicals and singlet oxygen. The results showed that Se treatment may not only increase the yield of forage plants but also improve their nutritive quality in many ways (Hartikainen 2005). Se acted as an antioxidant, inhibiting lipid peroxidation in ryegrass (*Lolium perenne*) in the concentrations 0.1 and 1.0 mg Se kg^{-1}. Senescence processes are partly delayed due to enhanced antioxidation, which is associated with an increase of GPX activity (Hartikainen et al. 2000). It is found that, in the senescing plants, the addition of Se strengthens the antioxidative capacity by preventing the reduction of tocopherol concentration and by enhancing superoxide dismutase (SOD) activity (Xue et al. 2001). Several studies have shown that a protective role of Se against the oxidative stress in higher plants coincided with enhanced GPX activity and decreased lipid peroxidation (Cartes et al. 2005) as reviewed by Hasanuzzaman et al. (2010).

Therefore, it could be concluded that, Se has a distinguished role in increasing resistance and antioxidant capacity of higher plants. Se strengthens the antioxidative capacity in higher plants by preventing the reduction of tocopherol concentration and by enhancing superoxide dismutase activity. After addition to forage plants, Se may not only increase the yield of plants but also improve their nutritive quality in many ways.

6.4.2 Role of Selenium Under Abiotic Stresses

As well known, the production of ROS is the important cause of damage to plants when subjected to salt stress, thus leading to the growth suppression (Nahar and Hasanuzzaman 2009). Research results obtained by different researchers showed the ability of Se to protect plants from salt stress-induced damages when applied at low concentration. It is reported that, Se is promoting antioxidant activity basically in plants subjected to any type of stress, while the possible action of this trace element (different application rates as well as forms selenate versus selenite) in the oxidative metabolism of non-stressed plants has hardly been documented (Hasanuzzaman et al. 2010). It could be revealed from these results that, in cucumber leaves, Se treatments at 5 and 10 μM significantly improved the growth rate and increased the photosynthetic pigments and proline contents when subjected to salt stress (Hawrylak-Nowak 2009). Additionally, Se enhanced the salt tolerance of seedlings by protecting the cell membrane against lipid peroxidation. The interaction of Se with soil salinity has also been studied by Terry et al. (2000). In Se-accumulators, selenate is taken up preferentially over sulfate. Chloride salinity had much less effect on selenate uptake than sulfate salinity (Wu and Huang 1991). Generally, there is a small decrease in shoot accumulation of Se with increasing salt levels as reviewed by Hasanuzzaman et al. (2010).

It is well known that, drought is a multi-dimensional stress, which causes various physiological and biochemical changes on plants (Hossain and Fujita 2009). One of the earliest responses of plants to drought is the accumulation of active oxygen species (ROS) such as $O_2^{\bullet}$, H_2O_2 and $^1O_2$ (Mittler 2002). Plants protect cell systems from the cytotoxic effects of these active radicals using enzymes such as SOD, APX, glutathione reductase (GR), CAT and non-enzymatic antioxidants: GSH, AsA and carotenoid (Car) (Foyer et al. 1997). However, there are few reports on the protective role of exogenous Se on drought stress in plant. A significantly higher actual photochemical efficiency of PSII was obtained in Se- and water-deficit plants, which was possibly due to improvement of plant water management during treatment. It could be also observed that, a significant interaction between the effects of water deficit and Se on respiratory potential (Hasanuzzaman et al. 2010).

It is suggested that optimal Se supply is favorable for growth of wheat seedlings during drought condition. The growth and physiological responses of seedlings were different, depending on the Se concentration. It is reported that, proline induces the expression of salt-stress-responsive proteins and improves the salt-tolerance in the desert plant *Pancratium maritimum* (Khedr et al. 2003). Increase of proline content in Se-treated soybean plants has also been reported by Djanaguiraman et al. (2005). However, the mechanisms and the reasons for proline accumulation in Se-supplied plants have not been fully investigated. It has been also studied the interaction between Se and soil salinity (Terry et al. 2000). It is hardly surprising that sulfate salinity drastically inhibits plant uptake of selenate, otherwise not all plant species are affected to the same extent of sulfate salinity (Zayed et al. 1998). In general, selenate is taken up preferentially over sulfate in Se-accumulator plants.

Table 6.1 Total Se, S, malondialdehyde (MDA) and H_2O_2 accumulation in lettuce plants subjected to different forms and concentrations of Se in nutrient solution

Added Se (μM)	Different accumulation in roots				Different accumulation in shoots			
	MDA	H_2O_2	Se	S	MDA[a]	H_2O_2	Se	S
Selenite, Se(IV)								
0	3.15 ab	3.37 b	1.1 h	7.64 a	4.86 b	4.39 a	0.7 i	3.59 b
2	2.78 b	3.30 b	48.5 d	7.67 a	6.52 ab	3.67 ab	3.7 h	3.30 b
4	2.56 b	3.74 ab	117.9 c	6.47 b	6.94 ab	3.41 ab	7.2 f	3.31 bc
6	2.67 b	3.28 b	127.9 b	6.56 b	7.32 a	3.93 ab	11.3 d	3.21 bc
15	4.97 a	4.71 a	201.4 a	6.96 b	7.37 a	3.75 ab	30.6 b	2.67 c
Selenate, Se(VI)								
0	3.15 ab	3.37 b	1.1 h	7.64 a	4.86 b	4.39 a	0.7 i	3.59 b
2	2.56 3b	2.71 b	1.9 g	6.56 b	5.82 b	3.71 ab	4.7 g	3.43 b
4	2.59 b	2.94 b	9.8 f	7.00 ab	6.48 ab	3.45 ab	9.2 e	3.82 b
6	2.35 b	3.03 b	21.1 e	5.90 b	6.62 ab	3.30 ab	14.6 c	3.88 b
15	2.67 b	2.83 b	44.2 d	6.10 b	6.04 ab	3.19 b	43.3 a	7.24 a

Adapted from Hawrylak-Nowak (2013)

[a]MDA and H_2O_2 concentration in nmol g^{-1} fresh weight (FW) in lettuce leaves, whereas concentration of Se and S in mg kg^{-1} dry weight (DW) and mg g^{-1} DW, respectively

On the other hand, chloride salinity had much less effect on selenate uptake than sulfate salinity, as mentioned by Wu and Huang (1991). Generally, there is a small decrease in shoot accumulation of Se with increasing salt levels (Banuelos et al. 1996) as reviewed by (Hasanuzzaman et al. 2010).

In a biofortified experiment, the oxidant status (levels of lipid peroxidation and H_2O_2 concentrations), as well as Se and S accumulation in lettuce plants were investigated. It is found that, Se concentration was higher for selenate presence compared to selenite in the edible parts of lettuce. The application of 15 μM Se as selenite caused a decline in the biomass and an intensification of prooxidative processes in the plant's tissues and as toxic should be excluded from further biofortification experiments. These results also indicated that an application of either selenate or selenite to the nutrient solution at concentrations below 15 μM can be used for biofortification of lettuce with Se (Table 6.1; Hawrylak-Nowak 2013)

There is no doubt that, although many heavy metals in trace amounts are essential for various metabolic processes in organisms, they create physiological stress leading to generation of free radicals when in high concentration (Hossain and Fujita 2010). Within last few years, several researches have been studied the role of Se on heavy metal stress tolerance in higher plants. It could be concluded that, during heavy metal stress Se might prevent its toxic effect in higher plants. It has been suggested that the protective effects of Se are due to the formation of non toxic Se-metal complexes (Vorobets 2006). It is also observed that, the proportion of α-tocopherol was similar in the control plants and in those supplied with Se separately or in combination with cadmium (Pedrero et al. 2008). It has been reported that an increase of α-tocopherol favors the stress tolerance of plants

as it favors the scavenging of singlet oxygen species in chloroplasts (Munne-Bosch 2005). Therefore, the increase of α-tocopherol in plants exposed to Se and Cd simultaneously, in comparison to those grown only in Cd, shows evidence that Se assists the plants in the adaptation. Under heavy metal stress, there are some possible mechanisms by which Se confers tolerance to stress (Hasanuzzaman et al. 2010).

Therefore, it could be concluded that, Se acted as an antioxidant, inhibiting lipid peroxidation via increased levels of thiols and GSH. It could be also suggested that, Se is either an antioxidant or it activates plant protective mechanisms, thereby alleviating oxidative stress and improving heavy metals or trace elements uptake in higher plants. Optimal Se supply is favorable for growth of some plants (like wheat seedlings) during drought condition. The growth and physiological responses of seedlings were different, depending on the Se concentration.

6.5 Selenium Hyperaccumulator Plants

It could be defined hyperaccumulation as the intriguing phenomenon through which some plant species accumulate one or more toxic elements to extraordinarily high concentrations, typically 100-fold higher than other vegetation on the same site (Baker et al. 2000). Whereas, hyperaccumulators are plants that accumulate toxic elements to extraordinary levels. Thus, Se hyperaccumulators can contain 0.1–1.5 % of their dry weight as Se without showing any symptoms of toxicity (White et al. 2007), whereas these levels are toxic to most other organisms. The criterion used to distinguish a hyperaccumulator ranges from 0.01 to 1.0 % of leaf dry matter, depending on the element (El Mehdawi and Pilon-Smits 2012). In brief, elements that can be hyperaccumulated include arsenic (As, >0.1 %), cadmium (Cd, >0.01 %), cobalt (Co, >0.1 %), copper (Cu, >0.1 %), lead (Pb, >0.1 %), manganese (Mn, >1 %), nickel (Ni, >0.1 %), selenium (Se, >0.1 %) and zinc (Zn, >1 %). It could be mentioned that, the element levels accumulated in these plants would be lethal to other organisms, yet cause no toxicity in hyperaccumulators. About 450 plant species from over 40 families have been reported to hyperaccumulate, but hyperaccumulation is most prevalent in the Brassicaceae (El Mehdawi and Pilon-Smits 2012).

Se is a naturally occurring element in most soils and can be found at very high levels in alkaline soils where Cretaceous shale or other seleniferous rocks are the soil parent material (Beath 1982). Non-seleniferous soils contain less than 1 mg Se kg^{-1}, whereas seleniferous soils can range between 2 and 100 mg Se kg^{-1} (Mikkelsen et al. 1989). Selenate is thought to be the predominant bioavailable form of Se in seleniferous soil (Zhao et al. 2005) and reviewed by Lindblom et al. (2012). The Se concentrations in hyperaccumulators are typically 1000-fold higher than those in seleniferous soil, and 100-fold higher than those in other vegetation on the same soil (Galeas et al. 2007).

As mentioned before, Se is an essential nutrient for many animals including mammals, yet is also toxic at higher levels, leaving a narrow margin between

deficiency and toxicity (Li et al. 2009). It is thought Se toxicity occur because of the similarity of Se to sulfur (S) (Terry et al. 2000). Where, Se is metabolized into selenocysteine (SeCys), or selenomethionine (SeMet); these Se-amino acids can be non-specifically incorporated into proteins instead of cysteine or methionine, causing the proteins to lose function (Brown and Shrift 1982). Plants that accumulate Se may be used to both clean up excess Se from the environment (phytoremediation) and to prevent Se deficiency in consumers (biofortification). Crop species that are particularly good Se accumulators and that may be used for biofortification are garlic, onion, and broccoli (Fairweather-Tait et al. 2011).

It is reported that, Se enters the food chain primarily through plants that inadvertently take up and assimilate selenate via S transporters and enzymes (Sors et al. 2005a). It is well documented that, plant species differ in their capacity to accumulate and tolerate Se, and most plant species that are Se hyper-accumulators occur within the genus *Astragalus*; examples are *Astragalus bisulcatus* and *Astragalus racemosus* (Beath et al. 1939). The genus *Stanleya* also contains at least one Se hyperaccumulating species, *Stanleya pinnata* (Feist and Parker 2001). Hyperaccumulators detoxify Se by adding a methyl group to SeCys via the enzyme SeCys methyltransferase (SMT); the resulting methyl-SeCys (MeSe- Cys) can be accumulated safely since it is a non-protein amino acid (Neuhierl and Bock 1996). Methyl-SeCys is the primary form of Se found in young and old leaves of *A. bisulcatus* as well as in young leaves and flowers of *S. pinnata* (Quinn et al. 2011). It is found that, within hyperaccumulator leaves, the Se predominantly exist in the periphery, such as the margins, epidermis, and trichomes (Freeman et al. 2006). On the other hand, non-hyperaccumulator plant species accumulate Se mainly in the vascular tissues, in the form of selenate (Van Hoewyk et al. 2005); since this form of Se is more toxic, these species are less Se tolerant. About Se speciation in plant roots, it is needed to identify in any plant species, including hyperaccumulators (Zhang and Gladyshev 2009).

It is appeared that, Se is a beneficial nutrient for many plants, especially hyperaccumulators, which can reach twofold higher biomass in the presence of Se (Pilon-Smits et al. 2009). Thus, the functional significance of Se hyperaccumulation may be to offer better growth, perhaps due to better oxidative stress resistance (Hartikainen 2005). It is worth to mention that, an additional benefit of Se hyperaccumulation is enhanced resistance to Se-sensitive herbivores and pathogens (Quinn et al. 2010). Thus, Se hyperaccumulation may be considered a form of elemental defense (Boyd 2010), whereas as with any plant defense, herbivores and pathogens are likely to overcome it over time. That means, there is indeed evidence of Se-tolerant herbivores and rhizosphere microbes (Wangeline et al. 2011). It is found that, Se-tolerant diamond back moth feed on *S. pinnata* containing 0.2 % Se dry weight without ill effects and it accumulated MeSeCys, like its host, explaining its tolerance. Mechanisms of Se-tolerant microbes include: Se reduction to insoluble, non-toxic elemental Se (Se^0), volatilization, or conversion to MeSe- Cys (Hunter and Manter 2009). Whereas, some plant-associated microbes have been shown to affect plant Se accumulation and volatilization (de Souza et al. 1998). The capacity of microbes to affect plant Se speciation is needed to investigate (Lindblom et al. 2012).

Table 6.2 Overview of physiological differences between Se hyperaccumulators and non-hyperaccumulators

Property	Se hyperaccumulators	Non-hyperaccumulators
Se uptake	Sulphur-independent	Inhibited by sulphur
Root-to-shoot Se translocation	Higher	Lower
Se accumulation and tolerance	1000–15,000 mg Se kg^{-1} DW	<1000 mg Se kg^{-1} DW
Se volatilization	Higher, as dimethyldiselenide	Lower, as dimethylselenide
Sequestration (organ level)	Highest in reproductive organs	Highest in leaves
Sequestration (tissue level)	Highest in epidermis, pollen, ovules	Highest in vascular tissues
Main Se form in tissues	Methyl-SeCys	Selenate
Seasonal fluctuations of Se and S	Highest in spring for Se, summer for S	Highest in summer for Se and S

Source: El Mehdawi and Pilon-Smits (2012)

It is well known that, hyperaccumulators differ from non-hyperaccumulators in several ways, including (i) selenate uptake by hyperaccumulators is not inhibited by high sulphate concentration (Feist and Parker 2001), (ii) hyperaccumulators tend to become enriched with Se relative to S: they have a higher Se/S ratio in their tissues compared to their growth medium (White et al. 2007), (iii) hyperaccumulators contain elevated S levels, compared to non-hyperaccumulators (El Mehdawi et al. 2011), (iv) Se hyperaccumulators show a relatively high degree of root/shoot Se translocation compared to other plants and (v) the Se speciation or form of Se accumulated by hyperaccumulator plants is mainly organic methyl-SeCys (Freeman et al. 2006), while in non-accumulators and accumulators, the majority of Se remains as selenate (Van Hoewyk et al. 2005). It could be summarized these physiological differences between non- hyperaccumulators and hyperaccumulators in Table 6.2.

It could concluded that, because of this difference in plant Se speciation, the tissue Se sequestration pattern is different for hyperaccumulators, where they store Se mainly in the leaf epidermis (sometimes in leaf hairs) and in reproductive tissues, particularly pollen, ovules and seeds (Quinn et al. 2011). About non-hyperaccumulators mainly store Se in vascular tissues of leaves, and have higher Se levels in leaves than flowers (Quinn et al. 2011). It could be converted methyl-SeCys to dimethyldiselenide, where it is the main form of volatile Se produced by hyperaccumulators. On the other hand, non-hyperaccumulators produce volatile dimethylselenide, using selenomethionine (SeMet) as a starting point (Terry et al. 2000). Plant levels of Se also show different seasonal fluctuations in hyperaccumulators and non-accumulators, where the leaf Se concentration is highest in the early spring for hyperaccumulators, but peaks in summer for non-hyperaccumulators (Galeas et al. 2007). The seasonal fluctuations in Se levels are correlated with S levels for non-hyperaccumulators, but not for hyperaccumulators as reviewed by El Mehdawi et al. (2012).

Table 6.3 Classification of plant Se toxicity according to de Filippis (2010)

Plant accumulator category	Concentration of Se toxicity level in plant shoots (mg Se kg^{-1} dry weight)	References
Primary accumulator plants	Se level: 2000 and 4000 mg Se kg^{-1} dry weight shoots	Galeas et al. (2007)
	Plant group: *Astragalus, Stanleya, Neptunia* and *Brassica*	
Secondary accumulator plants	Se level: 75 and 900 mg Se kg^{-1} dry weight shoots	Sharma et al. (2009)
	Plant group: clover, strawberry clover, bent grass, ryegrass, rice, buffalo grass, and alfalfa	
Non-accumulator plants	Se level: 2 and 25 mg Se kg^{-1} dry weight shoots	Sharmasarkar and Vance (2002)
	Plant group: wheat, rice, pea, mustard, kidney beans and alfalfa	

It could be followed the Se-tolerance mechanism of hyperaccumulators using microfocused X-ray fluorescence (μXRF) mapping and micro-X-ray absorption near edge structure (μXANES) spectroscopy, which revealed a stark contrast in spatial distribution and chemical speciation of Se between hyperaccumulators and nonaccumulators (El Mehdawi et al. 2012). On the other hand, there is a great variation in plants' capability to absorb Se from soils, especially from seleniferous ones. Thus, it could be divided these plants into three categories: (1) plants that are accumulators and contain high amounts of Se (>1000 μg kg^{-1}) and presumably require this element, (2) plants that absorb medium quantities, up to around 100 μg Se kg^{-1}, and (3) plants that are nonaccumulators, containing usually below 30 μg Se kg^{-1}, under field conditions (Table 6.3; de Filippis 2010).

A limited number of plants, especially from the Fabaceae and Brassicaceae can accumulate considerably higher levels of Se in leaves, and are often found on soils that are naturally enriched with Se or seleniferous soils. These accumulator plants can be further sub-divided into two groups (White et al. 2007):

(a) **Primary accumulators (hyperaccumulators)** – which have concentrations of Se in leaves (70–300 mg Se kg^{-1} dry weight) and discrimination coefficients (DC = [Se/S] plant/[Se/S] solution) between Se and S (Se/S) of more than 2.5 in solution culture, including various species like *Astragalus, Stanleya pinnata, Melilotus officinalis*. DC. Examples *Grindelia squarrosa, Neptunia amplexicaulis,* and *Bertholletia excelsa* (White et al. 2004).

(b) **Secondary accumulators** – which take-up Se in proportion to the amount of Se available in the soil and have a DC of less than 2.5. Tissue concentrations of Se are in the range of 5–30 mg Se kg^{-1} dry weight, including species of *Aster*, *Attriplex, Brassica juncea* and *Brassica napus* (canola) (White et al. 2004).

It is worth noting that although there is a relationship between higher Se accumulation and a higher DC ratio, this is not always true. For example, *B. juncea* and *B. oleracea* have moderate DC ratios of 1.50–1.75 yet contain high leaf Se content (21–33 mg Se kg^{-1} dry weight), but in contrast *B. gracilis,* and *S. melongena* have high DC ratios of 1.80–1.87 yet contain low leaf Se content (5.8–7.1 mg Se kg^{-1} dry weight) (Freeman et al. 2006).

It could be concluded that, elements that can be hyperaccumulated include As (>0.1 %), Cd (>0.01 %), Co (>0.1 %), Cu (>0.1 %), Pb (>0.1 %), Mn (>1 %), Ni (>0.1 %), Se (>0.1 %) and Zn (>1 %). It could be also mentioned that, the element levels accumulated in these plants would be lethal to other organisms, yet cause no toxicity in hyperaccumulators. The Se-tolerance mechanism of hyperaccumulators can be followed by using microfocused X-ray fluorescence (μXRF) mapping and micro-X-ray absorption near edge structure (μXANES) spectroscopy, which revealed a stark contrast in spatial distribution and chemical speciation of Se between hyperaccumulators and nonaccumulators.

6.6 Plant Selenium Toxicity and Tolerance

Concentration of Se in the plants tissues at which they begin to show symptoms of toxicity, such as stunting, chlorosis, and withering of leaves, range from 2 mg kg^{-1} in nonaccumulators, such as rice, and 330 mg kg^{-1} in white clover (Fig. 6.2; Mikkelsen et al. 1989), to several thousand mg kg^{-1} in the accumulator *Astragalus bisulcatus* (Shrift 1969). There are some factors determine the susceptibility of a particular plant to toxicity such as Se concentrations, levels of sulphate in the soil, the stage of growth, and the chemical form of Se accumulated. It is noticed that, both selenite (SeO_3^{-2}) and selenate (SeO_4^{-2}) are the major forms that are toxic to nonaccumulators because they are readily absorbed and assimilated by plants (Wu et al. 1988).

It is believed that, the major mechanism of Se toxicity is to be the incorporation of selenoamino acids, selenocysteine, and selenomethionine, into proteins in place of cysteine and methionine and then, alterations in tertiary structure, resulting from differences in size and ionization properties between S and Se atoms, probably have a negative effect on catalytic activity of certain important proteins (Brown and Shrift 1982). It is believed also that Se induces toxicity in plants by interfering with chlorophyll synthesis (Padmaja et al. 1989) as well as with nitrate assimilation (Aslam et al. 1990). There is also evidence that Se can interfere with production of glutathione, and thus reduce a plant's defense against hydroxyl radicals and oxidative stress (Bosma et al. 1991). It is appeared that, tolerance by accumulators towards levels of Se that would result in toxicity in nonaccumulators is largely due to the reduction of intracellular concentrations of selenocysteine and selenomethionine, thus preventing their incorporation into proteins. This is brought about by converting the Se into nonprotein selenoamino acids, such as selenocystathionine, or into the dipeptide γ-glutamyl-seleno-methyl-selenocysteine (Nigam et al. 1969). There is some evidence that it may, to some extent, be achieved by compartmentation of the element in the form of selenate, or perhaps as nonprotein selenoamino acids, in vacuoles (Terry et al. 2000) as reviewed by Reilly (2006).

It is reported that, the toxic Se concentrations of nonaccumulator plants, resulting in a 10 % reduction of yield, without visible symptoms, range from Se contents of 2–330 mg kg^{-1} in rice and white clover, respectively. In accumulator plants, Se concentration may reach 4000 mg kg^{-1}, without negative effects (Kabata-Pendias 2011). Tolerance mechanisms involve processes of exclusion of active Se amino acids, thus

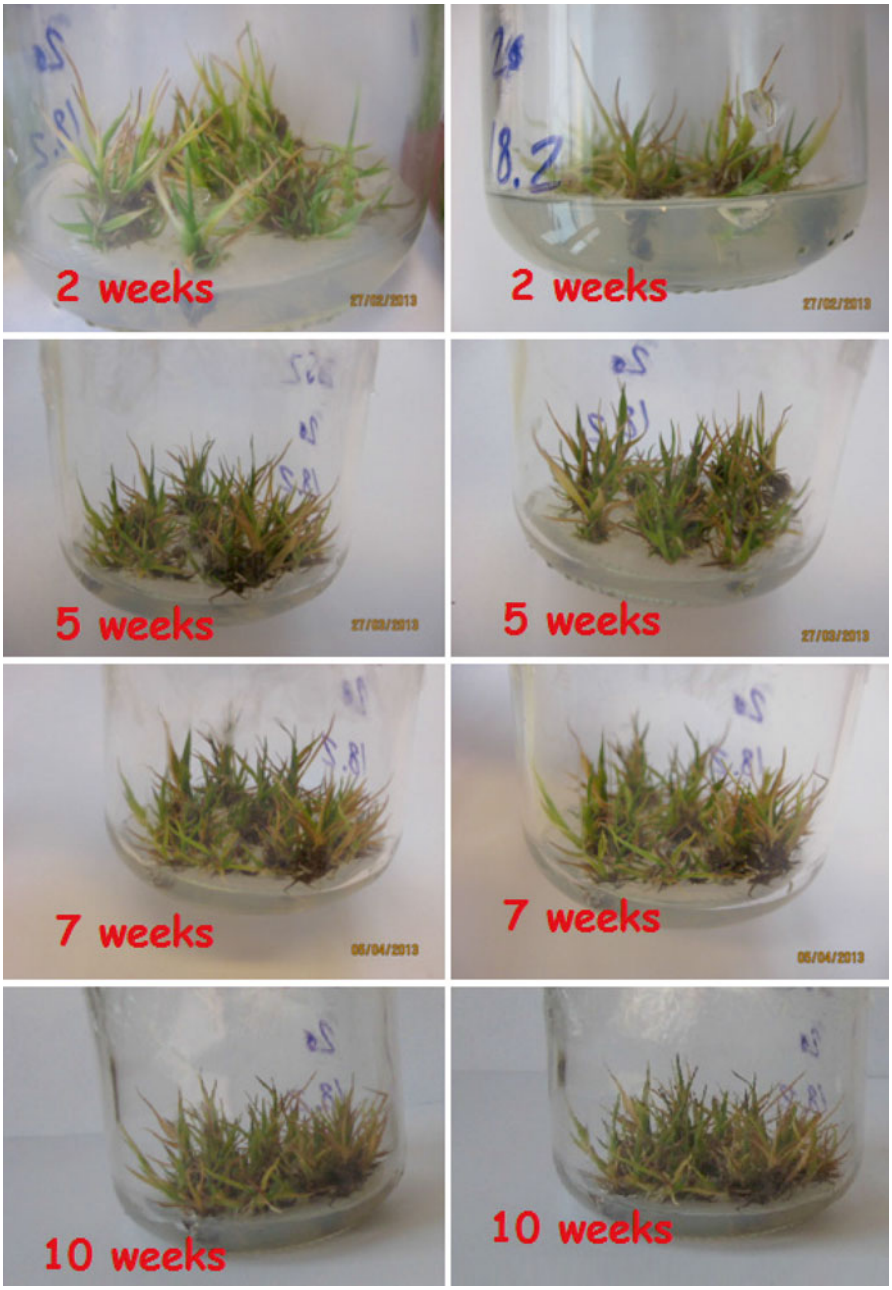

Fig. 6.2 Some toxicity symptoms and tolerant of Se on giant reed (*Arundo donax* L., Hungarian ecotype: 20SZ) in solid media after about **2, 5, 7 and 10 weeks** (from top to bottom, respectively) from clusters transfer at 20 mg Se kg^{-1} (Photos by H. El-Ramady)

preventing their incorporation into proteins and damaging effects on plant functions (Terry et al. 2000). The exclusion of Se from proteins in accumulator plants is the basis for their tolerance to Se. In general, food crops have a low Se tolerance; however, most other plants may accumulate amounts of Se that are toxic to humans and animals. In nontolerant plant species, an excess of Se may impair germination and growth, and cause chlorosis and black spots on leaves. Increased Se levels in plants suppress their concentrations of N, P, and S, as well as several amino acids, thus high Se concentrations inhibit the absorption of metals, mainly Mn, Zn, Cu, and Cd. These relationships are dependent on the ratio between the elements, and therefore stimulating effects of high Se levels on uptake of some trace elements may sometimes be expected. The application of N, P, and S is known to help in detoxifying Se, which may be a result either of depressing the Se uptake by roots or of establishing a beneficial ratio of Se to these elements as reviewed by Kabata-Pendias (2011).

It is reported that, when Se sensitive plants are exposed to elevated levels of Se in the soil root medium they may exhibit varying symptoms such as stunted growth, chlorosis, withering, drying of leaves and premature death of the whole plant (Mikkelsen et al. 1989). Generally, the threshold range in non-accumulator plants vary with plant age and S supply, where younger plants can be more susceptible to toxicity, and tolerance to Se toxicity increases with increasing sulphate supply (Brown and Shrift 1981). The threshold toxic value in non-accumulator plants also depends on the form of Se applied and with selenate and selenite being the main toxic forms to plants. This may be linked to both these forms of Se being readily absorbed and translocated in plants and assimilated in the inorganic forms (de Filippis 2010).

In a pot trial, a wide range of Se applications was tested, up to the high level of 132 mg kg^{-1} Se as selenate. It is found that, selenate's mobility and solubility was reflected in the high tissue Se concentrations, even at 1 mg kg^{-1}, the lowest level of applied Se (Table 6.4). This trial showed that a Se level above 2 mg kg^{-1} applied as selenate (which produced around 200 mg kg^{-1} Se in above-ground plant tissue) inhibited growth (Lyons et al. 2005). On the other hand, in a greenhouse experiment, 11 vegetable crops were investigated using alkaline clay (pH 8.25; EC, 0.20 dS m^{-1}; organic matter, 0.30 % and total Se content, 0.135 mg kg^{-1}). The soil was treated with four Se levels of 0, 1.25, 2.5 and 5.0 mg kg^{-1} added as $Na_2SeO_4 \cdot 5H_2O$ in solution form. Progressive restrictions in plant growth, size of leaves and burning of leaf margins were observed as visual symptoms in vegetable crops grown in the selenate-Se treated alkaline clay loam soil as presented in Table 6.5 (Dhillon and Dhillon 2009).

It could be concluded a number of possible modes of tolerance to toxic compounds, which described by Pilon-Smits (2005) and may involve any of six mechanisms; these include differences in adsorption, conjugation, sequestration, enzymatic modification, enzymatic degradation and volatilization. Tolerance in Se accumulator plants appears to be due to a number of mechanisms as follows:

- **Adsorption/transportation**: decrease in excessively high concentrations of Se being transported into cells of leaves.
- **Sequestration/enzymatic modification**: accumulation of Se in Se amino acids, but these seleno-amino acids are not incorporated into normal protein synthesis.

Table 6.4 Effect of available soil Se on plant yield and concentration of Se and S in whole tops of wheat grown for 22 days in Univeristy of California mix growth medium treated with sodium selenate in pot trial in greenhouse

Applied Se (mg kg^{-1} soil)	Shoot FW (g/plant)	Element concentration in wheat (mg kg^{-1} DW)		Se toxicity symptoms
		Se	S	
0	73	0.1	5200	None
1	72	85	9300	None
2	70	209	10,900	None
4	52	685	16,150	Stunting, mainly of 2nd youngest leaf; bleached 2nd and 3rd youngest leaves in some plants; leaf tip necrosis and exudate
8	28	1465	21,250	Stunting; wilting; chlorosis; tip necrosis and exudate; bleached 2nd and 3rd youngest leaves in most plants
16	20	1440	9850	Stunting; wilting and tip necrosis and exudate (but less than the 8 mg kg^{-1} plants); chlorosis; complete bleaching of the 2nd and 3rd youngest leaves
32	10	1745	6000	Severe stunting, wilting, chlorosis and bleaching
64	0.4	1810	3650	Severe stunting, leaf tip exudation and chlorosis; some bleaching on lower part of leaves
132	0.2	870	2900	Plants 2 cm tall; most dark green

Adapted from Lyons et al. (2005)
FW fresh weight, *DW* dry weight

Table 6.5 Selenium toxicity symptoms in some vegetable crops in alkaline clay loam soil in presence of added 5 mg kg^{-1} Se soil

	Added amount of selenate-Se applied to soil	
Vegetable crop	2.5 mg kg^{-1} soil	5.0 mg kg^{-1} soil
Radish, turnip and carrot	Leaves appeared more in number but smaller in size (Rosette appearance). Root growth severely restricted	Seeds germinated, but the seedlings died after 10–12 days of germination. In case of carrot 3–4 seedlings could survive till the end of the experiment
Spinach	Stunted growth	Burning of leaf tips and margins, stunted growth
Cauliflower	Stunted growth	Stunted growth, wilted look, drying of 3rd and 4th leaf, no fruit formation
Eggplant	Restricted growth	Plants turned yellow after 15 days of transplanting followed by drying up leading to death of the plants
Onion	Severely stunted growth	Burning of leaf tips, severely stunted growth
Garlic	Restricted growth	Burning of leaf tips, severely stunted growth
Pea	Severely stunted growth	Severely stunted growth, wilted look and no fruit formation
Tomato	restricted growth, leaves smaller in size	Severely stunted growth, wilted look and no fruit formation
Potato	—	No conspicuous symptoms were observed except restricted growth of shoots, tubers smaller in size

Adapted from Dhillon and Dhillon (2009)

Table 6.6 Selenium toxicity (or critical concentration of Se in used media, mg Se kg^{-1} DW) in some Se-nonaccumulator plants in different media (hydroponics, soil and *in vitro* solid media) sulphate concentration (mM) in the rhizosphere comparing with gaint reed plant

Plant (Scientific name)	Sulphate in rhizosphere (mM)	Critical concent. (mg Se kg^{-1} DW)	References
Hydroponics medium			
Bush bean (*Phaseolus vulgaris*)	4.0	25	Wallace et al. (1980)
Ryegrass (*Lolium perenne*)	1.875	320	Smith and Watkinson (1984)
White clover (*Trifolium repens*)	1.875	330	Smith and Watkinson (1984)
Alfalfa (*Medicago sativa*)	0.5, pH = 4.5	19	Mikkelsen et al. (1987)
Alfalfa (*Medicago sativa*)	0.5, pH = 7.0	94	Mikkelsen et al. (1989)
Bermudagrass (*Cynodon dactylon*)	0.25	73	Wu et al. (1988)
Buffalograss (*Buchloe dactyloides*)	0.25	340	Wu et al. (1988)
Ryegrass (*Lolium perenne*)	0.5	590–900	Hopper and Parker (1999)
Strawberry clover (*Trifolium fragiferum*)	0.5	490–840	Hopper and Parker (1999)
Soil medium			
Mustard (*Brassica juncea*)	ND	3.0	Tripathi and Misra (1974)
Pea (*Pisum sativum*)	ND	3.0	Tripathi and Misra (1974)
Wheat (*Triticum vulgare*)	ND	3.0	Tripathi and Misra (1974)
Rice (*Oryza sativa*)	ND	<2.0	Prasad and Arora (1980)
Rice (*Oryza sativa*)	8.9 mM (upland)	160	Mikkelsen et al. (1989a)
Rice (*Oryza sativa*)	ND (lowland)	81	Mikkelsen et al. (1989a)
***In vitro* solid medium**			
Giant reed (*Arundo donax* L.)[a]	ND	20	El-Ramady et al. (2013)
Giant reed (*Arundo donax* L.)[b]	ND	50	El-Ramady et al. (2013)

Source: White et al. (2004). The critical **shoot tissue** Se concentrations correspond to a 10 % reduction in the yield of plants grown in the presence of added selenate
[a]for the American ecotype (Blossom) and [b]for the Hungarian one (20SZ)

- **Sequestration**: compartmentation of Se as selenate in the vacuole and away from more sensitive cytoplasmic reactions.
- **Enzymatic modification**: increase ATP sulphurylase and SeCys methyltransferase activities to reduce inorganic Se to organic forms of Se, although other enzymes and reactions are also required.
- **Conjugation**: conjugation with glutathione (GSH) and an increase in anti-oxidation protective reactions. Conjugation with Se binding proteins and poly-peptides, decreasing inorganic Se content.
- **Volatilization**: increase volatilization of mainly organic forms of Se out of plant cells and tissues (de Filippis 2010).

As well known, plant species differ in their ability to accumulate Se and most plants contain less than 25 mg Se kg^{-1} dry matter and are termed non-accumulators. Such plants are incapable of tolerating high Se in the environment, and Se toxicity

occurs below about 10–100 mg Se kg^{-1} dry matter, although the exact value depends critically upon the selenate: sulphate ratio in the rhizosphere solution (Table 6.6; White et al. 2004). These plants tolerate low Se concentrations in the rhizosphere by restricting Se uptake and movement to the shoot (Wu et al. 1988). Several plant species can grow adequately in both seliniferous and non-seliniferous soils, and can contain up to 1000 mg Se kg^{-1} dry matter without consequence (White et al. 2004).

Therefore, it could be concluded that, both selenite (SeO_3^{-2}) and selenate (SeO_4^{-2}) are the major forms that are toxic to nonaccumulators because they are readily absorbed and assimilated by plants. Increased Se levels in plants suppress their concentrations of N, P, and S, as well as several amino acids, thus high Se concentrations inhibit the absorption of metals, mainly Mn, Zn, Cu, and Cd. These relationships are dependent on the ratio between the elements. The application of N, P, and S is known to help in detoxifying Se, which may be a result either of depressing the Se uptake by roots or of establishing a beneficial ratio of Se to these elements.

6.7 Plant Selenium Metabolism

A lot of research, in recent years, has been carried out on the uptake and metabolism of Se by plants , where the metabolic fate of Se taken up by different plants has been reviewed by different researchers (e.g., Sors et al. 2005b; Pilon-Smits and Quinn 2010; Nakamaru and Altansuvd 2014; Pilon-Smits et al. 2014; Malagoli et al. 2015; Pilon-Smits 2015). Where, the trigger for much of this work was the recognition, not only by scientists, but also by administrators, politicians, and, not least, the general public, especially in the USA, of the importance of Se as an environmental contaminant (Reilly 2006). As mentioned before, although there is no strong evidence that Se is an essential requirement for plant growth, it is nevertheless metabolized in a variety of ways once it is taken up into the plant tissues (Fig. 6.3). Selenate (SeO_4^{2-}) is the predominant form of bioavailable Se in oxic soils and selenite (SeO_3^{2-}) is more abundant in anoxic wetland conditions. Thus, both forms are readily taken up by plants. SeO_4^{2-} is taken up and distributed by means of sulfate-proton co-transporters. All of the sulfate transporters in plants likely can transport SeO_4^{2-} as well (Maruyama-Nakashita et al. 2004). SeO_4^{2-} assimilation takes place predominantly in the leaf chloroplasts (Pilon-Smits and Quinn 2010). The reduction of SeO_4^{2-} to SeO_3^{2-} appears to be a rate-limiting step in the Se assimilation pathway, since most plants supplied with SeO_4^{2-} accumulate predominantly SeO_4^{2-}, while plants supplied with SeO_3^{2-} accumulate organic Se (de Souza et al. 1998). The conversion of SeO_4^{2-} to SeO_3^{2-} involves the consecutive action of two enzymes (Fig. 6.3). ATP sulfurylase (APS) couples selenate to ATP, forming adenosine phosphoelenate (APSe) This is subsequently reduced to selenite by APS reductase (APR). There are isozymes for APS and APR in both chloroplast and cytosol, but most of the selenate reduction likely takes place in the chloroplast (Pilon-Smits and Quinn 2010).

The further reduction of SeO_3^{2-} to selenide (Se^{2-}) may happen exclusively in the chloroplast if it is mediated by sulfite reductase, in analogy with sulfite reduction. However, it has also been suggested that nonenzymatic reduction by reduced

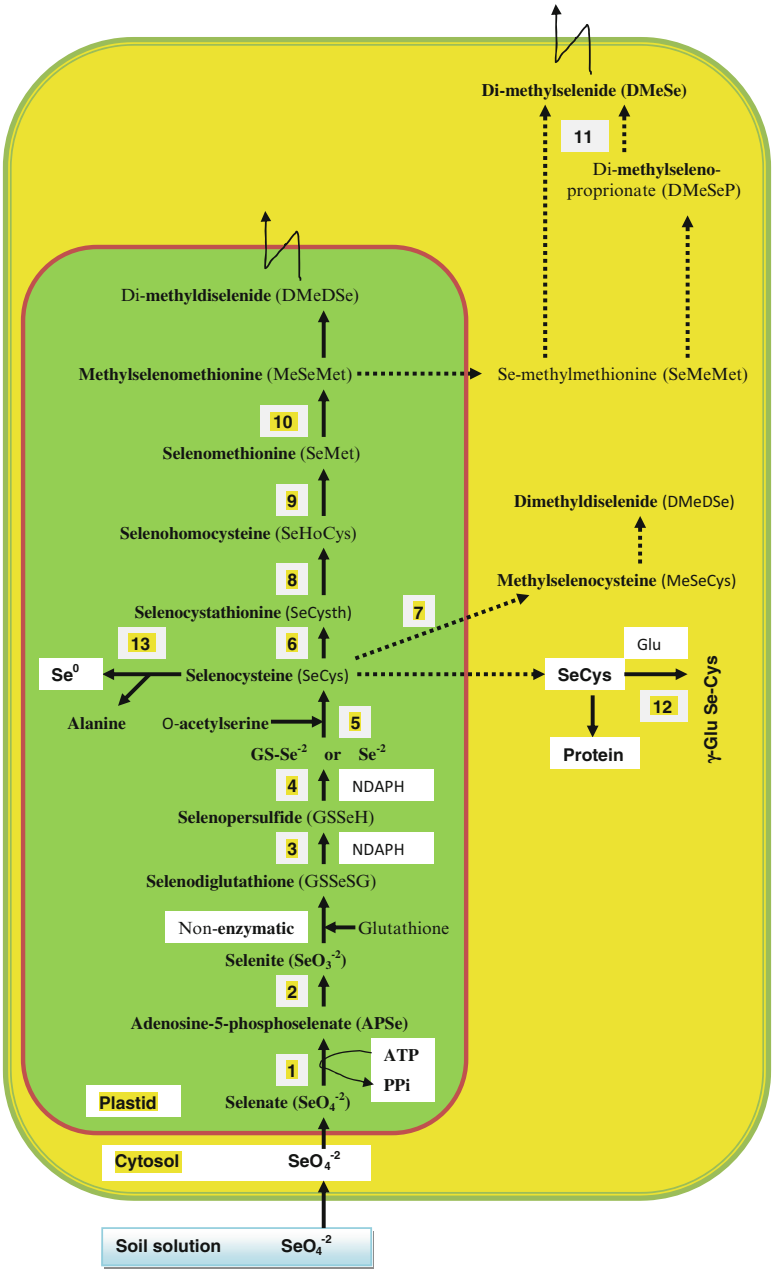

Fig. 6.3 Generalized overview of Se metabolism in plants. Numbers denote known enzymes. (1) ATP sulfurylase, (2) adenosine-5-phosphosulfate reductase, (3) glutathione or sulfite reductase, (4) glutathione reductase or O-acetylserine thiol lyase, (5) Selenocysteine methyltransferase, (6) Selenocysteine lyase, (7) cystathionine-g-synthase, (8) cystathionine-b-lyase, (9) methionine synthase, (10) methionine methyltransferase, (11) DMSP lyase, (12) g-glutamylcysteine synthetase (Source: Parker et al. (2003), Sors et al. (2005b), Pilon-Smits and Quinn (2010), Lindblom et al. (2012), Yu and Gu (2013), Pilon-Smits et al. (2014), Winkel et al. (2015) and Pilon-Smits (2015))

glutathione (GSH) may play a significant role in selenite reduction (Terry et al. 2000). Se^{2-} can subsequently be coupled to O-acetylserine (OAS) to form SeCys, by means of OAS thiol lyase (also called cysteine synthase). This enzyme activity is found in cytosol, chloroplasts, and mitochondria. OAS is synthesized by the enzyme serine acetyl transferase, and functions as a signal molecule that upregulates the activity of sulfate transporters and sulfate assimilation enzymes (Pilon-Smits and Quinn 2010).

It could be converted selenocysteine (SeCys) to selenomethionine (SeMet) and dimethylselenide (DMSe) – SeCys to SeMet via the action of three enzymes (Fig. 6.3). The first, cystathionine-γ-synthase (CγS), couples SeCys to *O*-phosphohomoserine to form Se-cystathionine. The second enzyme, cystathionine-β-lyase, converts Se-cystathionine into Se-homocysteine. These first two enzymes are thought to be chloroplastic. However, the next step occurs in the cytosol. Se-homocysteine is converted to SeMet via the action of Met synthase. SeMet has multiple possible fates, one of which is to be methylated to methyl-SeMet via the enzyme methionine methyltransferase. Methyl-SeMet can be further metabolized to volatile DMSe, which is cleaved off of the intermediate, dimethylselenopropionate (DMSeP), by DMSeP lyase (Pilon-Smits and Quinn 2010).

It could be also noticed that, since roots volatilize Se at a much faster rate than other tissues, DMSe precursors, which are synthesized in chloroplasts, must be transported downwards from the leaves for this to occur (Zayed and Terry 1994). Otherwise, the initial steps in Se uptake and conversion to selenocysteine are believed to be the same in both Se accumulators and non-accumulators, subsequent metabolic pathways differ. Unlike nonaccumulators, accumulators metabolize selenocysteine primarily into different nonprotein selenoamino acids (Brown and Shrift 1982). Among these are selenomethylselenocysteine (SemethylSeCys), selenocystathione, and the dipeptide, γ-glutamyl-seleno-methylselenocysteine (Terry et al. 2000) as reviewed by Reilly (2006).

As well known, the metabolic pathway of Se assimilation in higher plants has been reviewed by different researchers such as Sors et al. (2005b) and Pilon-Smits and Quinn (2010). About Se speciation in plant tissues, it is very important from the perspectives of understanding the metabolic pathways and human nutrition, and it has been investigated in several plant species. Thus, Se speciation varies with plant species and the form of Se fed to the plant. For example, in Indian mustard (*Brassica juncea*), the main Se species is selenate when the plant is fed with SeO_4^{2-}, whereas in plants fed with SeO_3^{2-}, SeMet and SeOMet dominate (Kápolna and Fodor 2006). On the other hand, SeMeSeCys is also a major Se compound in Se-enriched garlic (*Allium sativum*), onion (*Allium cepa*), leek (*Allium ampeloprasum*) and broccoli (*Brassica oleracea*), accounting for approximately half of the total Se. By contrast, SeMet is the predominant Se species in most grains, such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare*) and rye (*Secale cereale*), accounting for ~60–80 % of the total Se (Stadlober et al. 2001). However, the speciation might differ in grains with exceptionally high concentrations of Se as reviewed by Zhu et al. (2009).

Therefore, it could be concluded that, plant Se metabolism is of great importance for the Se nutrition of humans and animals. Because of the chemical similarity of Se and S, and as a result, plants and other organisms readily take up and metabolize

Se via S transporters and pathways. While, higher plants do not appear to require Se, they readily take it up from their environment and incorporate it into organic compounds using S assimilation enzymes. In brief, inorganic SeO_4^{2-} is reduced and assimilated into organic Se and the first organic form of Se produced is SeCys. SeO_4^{2-} and SeO_3^{2-} can be assimilated to SeCys and SeMet after plant uptake and non-specifically incorporated into any S compound.

6.8 Selenium Uptake and Transport by Plants

The uptake of Se by plants is affected, in addition to plant-specific ability, by soils factors, of which the most significant are pH, Eh, water regime, clay content, soil organic matter, cation exchange capacity, nutrient balance, and concentration of other trace elements (Kabata-Pendias 2011). Climatic conditions also are shown to influence the rate of Se uptake, which may be partly an indirect impact due to the water flow phenomenon. In general, a higher ambient temperature influences a greater uptake of Se by plants. The uptake of Se by plants depends also on several factors, but when Se is present in soluble forms, it is readily absorbed by plants, although differences between plant species are very pronounced. It is reviewed about the Se metabolism in plants and concluded that regardless of the common traits of these processes, different plant capacities to extract and accumulate this element is evidently related to different metabolic strategies (Di Gregorio 2008). In most cases, there is a positive linear correlation between Se in plant tissues and Se contents of soils. Otherwise, the complex impact of variable factors on Se uptake by plants can significantly alter the relation between Se in plants and soils. When present in soluble forms, Se is readily absorbed by plants, although differences between plant species are commonly observed. The availability of soil Se is also controlled by several soil factors, among which pH is believed to be the most pronounced as reviewed by Kabata-Pendias (2011).

It is well known that, plants mainly take up Se from soil solution in the form of selenate (SeO_4^{-2}), which is taken up inadvertently via sulfate transporters, and metabolized via the S assimilation pathway (Sors et al. 2005a). In this pathway, selenate is reduced to selenite (SeO_3^{-2}), which can undergo further reduction to selenide (Se^{-2}). This may be incorporated into the organic forms, selenocysteine (SeCys), selenocystathionine (SeCysth) and selenomethionine (SeMet). Thus, plant species differ in their capacity to accumulate Se. While, most plant species accumulate Se to concentrations below 100 mg Se kg^{-1} DW, even when growing on Se-rich (seleniferous) soils, some plant species native to seleniferous soils can accumulate Se to concentrations as high as 10,000 mg Se kg^{-1} DW (Galeas et al. 2007). These are called Se hyperaccumulators; examples are *Astragalus bisulcatus* (Fabaceae) and *Stanleya pinnata* (Brassicaceae) as reviewed by El Mehdawi et al. (2012b).

On the other hand, SeO_3^{-2} uptake may not be mediated by membrane transporters, as hydroxylamine a respiratory inhibitor inhibits SeO_3^{-2} uptake by only about 20 %, however hydroxylamine inhibited SeO_4^{-2} uptake by 80 % (Arvy 1997). It is well

known that, translocation of Se from the roots to the shoots is highly dependent on the form of Se supplied, where SeO_4^{-2} is transported more readily than SeO_3^{-2} or organic Se compounds. For example, more than 50 % of Se was transported from the roots to the shoots within 3 h when SeO_4^{-2} was added. Whilst, less than 10 % Se was transported from the roots to the shoots when SeO_3^{-2} or organic Se was added (Shrift and Ulrich 1976). The reason may be that SeO_3^{-2} is more easily converted to organic Se than SeO_4^{-2}, and SeO_4^{-2} is more strongly retained in the roots after transportation from the soil to the root by HAST. As well, the other conclusion could be that only SeO_4^{-2} is readily available in the roots for transportation to the leaves by LAST. The distribution of Se in plants also differs with the type of Se accumulating plant species under investigation as reviewed by de Filippis (2010).

It is well documented that, selenate is taken up by plant roots from soil solution by a process of active transport (Brown and Shrift 1982). It competes with sulfur for uptake, both anions using a sulfate transporter in the root plasma membrane (Arvy 1993). Organic forms of Se, such as selenomethionine, are also taken up actively by plant roots. In contrast, transport of selenite does not appear to require the use of a sulfur transporter (Abrams et al. 1990). Subsequent translocation of Se within the plant is related to the form in which the element is supplied to the root. SeO_4^{-2} is more easily transported from the roots and much more is accumulated in the leaves than either SeO_3^{-2} or organic selenium. Much of the SeO_3^{-2} is retained in the roots where it is rapidly converted into organic forms, particularly selenomethionine (Zayed et al. 1998). Distribution of Se in various tissues differs between accumulator and nonaccumulator plants. In the former, the Se is accumulated especially in young leaves, but later appears at higher levels in seeds than in other tissues, while, in nonaccumulators, such as cereals, levels in seeds and roots are usually the same as reviewed by Reilly (2006).

The uptake of Se by plant roots is influenced by the chemical form and concentration of Se in the soil solution, soil redox conditions, the pH of the rhizosphere, and the presence of competing anions such as sulfate and phosphate (Wu 2004). Plant roots can take up Se as SeO_4^{-2}, SeO_3^{-2}, or organoselenium compounds. Roots take up SeO_4^{-2} faster than SeO_3^{-2} at the same concentration (Zhao et al. 2005), but aquire organoselenium compounds, such as selenocysteine (SeCys) and selenomethionine (SeMet), most avidly (Montes-Bayon et al. 2003). The presence of sulfate in the rhizosphere inhibits SeO_4^{-2} uptake and accumulation, suggesting direct competition between SeO_4^{-2} and SO_4^{-2} for transport (White et al. 2004), but paradoxically, increasing SeO_4^{-2} concentration in the rhizosphere often increases shoot S concentrations (Lyons et al. 2005). The latter observation has been interpreted as the consequence of either SeO_4^{-2} or organoselenium compounds interfering with the regulation of sulfate uptake by plant S status (White et al. 2004). Indeed, changes in the root transcriptome in response to SeO_4^{-2} in the rhizosphere mimic those observed during S starvation (van Hoewyk et al. 2005). Sulfate uptake is regulated at the level of gene transcription (Hawkesford and De Kok 2006), and both the downregulation of sulfate transport capacity by sulfate, cysteine, or glutathione, and the upregulation of sulfate transport capacity by increased *O*-acetylserine or decreased sulfide concentrations, have been proposed (White et al. 2007).

Less is known about the mechanisms of plant uptake of SeO_3^{-2}, which might be prevalent in acidic to neutral soils or under reduced soil conditions, such as paddy soils. It has been suggested that the mechanism of SeO_3^{-2} uptake by plant roots is not metabolically dependent (passive uptake). However, Li et al. (2008) reported that SeO_3^{-2} uptake by wheat was suppressed by the metabolic inhibitor carbonyl cyanide mchlorophenyl hydrazone (CCCP), inhibited by phosphate in the nutrient solution and enhanced by phosphorus deficiency. It was argued that inconsistency in the rate of selenite uptake by plants could be ascribed to different phosphate concentrations present in the growth solutions used for different studies. Thus, SeO_3^{-2} uptake mechanisms require further investigation at both the physiological and molecular levels. In soil, SeO_3^{-2} is less bioavailable to plants than is SeO_4^{-2} because the former is more strongly adsorbed by iron oxides and/or hydroxides (Zhu et al. 2009). Translocation of Se from root to shoot depends on which Se species is supplied to the plant. In plants fed with SeO_4^{-2}, Se is readily translocated to the shoot, and SeO_4^{-2} is the predominant species in the xylem sap. By contrast, in SeO_3^{-2}-treated plants, most of the Se stays in the roots, with little SeO_3^{-2} being detected in the xylem sap (Li et al. 2008). SeO_3^{-2} taken up by roots is readily converted to other forms, including selenomethionine (SeMet) and selenomethionine Se-oxide hydrate (SeOMet), but mostly into unidentified and water-insoluble forms. Thus, in general, Se translocation from root to shoot is lower in plants fed with SeO_3^{-2} than in those fed with SeO_4^{-2} as reviewed by Zhu et al. (2009). The chemical reduction from SeO_4^{2-} to SeO_3^{2-}, and further SeCys likely occurs at chloroplasts in leaves, while the production of SeMet and methylation of SeMet likely takes place in the cytosol (Lin 2011).

Therefore, it could be concluded that, plants mainly take up Se from soil solution in the form of selenate (SeO_4^{-2}), which is taken up inadvertently via sulfate transporters, and metabolized via the S assimilation pathway. In this pathway, selenate is reduced to selenite (SeO_3^{-2}), which can undergo further reduction to selenide (Se^{-2}). This may be incorporated into the organic forms, selenocysteine (SeCys), selenocystathionine (SeCysth) and selenomethionine (SeMet). Selenate is taken up by plant roots from soil solution by a active uptake, where it competes with S for uptake, both anions using a sulfate transporter in the root plasma membrane. On the other hand, organic forms of Se, such as selenomethionine, are also taken up actively by plant roots. In contrast, transport of selenite does not appear to require the use of a sulfur transporter (passive uptake).

6.9 Volatilization of Selenium by Plants

It could be defined a Se-tolerance mechanism as the ability of plants to convert Se into volatile compounds that are then released into the atmosphere. Thus, reducing their Se load is an important metabolic activity of a variety of different plant types. Rates of volatilization vary substantially between plant species and are related to a number of factors, including the concentration and chemical form of Se and of S in

the soil, as well as to time of the year. The rate of Se volatilization varies widely amongst plant species (Reilly 2006). In a laboratory study of the process in different crop species grown in solution culture, the highest rates of volatilization, between 300 and 350 μg Se m^{-2} leaf area day^{-1}, were in rice, broccoli, and cabbage, while in beet, bean, lettuce, and onion they were >15 μg m^{-2} day^{-1} (Terry et al. 1992). In trials, wetland plants showed a 50-fold variation in Se volatilization, with a low rate of 1 mg Se kg^{-1} dry weight day^{-1} attained for SeO_4^{2-}, to a higher rate of 4 mg Se kg^{-1} dry weight day^{-1} for SeO_3^{2-} in *Azzola*. The plant *Salicornia bigelovii* had a high rate of Se volatilization of 420 μg Se m^{-2} soil day^{-1}, and was between 10 and 100 times greater than other species tested; including salt grass, cord grass, cotton, *Eucalyptus* and canola (Terry and Lin 1999) as reviewed by de Filippis (2010).

It is well documented that, the ability of plants to volatilize Se is influenced by the concentration of Se around the roots and the chemical form of Se supplied. There was a direct linear relationship between an external Se concentration and internal plant tissue concentration of Se in Indian mustard supplied with SeO_4^{2-} or SeO_3^{2-} (De Souza et al. 1999). It is found that, Se volatilization was also correlated to plant tissue concentrations, and SeO_3^{2-} treated plants released 10–15 times more Se than plants supplied with SeO_4^{2-}. However, plants supplied with SeMet volatilized Se at an even higher rate; but plants supplied with DMSeP volatilized Se at the highest rate recorded (Terry et al. 1992). An important environmental factor in volatilization of Se is the concentration of SO_4^{2-} compared to SeO_4^{2-} in the soil. Se volatilization can be inhibited strongly by the presence of SO_4^{2-} in the range of 0.25–10 m*M*. Rates of volatilization decreased from 97 to 14 μg Se m^{-2} leaf area day^{-1} with the higher SO_4^{2-} supply (Zayed et al. 1998). Generally, the rate of inhibition decreases with an increase in the S:Se ratio in plant tissue. The inhibition of volatilization suggests that S compounds out compete Se compounds for the active sites of the enzymes responsible for Se volatilization. In wetlands, Se volatilization is dependent on many parameters, like Se concentration, water sediment, the plant used, microbial biomass in sediment, pH, dissolved oxygen, salinity, depth and temperature. However, the most important factors appear to be water temperature, Se concentration in roots and microbial biomass in the sediment as reviewed by de Filippis (2010).

After SeMet is synthesised it can be methylated and converted to dimethylselenide (DMSe), which is the major volatile Se compound in non-Se accumulating plants. The enzymatic steps are well known, however no detail knowledge of the enzymes, except SeMet hydrolase at the molecular level have been investigated (Bourgis et al. 1999). Plants can also volatilise Se as dimethyldiselenide (DMDSe) via oxidative and subsequent methylation with an intermediate DMSeP which is also volatile. The enzymatic and biochemical steps are also well known but no molecular biology knowledge is available (de Filippis 2010).

It is well known that, bacteria, fungi and algae can assimilate and volatilize Se independently of plants; and the rates achieved can be considerably higher than in plants. Therefore, the role of the rhizosphere microbes appeared to be somewhat specific for SeO_4^{2-} and its uptake, by producing heat labile compounds that were proteinaceous in nature; possibly the amino acid derivative o-acetylserine and

the amino acid serine which can stimulate the uptake of SeO_4^{2-} by the sulphate transporters (Zayed et al. 1998). There was no such stimulation with SeO_4^{2-} supplied plants, and indications were that the rhizosphere organisms aided in the production of organic Se compounds like SeMet, which can be converted to DMSeP and DMSe, and both of these compounds are more readily volatilized (de Filippis 2010).

Biological volatilization of Se plays an important role in the biogeochemical cycling of Se, in respect to its toxicity, remediation, deficiency, or biofortification (Lin 2011). On a global scale, biogenic volatile Se emission from the ocean to the atmosphere is on the order of $5–8 \times 10^6$ kg per year, while approximately 1.2×10^6 kg per year emits from the terrestrial ecosystems (Frankenberger and Karlson 1994). Biochemical reduction and biomethylation of Se in plants and microbes of soil-plant systems are the two primary mechanisms controlling biological Se volatilization process. The pathway of Se assimilation and volatilization in plants has been proposed by Terry et al. (2000). It is found that, biogenic volatilization of Se varies substantially under field conditions and as a biological process, Se volatilization in soil-plant systems can be significantly alternated by the presence of different plant species and soil microbes (Lin 2011).

The rate of Se volatilization significantly correlates with total and bioavailable Se concentration in the substrate and plant tissues. It is demonstrated that, in laboratory experiments, volatilization of Se from different chemical forms varies substantially with the following order of magnitude: SeMet > SeO_3^{2-} > SeO_4^{2-}, indicating that the production of DMSe from SeO_4^{2-} is less favorable energetically than from SeO_3^{2-} or SeMet (Lin 2011). It could be transformed the chemical forms of Se in plants and soil microbes, and some plant species, where microbial strains have a greater ability of bio-transforming SeO_4^{2-} to SeMet compared with others. On the other hand, dimethyl selenide is the major volatile Se compound produced by microbes and most higher plants, along with trace amounts of dimethyl diselenide (DMDSe), dimethyl selenone, methane selenol, dimethyl selenenyl sulfide and others. Otherwise, DMDSe may become dominant with Se-hyperaccumulators, such as *Astragalus bisulcatus* and *Stanleya pinnata*, because these plants metabolize SeCys to methylSeCys, and further DMDSe as reviewed by Lin (2011).

Therefore, it could be concluded that, in laboratory experiments, volatilization of Se from different chemical forms varies substantially with the following order of magnitude: SeMet > SeO_3^{2-} > SeO_4^{2-}. Biological volatilization of Se plays an important role in the biogeochemical cycling of Se, in respect to its toxicity, remediation, deficiency, or biofortification.

6.10 Biogeochemistry of Selenium

It is well documented that, Se is found in virtually all materials on earth as reviewed by McNeal and Balistrieri (1990). That means processes of distribution of Se through the environment involve a variety of physical, chemical, and biological activities including combustion of fossil fuels, volcanic activity, weathering of rocks

and soils, groundwater transport, soil leaching, plant and animal uptake and release, adsorption and desorption, chemical and biological reduction and oxidation reactions, and mineral formation; the importance of a given process is determined by the particular speciation of Se (Nrigau 1989). As well known, weathering of rocks is the major source of environmental Se and in general, limestone and sandstone contain lower concentrations of Se (<0.1 mg kg^{-1}), whereas, as reviewed by Wu (2004), shale tend to contain higher Se concentrations (0.6 mg kg^{-1}).

The biogeochemistry of Se is characterized by several reviews (e.g., Hartikainen 2005; Di Gregorio 2008; Fordyce 2013; El-Ramady et al. 2014; Sharma et al. 2015; Nancharaiah and Lens 2015; El-Ramady et al. 2015a; Gojkovic et al. 2015; Winkel et al. 2015). These biogeochemical processes have many distinct features including (1) the substitution of Se for S decreases if the oxygen fugacity increases; (2) the stable SeO_4^{2-} are rapidly oxidized in surface conditions; (3) the adsorption of SeO_4^{2-} on soil minerals is nearly quantitative (Wang and Gao 2001). The biological and environmental factors that affect the biogeochemical cycling of Se in the environment have a profound influence on its subsequent availability and toxicity to different organisms (Amweg et al. 2003). These factors include: (i) Se oxidation states, (ii) Se biotransformations between inorganic and organic forms as a result of biotic and abiotic processes, and (iii) Se bioaccumulate in aquatic food webs (Germ et al. 2007). Furthermore, Se occurs in several different oxidation states in the aquatic environment that include oxidized forms of Se, selenates (Se^{+6}) and selenites (Se^{+4}), elemental selenium (Se^0) and the reduced form of Se, selenides (Se^{-2}). About Se^0, the primary form found in sediments, has little toxicological significance for most organisms. Se^{6+} and Se^{4+} are both water soluble inorganic species, found typically in aerobic water sources. Selenite is more bioavailable and approximately 5–10 times more toxic for organisms than selenate (Lemly et al. 1993). Organic Se, bound in organic compounds such as Se amino acids, is the most bioavailable form, and is taken up by algae 1000 times more readily than inorganic forms (Amweg et al. 2003). Since traditional laboratory chronic toxicity tests rarely include realistic exposures to the diet, they are less relevant for directly assessing the toxicity of Se in natural settings (Sappington 2002). As far as Se ecotoxicology is concerned, a critical point is that chronic toxicity resulting from dietary Se uptake and food chain transfer constitutes a far greater problem than acute toxicity associated with direct water exposure. In addition, extensive biotransformations and food chain transfer make it difficult to predict Se risk based on waterborne Se concentrations alone (Fan et al. 2002) as reviewed by Germ et al. (2007).

It is well known that, plants play a unique role in recycling and delivering Se from the soil to the food chain, where plant roots take up Se from soil water in either the SeO_4^{2-} or the SeO_3^{2-} ionic forms. The concentration of Se in agricultural products and fodder depends on the content of Se in the soil and its bioavailability. Thus, availability of Se is restricted in soils and its content is relatively low as a result of reduced weathering status and acidity (Ylaranta 1985). In higher plants, metabolism of Se is closely related to that of S due to their chemical similarity. Thus, quantities in the soil solution are governed by the solubility of adsorbed forms and by the biological transformation of organic forms (Fig. 6.4; Hasanuzzaman et al. 2010).

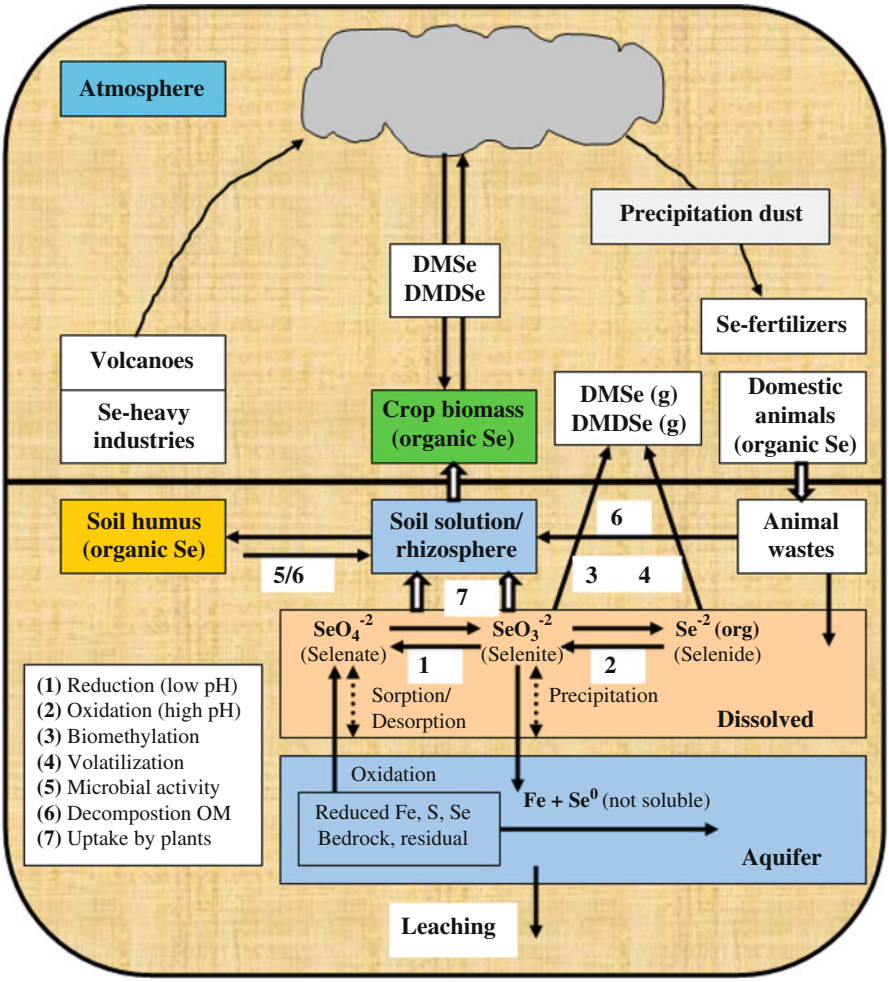

Fig. 6.4 Possible biogeochemical cycles of Se under field conditions or Se in the soil-plant-water consumer system. Plant roots take up selenate or selenite forms of Se from the soil water. The Se concentration in the soil solution depends on the solubility of the forms of Se present and the biological transformation of organic forms (Source: from Gissel-Nielsen and Gupta (2004), Hasanuzzaman et al. (2010) and Bailey et al. (2012))

On the other hand, Se speciation, mobility and bioavailability are highly affected by the presence of microorganisms in the environment. Their main influence on the bioavailability is through the control of Se oxidation state, which directly relates to the solubility of different Se compounds. Different biotic pathways have been identified nowadays by which a Se oxyanion can be reduced to Se^0 or Se(−II) (Fernández-Martínez and Charlet 2009).

Bacteria can use SeO_4^{2-} and SeO_3^{2-} as terminal electron acceptors in energy metabolism (dissimilatory reduction) and they can also reduce and incorporate Se

into organic compounds (assimilatory reduction). Other relevant processes are alkylation, dealkylation and oxidation. As it is well known, the bioavailability of a trace element is related to the factors that make it available to an organism, that is, in a form that can be transported across the organism's biological membrane (Reeder et al. 2006). However, this concept is not very precise, as a substance can be adsorbed on a colloidal particle small enough to pass through the membrane. Bioavailability and bioaccessibility rely then on a variety of entangled physico–chemical factors affecting mainly solubility of the substances, like redox potential, speciation, ionic strength, or pH. Usually, Se^0 is considered to have little toxicological significance to most organisms (Combs et al. 1996), although biological activity has been reported for elemental selenium nanoparticles (Zhang et al. 2005).

SeO_3^{2-} and SeO_4^{2-} are both water soluble inorganic species typically found in aerobic water sources. Selenite (SeO_3^{2-}) is both more bioavailable and ~5–10 times more toxic than SeO_4^{2-} (Lemly 1993). Organic Se, in the form of selenide, Se(−II), is the most bioavailable form, and it is taken up by algae 1,000 times more readily than inorganic forms (Maier et al. 1993). Thus, Se bioavailability is influenced by different factors include adsorption properties of soils, speciation in aqueous solution; sediments and aquifer substrates; mobility of the different species and solubility with respect to solid phases as reviewed by Fernández-Martínez and Charlet (2009). While, the principal source of Se for most individuals is the daily diet, intake with food and drinking water is ultimately dependent on geochemical factors. The biogeochemical Se cycle begins and ends with soil, and the chemical forms (fixed in the mineral lattice, adsorbed on the oxide surfaces, dissolved in soil solution) and concentrations of Se in soil determine its bioavailability and thus the need for dietary supplementation. Theoretically, Se exhibits a broad range of oxidation states: +6 in selenates, +4 in selenites, 0 in elemental Se, and −2 in inorganic and organic selenides. It also forms catenated species, such as volatile diselenides (RSeSeR). SeO_4^{2-}, which is weakly adsorbed on oxide surfaces and thus the most mobile Se form, can be expected to occur under high oxidative conditions (White and Dubrovsky 1994). Under low redox potential, it can be reduced to SeO_3^{2-}, which has a much higher adsorption affinity. It is strongly retained by ligand exchange on oxide surfaces, especially at low pH, which reduces its bioavailability. About volatile Se, it is lost to the atmosphere from plants or through microbial activity, but Se also returns to the soil from the atmosphere with precipitation (Hartikainen 2005).

It is well documented that, since redox conditions affect the pH of soils, inorganic Se in soils with high pH occurs mainly as selenate (Se^{6+}), which is fixed very little in the soil and is, therefore, highly available to plants. Consequently, if precipitation and leaching are low, crops will be rich in Se. On the other hand, a low pH favors the selenite form (Se^{4+}), which is fixed strongly to the soil clay particles and iron hydroxides. Consequently, soils with similar Se content will produce crops with less Se on the low pH soils than on the high pH soils. This is illustrated in Fig. 6.4, where the adsorption of selenite to clay minerals and organic matter is shown, along with a very strong fixation to iron hydroxides. Moreover, volatile Se is lost to the atmosphere through microbial activity, but Se also returns to the soil

from the atmosphere through precipitation. Thus, this leaves only a minor part of Se in the cycle to pass through the plant-animal system (Gissel-Nielsen and Gupta 2004). Furthermore, the Se concentration of plants depends on the time of sampling. The release of Se from clay and organic matter is a very slow process. Se released during the winter is available for the crops in the spring when the yield of grass is low, giving a relatively high Se concentration. However, when the growth of grass increases during the summer, less Se will be taken up and distributed over more dry matter yields, and therefore, the Se concentration drops as a result of biomass dilution (Gissel-Nielsen and Gupta 2004).

Therefore, it could be concluded that, plants play a unique role in recycling and delivering Se from the soil to the food chain, where plant roots take up Se from soil water in either the SeO_4^{2-} or the SeO_3^{2-} ionic forms. Thus, quantities of Se in the soil solution are governed by the solubility of adsorbed forms and by the biological transformation of organic forms. Moreover, Se bioavailability is influenced by different factors include adsorption properties of soils, speciation in aqueous solution; sediments and aquifer substrates; mobility of the different species and solubility with respect to solid phases.

6.11 Selenium and Its Relationship with Sulfur

As it is well known, S and Se have received little attention with respect to biotechnology based crop improvement, at least when compared with nitrogen or phosphorus. In pursuit of higher yield, better nutritional value, and quality in combination with sustainable plant management, several biotechnological approaches have attempted to improve crop plants in recent years (Khan and Hell 2008). Plant nutritional aspects may be the major reason for this lack of interest of S and Se. Furthermor, S is the least required among the six macronutrients and is often sufficiently available in soils of arable land. Its mineral fertilization is relatively affordable, mostly combined with chemically reduced nitrogen (ammonium sulfate), and even S contaminations of nitrogen and phosphate mineral fertilizers may be sufficient to support crop growth in some cases (Pasricha and Abrol 2003). On the other hand, Se still has not been identified as an essential nutrient for plants (not regarding algae) and only plays a role as a potentially deleterious component in small agricultural areas with high selenate content in soil. However, many reduced Se compounds, such as methionine and several vitamins (Hell 1997), are essential in the human diet as is selenide in a steadily increasing number of specialized enzymatic functions (Sors et al. 2005b).

Due to the physical and chemical similarities of Se and S, it could be explained the intimate association between Se and S metabolism in plants (Table 6.7). Both S and Se form part of group 16 in periodic table, with the most common valence states of S and Se being −2, 0, +2,+4, +6, with Se occurring as Se^{2-} (selenite), Se^0 (elemental selenium), $Se_2O_3^{2-}$ (thioselenate), SeO_3^{2-} (selenite), and SeO_4^{2-} (selenate), respectively. The predominant forms of S and Se available to plants are SO_4^{2-},

Table 6.7 Selected properties of selenium comparing with sulfur for some physical, chemical and biological properties

Properties or items (unit)	Sulphur (S)	Selenium (Se)
Name origin	From the Latin word *sulfur* (brimstone)	From Greek word *Selênê* (Moon)
Discovery or discoverer of essentiality (year)	von Sachs, Knop (1865)	J. Berzelius (1817)
World mine production in 2014 (metric tons)	70,400,000	2,275
Abundance in the Earth's crust	0.06–0.1 (%)	0.05 (mg kg^{-1})
Abundance or usual soil content	0.01–0.1 (%)	0.33 (mg kg^{-1})
Ranking in order of abundance in earth crust	14	69
Most important minerals	Gypsum ($CaSO_4$ $2H_2O$), Pyrite (FeS_2), Chalcopyrite ($CuFeS_2$), Galena (PbS)	Klockmanite (CuSe), Clausthalite (PbSe), Tiemannite (HgSe)
Most important sources	Iron sulfide and sulfate	Refining of lead, copper, nickel
Most important uses	Matches, gunpowder, medicines	Photoelectric cells, TV cameras
Common valence states	−2, 0, +2,+4, +6	−2, 0, +2,+4, +6
Ionic Radius (A°), where 1 Å = 100 pm	0.37	0.50
Electronegativity (according to Pauling scale)	2.58	2.55
Atomic Number	16.00	34.00
Atomic Mass (atomic mass unit)	32.06	78.96
Atomic Radius (picometres or pm)	88.00	122.00
Density at 20 °C (g cm^{-3})	2.07	4.79
Boiling point (°C)	444.60	684.90
Melting point (°C)	112.80	217.00
Crystal Structure	Orthorhombic	Hexagonal
Principal forms for plant uptake	SO_4^{-2}	SeO_4^{2-} or SeO_3^{2-}
Essentiality for animals and plants	Essential for both	Essential for animals & beneficial for plants
Critical or sufficient level in plant leaf (DW)	0.1–0.5 (%)	0.1–2.0 (mg kg^{-1})
Toxic level in plant leaf (DW, dry weight)	0.5–0.7 (%)	5.0–30 (mg kg^{-1})
Uptake by plants	Active (SO_4^{-2})	Passive (SeO_3^{2-}) and active for (SeO_4^{2-}) & SeMe
Major antagonistic elements	As, Fe, Pb, Mo, and Se	Hg, Mn, Zn, Cu, and Cd
Mobility in plant	Moderately mobile	Moderately mobile
Movement in soil	Mass flow (SO_4^{-2})	Very mobile in soil by mass flow (SeO_4^{-2})

SeMe selenomethionine

SeO_4^{2-} and SeO_3^{2-} (Sors et al. 2005a). These elements have some chemical differences, from which one can infer that some biochemical processes involving Se may be excluded from those associated with S. As observed from the periodic table, the Se atom is larger than S with a radius of 0.5 A° compared to 0.37 A°, for S. Consequently, the bond between two Se atoms is approximately one-seventh longer and one-fifth weaker than the disulfide bond (Sors et al. 2005b).

Historically, it was observed that SO_4^{2-} and SeO_4^{2-} competed for influx to plant roots (Shennan et al. 1990), and exhibited similar Michaelis constants (K_m) for high-affinity transport ($K_m = 15–20$ μM). However, when plants are supplied with mixtures of SO_4^{2-} and SeO_4^{2-}, the Se/S concentration ratio in shoot tissues is rarely identical to the Se/S concentration ratio in the rhizosphere (White et al. 2004). Indeed, there is often no correlation between the shoot Se and S concentrations of different plant species (or even ecotypes of the same species) growing in the same environment (Feist and Parker 2001), although strong correlations between shoot Se and S concentrations have been reported when the analysis is limited to Se nonaccumulator crop plants (Hurd-Karrer 1937) as reviewed by White et al. (2007).

The Se/S accumulation ratio is increased by S supply, suggesting that the sulfate transporters induced by S deficiency are more selective for SO_4^{2-} than the sulfate transporters present constitutively. Taken together, these observations suggest that several sulfate transporters, with contrasting anionic selectivities, facilitate the uptake of SO_4^{2-} and SeO_4^{2-} by plant roots, and that the complement of these is determined genetically and may be regulated by plant nutritional status. However, the structural basis of the anionic selectivity of different sulfate transporters is unknown. Following uptake by root cells, S and Se are converted to SO_4^{2-} and SeO_4^{2-}, which are then loaded into the xylem and transported to the shoot, where they are assimilated into organic compounds. Most SO_4^{2-} assimilation occurs in the shoot, and the enzymes responsible are generally encoded by extensive gene families whose products are directed to different intracellular compartments (Hawkesford 2005). An increase in the expression of genes encoding these enzymes is commonly observed during S starvation (White et al. 2007).

Selenate is accumulated in plant cells against an electrochemical potential (or gradient) by active transport driven by ATP (ATPase). SeO_4^{2-} readily competes with the uptake of SO_4^{2-}, and both anions appear to be taken-up by a number of sulphate transporters in the root plasma membrane (Abrams et al. 1990). The sulphate transporters modulate Se uptake in bacteria and yeasts, and at least two types of these transporters are also present in plants. The S/Se transporters described belong to two main classes (de Filippis 2010):

- **Transporters that have high affinity for sulphate (HAST)**: this is likely to be the primary transporter involved in sulphate uptake from the soil, and is expressed mainly in roots with a K_m for sulphate of 7–10 μM. HAST is also considered to be involved in selenate uptake; and
- **Transporters with a low affinity for sulphate (LAST)**: this secondary transporter is more likely to be involved in intercellular transport of sulphate, expressed in both the roots and shoots with a K_m for sulphate of 100 μM. LAST is also considered to be involved in selenate uptake (Cherest et al. 1997).

Therefore, it could be concluded that, physical and chemical similarities of Se and S help to explain the intimate association between Se and S metabolism in plants. Both S and Se form part of group 16 in periodic table and the Se atom is larger than S with a radius of 0.5 A° compared to 0.37 A°, for S. Several sulfate transporters, with contrasting anionic selectivities, facilitate the uptake of SO_4^{2-} and SeO_4^{2-} by plant roots, and that the complement of these is determined genetically and may be regulated by plant nutritional status.

6.12 Application of Se-Containing Fertilizers

It is well documented that, inorganic Se fertilization at a national scale has proven effective in Finland since 1984 when the incorporation of Se into all multi-element fertilizers became mandatory. Se concentrations in Finnish foods items have since risen dramatically (Ekholm et al. 2007). Because soil, climatic and cropping conditions will affect the efficiency of Se biofortification, experience gained in Finland and elsewhere may not be applicable to other regions. Another important factor to consider is that the window of Se intake from deficiency to toxicity is rather narrow, necessitating detailed studies on the efficacy of Se biofortification through fertilisation if this approach is to be adopted on a commercial scale (Broadley et al. 2010).

In controlled environment studies, growth stimulations induced by SeO_4^{2-} fertilization have been reported in ryegrass (Xue and Hartikainen 2000**;** Cartes et al. 2010), lettuce (Ríos et al. 2009), potato (Turakainen et al. 2004), arabidopsis (White et al. 2004) and soybean (Djanaguiraman et al. 2005). Growth or yield stimulation might be due to selenate-induced antioxidant production, such as ascorbate and glutathione (GSH) peroxidases which detoxify H_2O_2 and improve stress resistance (Ríos et al. 2009). Selenate-induced upregulation of sulphate-transport and assimilation is also likely to occur (Van Hoewyk et al. 2008). Therefore, whilst Se is probably beneficial to vascular plants, no increases in yield or stress resistance have been reported in Se-enriched field-grown crops to our knowledge Broadley et al. (2010).

Different ways of raising Se concentrations have been investigated over the years to overcome the naturally low Se content of crops in some areas, and this subject has been discussed in a number of reviews (Gissel-Nielsen and Gupta 2004). It is well documented that, in 1984 in Finland, Se-containing fertilizers came into general use. Sodium selenate is added to the fertilizer slurry in order to obtain a uniform Se concentration in the granules during the manufacturing process (Hartikainen 2005). Since, the commencement of Se fertilization, its impact has been regularly monitored by analyzing Se in agricultural soils, water and plants, all types of feeds, plant and animal foods, and human sera, the results of these studies appearing in numerous publications such as Ekholm et al. (1994) and Eurola et al. (2003). The Se level in fertilizers has been adjusted on the basis of these findings. The initial level of 16 mg kg^{-1} used for cereal crop fertilizers was reduced to

6 mg kg^{-1} (in 1991). Since this measure had an adverse effect on the crop quality, the Se concentration was raised to the present level of 10 mg kg^{-1} (in 1998). Fertilization induced drastic changes in the Se concentration in agricultural products. For example, in spring cereals the increase was generally 20–30 fold during the first years of supplementation. The Se level in 2005 is about 13 times higher than in the mid- 1970s. In winter cereals, the Se levels increased first 2–5 fold to 0.07 mg kg^{-1} dry weight in 1990, the present level being about 10–12 times higher than that in the 1970s as reviewed by Hartikainen (2005).

Among the different ways of increasing Se concentration in crops, application to the soil and foliar application of SeO_4^{2-} or SeO_3^{2-} are those of practical importance. Pre-sowing treatment of barley seeds with selenite, and selenate treatment of soybean seeds have also been tested and more details about application of Se-containing fertilizers as follows:

6.12.1 Soil Application

The first studies on soil application of Se in the 1960s involved the spraying of selenite or selenate solutions onto the soil surface. These relatively simple experiments from New Zealand and USA showed promise in increasing Se content by such treatments, and they have been followed by comprehensive studies worldwide (Gissel-Nielsen and Gupta 2004). On the other hand, a large-scale field experiment involving an annual addition of 60 and 120 g Se ha^{-1} for 5 years (as Na_2SeO_3) incorporated into a NPK compound fertilizer was carried out in 21 farms covering the common Danish soil types. The soils differed in their content of organic matter, clay, previous cropping, etc., but were all glacial deposit mineral soils with a pH of 5–7. The 120 g Se treatment raised the native Se concentration of 0.02–0.04 mg kg^{-1} of wheat, barley, rye grass, clover, and fodder beets (0.09 mg kg^{-1} in the beet top) to 0.08–0.13 mg Se kg^{-1} that is considered a sufficient and safe level for animal nutrition (Gissel-Nielsen and Gupta 2004). It is reported that, field trials were conducted at two South Australian sites, Charlick and Minnipa, in 2002, where Se was applied as sodium selenate at rates from 0 to 120 g ha^{-1} Se either to the soil at seeding or as a foliar spray after flowering (Table 6.8; Lyons et al. 2005).

In areas with low soil Se, applications of Na_2SeO_3 to soils or as a foliage spray are proposed for correcting Se nutritional deficiencies. However, in view of the toxic properties of Se salts, these practices should be carefully controlled and the addition of Se to soil, at 10 g ha^{-1} affected its contents in grains of barley and oats, from 0.019 to 0.26 mg kg^{-1} and from 0.032 to 0.44 mg kg^{-1}, respectively (Table 6.9; Gupta and Gupta 2000).

Generally, soil applications are recommended, especially for crops prone to late-season moisture or heat stress (Lyons et al. 2005), although foliar applications can also be effective (Ducsay and Ložek 2006) due to the mobility of Se in plants (Broadley et al. 2010).

Table 6.8 Comparison of surface soil characteristics at two South Australian sites (Charlick and Minnipa) and effects of soil and foliar applications of Se as selenate on wheat grain Se concentration and yield in 2002

Property or item	Charlick location	Minnipa location
Texture	Clay-loam soil	Calcareous sandy-loam soil
Soil EC (dS m^{-1})	0.05	0.11
pH (H_2O)	6.6	8.6
Organic Carbon (%)	13.3	7.8
Total S in soil (mg kg^{-1})	4.4	2.4
Total Se in soil (μg kg^{-1})	<200	<200

Effects of soil and foliar applications of Se as selenate on wheat grain Se concentration and yield

	Charlick location				Minnipa location			
	Soil application		Foliar application		Soil application		Foliar application	
Se rate (g ha^{-1})	Se (μg kg^{-1})	Yield (t ha^{-1})	Se (μg kg^{-1})	Yield (t ha^{-1})	Se (μg kg^{-1})	Yield (t ha^{-1})	Se (μg kg^{-1})	Yield (t ha^{-1})
0	57	1.89	65	1.89	600	1.41	650	1.44
4	205	1.82	145	1.80	1050	1.48	888	1.43
12	355	1.82	354	1.80	940	1.41	1130	1.48
40	2125	1.83	597	1.76	2730	1.40	1780	1.42
120	8325	1.79	1240	1.88	11,950	1.46	3580	1.49

Source: Lyons et al. (2005)
Total soil Se determined by inductively coupled plasma mass spectrometry (ICP-MS) after digestion with nitric/perchloric acid
EC electrical conductivity

6.12.2 Foliar Application

It is well documented that, the advantage of foliar application compared with soil fertilization with Se for biofortification is that losses caused by soil adsorption, chemical or microbiologically mediated conversions or losses are not likely to occur. Furthermore, the direct foliar uptake route ensures a high degree of assimilation by the plant, which is beneficial when only a small amount of a costly enriched isotope is available for biofortification (Kápolna et al. 2012). In order to avoid the complication of soil effect on the availability of added Se, foliar application was considered back in the mid-1960s. Experiments in New Zealand had shown that SeO_3^{2-} sprayed onto pasture plants had a much greater effect than when it was sprayed onto the soil surface. In 1972–1973 foliar applications of up to 50 g Se ha^{-1} as selenite was tested in field experiments with barley (Table 6.9; Gissel-Nielsen and Gupta 2004).

As well known that, foliar application, performed manually only on plants, is the most appropriate way to add Se, since contamination of the soil is minimal. Foliar application to barley at 10 and 20 g Se ha^{-1}, as sodium selenate, increased the Se contents of barley grain and straw and red clover forage (Kabata-Pendias 2011).

Table 6.9 Effect of soil applied selenate fertilizers on Se concentration in a variety of crops under field conditions comparing with spraying of sodium selenate under micro-farm systems

Crop	Applied Se rate (g ha^{-1})	Se concentration in crop (mg Se kg^{-1})	References
Field conditions			
Pasture herbage	140	11.6	Archer (1983)
Timothy tops	40	1.03	Gupta and Winter (1989a, b)
Wheat Flour	10	0.12	Stephen et al. (1989)
Alfalfa tops	40	0.79	Gupta et al. (1993)
Barley grain	10	0.45	Gupta and MacLeod (1994)
Oat grain	10	0.56	Gupta and MacLeod (1994)
Soybean seeds	10	1.47	Gupta and MacLeod (1994)
Wheat grain, soil application[a]	120	8.325	Lyons et al. (2005)
Wheat grain, foliar application	120	1.240	Lyons et al. (2005)
Wheat grain, soil application[b]	120	2.730	Lyons et al. (2005)
Wheat grain, foliar application	120	11.950	Lyons et al. (2005)
Wheat grain	20	0.266	Stroud et al. (2010b)
Wheat straw	20	0.104	Stroud et al. (2010b)
Micro-farm conditions			
Wheat (roots + seeds)	2 mg Se L^{-1}	13.30	El-Ramady et al. (2015)
Wheat (shoots)	2 mg Se L^{-1}	18.98	
Millet (whole plant)	2 mg Se L^{-1}	41.28	
Radish (whole plant)	2 mg Se L^{-1}	69.20	
Alfalfa (whole plant)	2 mg Se L^{-1}	89.58	

Complied from Gissel-Nielsen and Gupta (2004), Lyons et al. (2005) and Stroud et al. (2010b)
[a]Charlick and [b]Minnipa location, respectively in Australia

Foliar application of Se on potatoes resulted in its increased level in tubers, from 0.465 mg kg^{-1} in control up to 1068 mg kg^{-1} in experimental plants (Hlušek et al. 2006). It is observed an increase of Se contents of winter wheat grains after the Se foliar application (Se doses 10 and 20 g ha^{-1}) from 0.094 to 0.192 mg kg^{-1} and concluded that the Se dose 10 g ha^{-1} is sufficient for reaching the required Se content in wheat grains (Duscay et al. 2006). Enriched Se content of garlic has often been reported as an important dietary supplement (Ip et al. 2000). It is observed commonly increased levels of Se in Brazil nuts (*Bertholletia excelsa*) may highly vary, depending on the growth conditions. For instance, nuts that were grown in soils derived from Cretaceous sediments enriched with Se by volcano emissions contain more Se than those grown in lower Se soils (Kabata-Pendias 2011). It could be concluded that, a stimulatory effect of foliar application of Se on growth has been reported for ryegrass (Hartikainen et al. 2000), lettuce (Xue et al. 2001), green tea leaves (Hu et al. 2003), potato (Turakainen et al. 2004) and pumpkins (*Cucurbita pepo*) (Germ et al. 2005). Se affected plant growth promotion might be the result of increased starch accumulation in chloroplasts (Pennanen et al. 2002) and that protected cell content (Xue et al. 2001).

In potato plant, Se increased carbohydrate accumulation in the young upper leaves and in stolons, roots and tubers at maturity. It could not be explained, however, by increased production of photoassimilates as net photosynthesis did not differ among Se treatments. Increased yield of Se treated plants suggested that Se may enhance the allocation of photoassimilates for tuber growth, acting as a strong sink for both Se and for carbohydrates. It was also observed that, Se improves the processing and storage quality of potato tubers (Turakainen 2007). The positive impact of Se on the yield of potato plants could be related to its antioxidative effect in delaying senescence as reviewed by Hasanuzzaman et al. (2010).

6.12.3 Seeds Treatment with Selenium

It could be concluded that, among the three main methods of Se enrichment, seed treatment has been researched the least. Field experiments have shown that seed treatment with Se offers great promise for enriching soybeans (*Glycine max* Merr L.), which are rather high accumulators of Se. Recent data showed that at similar rates of seed-applied Se, soybean grain contained higher Se than a number of other feed and food crops (Gissel-Nielsen and Gupta 2004). The effects of various rates of seed-applied Se for two soybean cultivars have been tested. The results indicated that increasing Se from 10 to 100 g ha^{-1} proportionately increased the Se concentration in the grain. Therefore, grains containing up to 7.5 mg Se kg^{-1} obtained at an application rate of 100 g Se ha^{-1} should not pose a toxicity hazard. Due to the higher capacity of soybeans to mobilize Se into the grain, seed treatment with Se offers an alternative for producing crops with the desired Se levels (Gissel-Nielsen and Gupta 2004).

Therefore, it could be concluded that, considering all previous experiments, the overall conclusion is that foliar application of about 5 g Se ha^{-1} as SeO_3^{2-} or SeO_4^{2-}, soil fertilization using about 10 g Se ha^{-1} as SeO_4^{2-}, or about 120 g Se ha^{-1} as SeO_3^{2-}, and 10 g SeO_4^{2-} Se ha^{-1} as seed treatment are effective annual treatments for raising the Se content of annual crops to a desirable level for human and animal nutrition. The effect of Se is enhanced when it is used with a detergent for foliar application. For all treatments, the effect is greatest when carried out on a well-established crop. The residual effect of these treatments is very small and a somewhat higher amount is needed for pasture crops, but this gives a residual effect lasting 2–3 years.

6.13 Selenium in Edible Plants

It is well known that, Se concentrations in plant foods, such as rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.), can vary greatly between countries and regions; thus, to avoid Se deficiency and toxicity, it is important to monitor and optimize crop Se concentrations (Zhu et al. 2009). In 2009, a global survey of Se in rice purchased from retail outlets, it was highlighted that Se levels in major rice-producing

and -consuming countries, such as Egypt, China and Thailand, are low, whereas they were higher in rice from the USA and India. The concentration of Se in wheat also shows large regional variation (Hawkesford and Zhao 2007). Where both rice and wheat are produced (e.g. India, China and Egypt), the Se concentrations of wheat and rice tend to be similar. Offsetting regions with inadequate Se by sourcing Se-rich grain is a practical solution to curtail the problem, but further characterization of both rice and wheat grain Se concentrations is needed (Williams et al. 2009).

As mentioned before, the Se content of crops received recently much attention because of its importance in the food chain. Thus, most data that are available are for food and fodder plants (Table 6.7). Generally, mean concentrations of Se in grains are higher in countries from arid climates than in countries from humid climates. The common range of mean Se levels varies from 0.34 to 0.92 mg kg^{-1} for countries with high Se levels in grains, and from 0.014 to 0.042 mg kg^{-1} for countries with low Se levels in grains. These variations do not indicate a significant impact of climatic conditions, because several other factors also control the Se absorption by plants (Kabata-Pendias 2011). It is found that, the environmental effect on the Se concentration in broccoli was about 10 times larger than genotype impact (Farnham et al. 2007). A variation in the Se uptake by various species of the same plant (*Astragalus*) is described by Somer and Caliskan (2007). It is also found that, most plants contain rather low Se levels, around 25 μg kg^{-1}, and rarely exceed 100 μg kg^{-1}. However, some plants reveal a great capability to accumulate Se and they may concentrate Se to extremely high levels that may be toxic to humans and animals. As mentioned before, although Se is not an essential element for plants, with some exceptions, it is being added to soil to ensure that both food and feed products contain adequate amounts for the dietary needs as reviewed by Kabata-Pendias (2011).

It could be followed the concentration of Se in edible plants within different Se soil content. There are three categories could be distinuished as follows:

1. ***Crops grown on low-Se soils***

As well known, in some regions of the world where the bioavailability of soil Se is low or declining like rice paddies, there is a potential risk to animal and human health due to Se deficiency (Zhang et al. 2006). Thus, increased dietary Se could be accomplished in rice through enhancing Se uptake into plants through the use of Se-fertilizers (Chen et al. 2002), but water management practices in rice paddy fields can cause changes in the soil redox and pH conditions that can change the bioavailability of soil Se (Premarathna et al. 2010). Low-Se agricultural soils have been reported in different countries, where soil Se levels are low, or the element occurs in a form that is not readily available for absorption by the roots, uptake by crops will be limited. Until steps were taken to improve soil levels by addition of sodium selenite to fertilizers, grazing on such lands resulted in Se deficiency diseases in sheep and cattle (Reilly 2006). On the other hand, low Se-levels in plant foods used directly for human consumption are implicated in serious health problems in areas of Se-deficient soils in central and western China and neighboring regions. There, the two best-known selenium deficiency-related conditions in

Table 6.10 The variation of Se in soil and maize comparing with Se concentrations in stream water during the last past 40 years in Enshi area, China (All data on basis of dry weight)

Concentration of Se in soil (mg kg^{-1}) Mean (min-max)	Se contentration (mg kg^{-1}) Mean (min-max)		Se in stream water (μg L^{-1}) Mean (min-max)[a]	Time in year	References
	Maize	Rice*			
9.68 (0.08–45.5)	8.66 (0.5–44.0)	3.96	56.0 (0–158)	1966	Yang et al. (1981)
3.45 (1.92–4.98)	14.07	–	–	1987	Mao et al. (1997)
5.48 (0–11.89)	4.17 (0.77–7.57)	–	–	1989	Zheng et al. (1993)
4.06 (2.82–5.30)	6.47 (2.18–10.7)	–	–	1992	Zhu and Zheng (2001)
4.99 (2.61–7.37)	1.38 (0.182–5.6)	1.26	40.4	1996	Fordyce et al. (2000)
4.75 (0–12.18)	1.48 (0.07–2.9)	–	58.4 (41.6–75.2)	1999	Zhu et al. (2008)
27.81 (3.76–79.08)	0.37 (0–0.79)	1.04	52.6 (15.1–192.7)	2010	Huang et al. (2013)

Yuan et al. (2012) and Huang et al.(2013)
Time is coresponding to sampling time
[a] Yuan et al. (2012)

humans—Keshan and Kashin-Beck diseases—are endemic (Yang 1991). Dietary intakes of Se as low as 7 μg day^{-1} occur, 10–20 times less than intakes in many other countries in which Se responsive diseases in humans do not normally occur as reviewed by Reilly (2006).

2. ***Crops grown on adequate-Se soils***

It is reported that, Se content of food plants grown on soils with an average level of Se in available form which is approximately 0.5–1.0 μg g^{-1}, according to what is known as the Wells rating scale (Wells 1967), will generally be in a relatively narrow range of approximately 0.1–1.0 μg kg^{-1}. This range will be varied somewhat between countries, depending on local soil conditions. For instance, in Australia, average Se levels in wheat of 0.15 μg g^{-1} have been reported by Tinggi et al. (1992), compared to North American levels of 0.33 μg g^{-1} (Ferretti and Levander 1974). In vegetables and fruits, Australian figures were 0.001–0.022 μg g^{-1}, somewhat lower than American findings of 0.004–0.063 μg g^{-1} in similar foods (Schubert et al. 1987) as reviewed by Reilly (2006).

3. ***Crops grown on high-Se soils***

It is well documented that, excessive soil Se concentrations (>3 mg kg^{-1}) occur in areas of North America, China and Ireland, whereas deficient soil Se concentrations (<0.125 mg kg^{-1}) occur in Siberia, New Zealand and the Keshan area of China (Table 6.10; Broadley et al. 2006). The most important factors in soil Se supply to

plants include the Se content of parent rocks (Spadoni et al. 2007), which controls soil Se concentrations and plant availability as Se bioavailability generally decreases with decreasing pH and the increased content of organic matter, clay minerals and iron hydroxides (Gissel-Nielsen et al. 1984) as reviewed by Stroud et al. (2010a). Under certain conditions, cereals and other farm crops grown on Se-rich soils may, accumulate high levels of Se and even pose a threat of toxicity to consumers. It is found that, samples of cereals from seleniferous regions of South Dakota in the USA contain up to 30 μg Se g^{-1} (Byers 1936). Whereas, in seleniferous regions of Enshi County in China, rice containing 2.5 μg g^{-1}, maize flour 7.5 μg g^{-1}, and leafy vegetables up to 7.6 μg g^{-1} of Se as reported by Yang et al. (1989). However, even on seleniferous soil, not all crops will take up toxic levels of Se, whereas the average Se content of wheat plants sampled from an area of high-Se soil in Montana, USA, was 1.9 μg g^{-1}, with a maximum of 8 μg g^{-1}, even though there were a number of wild accumulator plants containing more than 1,000 μg Se g^{-1} (UCAIC 1988). Even in the Chinese study of foods grown on high-Se soil, several of the plants analyzed had less than 1 μg Se g^{-1} (Yang et al. 1989) as reviewed by Reilly (2006).

Therefore, It could be concluded that, Se concentrations in plant foods can vary greatly between countries and regions; thus, to avoid Se deficiency and toxicity, it is important to monitor and optimize crop Se concentrations. The Se content of crops received recently much attention because of its importance in the food chain, where most data that are available are for food and fodder plants. Generally, mean concentrations of Se in grains are higher in countries from arid climates than in countries from humid climates.

6.14 Red Elemental Selenium Nanoparticles in Higher Plants

It is well decumented that, particles of elemental Se (Se^0) formed from some bacterial strains (such as *Lactobacillus* sp. and *Pseudomonas* sp.), and the redox system of glutathione or ascorbate and selenite has a very low bioavailability (<5 %). It is also observed that red elemental Se, formed in the redox system of selenite and glutathione or other reducing agents, was unstable, and could further aggregate into gray and black Se^0 if there were no controlling factors. Whereas, protein presented in the redox system could affect the aggregation of red elemental Se and the size of red elemental Se formed was dependent on the amount of protein in the redox system as reviewed by El-Ramady et al. (2015b). Furthermore, Se^0 has a very low biological availability and therefore a low toxicity (Hunter and Manter 2009).

It is emerged elemental Se nanoparticles as a novel Se source with the advantage of reduced risk of Se toxicity, where the Se nanoparticle solution may be added to functional foods, the final products of which may be subjected to heat during processing (Zhang et al. 2012). An important feature of nanoparticles is their high surface to volume ratio. As the nanosize decreases, surface energy, surface atomicity,

and surface binding energy all increase quickly; as a result, the surface atoms become more prone to diffusion, with an inherent tendency to combine with other atoms for energy dissipation. Hence, the thermodynamic stability and properties of nanoparticles should be affected by particle size and heat treatment (Zhang 2009). Otherwise, the thermal stability of Se nanoparticles as a type of nanoscale biomaterial remains unknown. Thus, the thermostability of Se nanoparticles is size-dependent, smaller Se nanoparticles being more resistant than larger Se nanoparticles to transformation into nanorods during heat treatment as reviewed by Zhang et al. (2012).

In a number of experiments, soil application of Se^0 was tested. This was a slow-release form of Se and was intended as a treatment having a long-term effect. However, Se^0 has to be oxidized to Se^{4+} or Se^{6+} before it becomes available to plants, and many environmental factors, such as pH, humidity, microorganisms etc., have an impact on the rate of oxidation. Consequently, it is very difficult to predict the effect of soil supplementation using elemental Se on the Se concentration in plants (Gissel-Nielsen and Gupta 2004).

It could be defined nano elemental selenium (Nano-Se), which is bright red, highly stable and of nano size in the redox state of zero (Se^0), is nanoparticles manufactured for use in nutritional supplements and developed for applications in medical therapy (Gao et al. 2002). It has been reported that Nano-Se have a higher efficiency in upregulating selenoenzymes and exhibit less toxicity than selenite (Wang et al. 2007). Furthermore, nano-materials exhibit novel properties, such as great specific surface area, high surface activity, a lot of surface active centers and high catalytic efficiency (Gao and Hiroshi 2005). Due to the advantage of size effect and high surface reactivity, nanoparticle has been already used in pharmaceutical applications to increasing the bioavailability of drugs and targeting therapeutic agents to particular organs (Davda and Labhasetwar 2002). It has been reported that nanoparticle showed new characteristics of transport and uptake and exhibited higher absorption efficiencies (Liao et al. 2010). However, there is little data on intestinal absorption and Se retention of Nano-Se (Hu et al. 2012).

It is well known that, Se^0 is rare, occurring mostly in sedimentary rocks (White et al. 2004). From three allotropes of Se^0 the gray and the black one are biologically inert, which may due to their insolubility (Huang et al. 2003). The red allotrope has been produced by several kinds of bacteria from selenite (for example, Prokisch et al. 2008). It was found that the red Se^0 particles of nano-size scale have good free radical scavenging effects on different free radicals *in vitro* (Huang et al. 2003). The nano-sized red nano-Se was shown lower acute toxicity as compared with selenite in mice; however bioavailability to selenite was similar in terms of inducing selenoenzymes in cultured cells and in Se-deficient rats (Zhang et al. 2001). In intact plants or in tissue cultures biological/biochemical effects of nanoSe have not been studied (Domokos-Szabolcsy et al. 2012).

On the other hand, results from field trials in Oregon in the use of Se amendment of fertilizers to prevent Se deficiency in plants and subsequently in animals consuming those plants have shown the treatment to be effective and safe. SeO_4^{2-} has been

shown to be more effective than SeO_3^{2-}. One trial with Se^0 has shown it to be only sparingly available and its use is not recommended. The level of 5–10 g per acre of SeO_4^{2-} has given good results and use of higher levels may bring about a transitory excess, which should be avoided (Hathaway et al. 2004).

As mentioned before, the solubility of Se in the soil and Se availability to plants depends on Se speciation, where Se speciation in the soil is mainly determined by the pH and redox conditions. SeO_4^{2-} species are soluble and are the major form of Se in aerobic soils, whereas a large fraction of Se in suboxic soils may occur as SeO_3^{2-} species (El-Rashidi et al. 1989). Se^0 is reported as a transitory compound in reduced soils, whereas SeO_3^{2-} reduces to selenide (Se^{2-}) species under acidic pH conditions (El-Rashidi et al. 1989). The potential availability and partitioning of Se added to three soils as Se^0, sodium selenite (SeO_3^{2-}), and sodium selenate (SeO_4^{2-}) were measured by isotopic dilution using either $^{75}SeO_3^{2-}$ or $^{75}SeO_4^{2-}$ (Premarathna et al. 2010). The soils were kept either submerged or at 80 % water-holding capacity for either 60 d. The availability of Se^0 as measured by concentrations of labile Se species was low due to limited oxidation to SeO_3^{2-} or SeO_4^{2-}. Therefore, Se^0 is not suitable for preplant Se fertilization of lowland rice (*Oryza sativa* L.) because it is not readily oxidized. In the submerged soils, concentrations of labile SeO_3^{2-} and SeO_4^{2-} were also low, with >80 % of the Se added as either SeO_3^{2-} or SeO_4^{2-} being fixed into nonlabile pools, probably through reduction to Se^0. Rates of oxidation of Se^0 will play a critical role in determining whether reduced Se^0 formed in submerged soils after fertilization will contribute to plant Se uptake through oxidation either during field drainage before harvest or in the rice rhizosphere (Table 6.11; Premarathna et al. 2010).

It is clearly indicated that, red nanoSe was taken up by tobacco (*Nicotinia tabacum* L.) callus cultures and rooted tobacco plantlets. The roots of regenerated plantlets accumulated Se in very high concentrations (2947 mg kg^{-1} DW) from the medium containing 530 μM nanoSe. The biological effects of nanoSe were different from the selenate ion (SeO_3^{2-}) in plant tissue culture. It is foulf that, concentration range of nano-Se 265–530 μM stimulated the organogenesis and the growth of root system significantly (~40 %), while selenate did not show these effects at any concentration moreover inhibited both callus growth and root regeneration totally in 265–530 μM concentrations (Table 6.12; Domokos-Szabolcsy et al. 2012) (Fig. 6.5).

It could be concluded that, there are major differences between biological effect of SeO_4^{2-} and nanoSe in tobacco tissue culture. The red elemental nanoSe in 265–532 μM range stimulated the callus initiation and microshoot formation on callus surface as well as root regeneration. Whereas the same previous concentration range (265–532 μM SeO_4^{2-}) inhibited both callus and root formation. The reason of these results can derive from the nature of two Se forms (Domokos-Szabolcsy et al. 2012). The selenate ion can get into plant tissue and in excess as a pro-oxidant can damage directly and/or indirectly the RC growth and regeneration of explants. That means, from the elemental Se-nanoshperes SeO_3^{2-} and selenide (selenide is a product of reduction while selenate is a product of oxidation) ions are gradually released

Table 6.11 Selected physical and chemical properties of the soils used after 60 days at field capacity or submerged conditions from rice-growing regions in Sri Lanka from 0 to 25-cm depth

Property or item	Mahailluppallama soil (MI)	Kiribath kumbura soil (KK)	Benthota soil (BT)
Texture	Sandy clay	Sandy clay	Sandy loam
FAO soil classification	Eutric Gleysol	Eutric Gleysol	Thionic Histosol
Soil drainage status	Poorly drained soil	Poorly-very poorly drained	Poorly drained soil
pH (water)	5.9	5.5	5.4
CEC ($cmol_c\ kg^{-1}$)	8.8	8–10	100–200
Organic Carbon (%)	0.7	1.0	4.3
Total Se ($\mu g\ kg^{-1}$)	215	395	118

Average concentrations of Se species in solution ($\mu g\ L^{-1}$) at field capacity (FC) and at submerged conditions

		At field capacity (FC)		At submerged conditions	
Soil/Added Se species		SeO_3^{2-} concentration	SeO_4^{2-} concent.	SeO_3^{2-} concent.	SeO_4^{2-} concent.
KI	SeO_3^{2-} (1 mg kg^{-1})	0.6	26.3	1.1	0.9
	SeO_4^{2-} (1 mg kg^{-1})	0.1	19.9	0.2	5.3
	Se^0 (1 mg kg^{-1})	2.2	3.2	0.2	0.2
KK	SeO_3^{2-} (1 mg kg^{-1})	3.2	13.3	0.3	1.8
	SeO_4^{2-} (1 mg kg^{-1})	0.9	19.3	0.3	2.7
	Se^0 (1 mg kg^{-1})	0.6	1.5	0.2	0.2
BT	SeO_3^{2-} (1 mg kg^{-1})	6.5	7.4	0.2	0.2
	SeO_4^{2-} (1 mg kg^{-1})	0.5	34.8	0.2	0.2
	Se^0 (1 mg kg^{-1})	0.9	0.3	0.2	0.2

The soils in this study were spiked with individual species at 1 mg kg^{-1} (equivalent to 2.94 kg ha^{-1})
CEC Cation exchange capacity

Table 6.12 Total Se content of *in vitro* explants regenerating calli (RC) at various nanoSe/selenate treatment levels (results based on dry weight)

Treatments	Total Se (mg kg^{-1})		Number of microshoots/RC		Shooty part FW (g)		Callus part FW (g)	
(mg kg^{-1})	Selenate	NanoSe	Selenate	NanoSe	Selenate	NanoSe	Selenate	NanoSe
0.0	0.23	0.23	6.7	6.7	0.76	0.76	0.93	0.93
0.1	3.88	0.71	9.2	7.1	0.87	0.48	2.36	0.93
1.0	16.0	3.05	9.1	7.7	1.19	0.94	1.45	1.47
10.0	300	40.9	10.8	9.9	1.14	1.23	1.11	1.17
50.0	n.g[a]	164	n.g[a]	10.6	n.g[a]	1.11	n.g[a]	1.52
100.0	n.g	391	n.g[a]	11.0	n.g[a]	1.71	n.g[a]	0.58

Source: Domokos-Szabolcsy et al. (2012)
Average number of microshoots and fresh mass of shooty and callus parts of RC at various nanoSe/selenate concentrations, results based on fresh weight
[a]n.g, No growth, that means, the plant materials were not grown in that concentrations of selenate

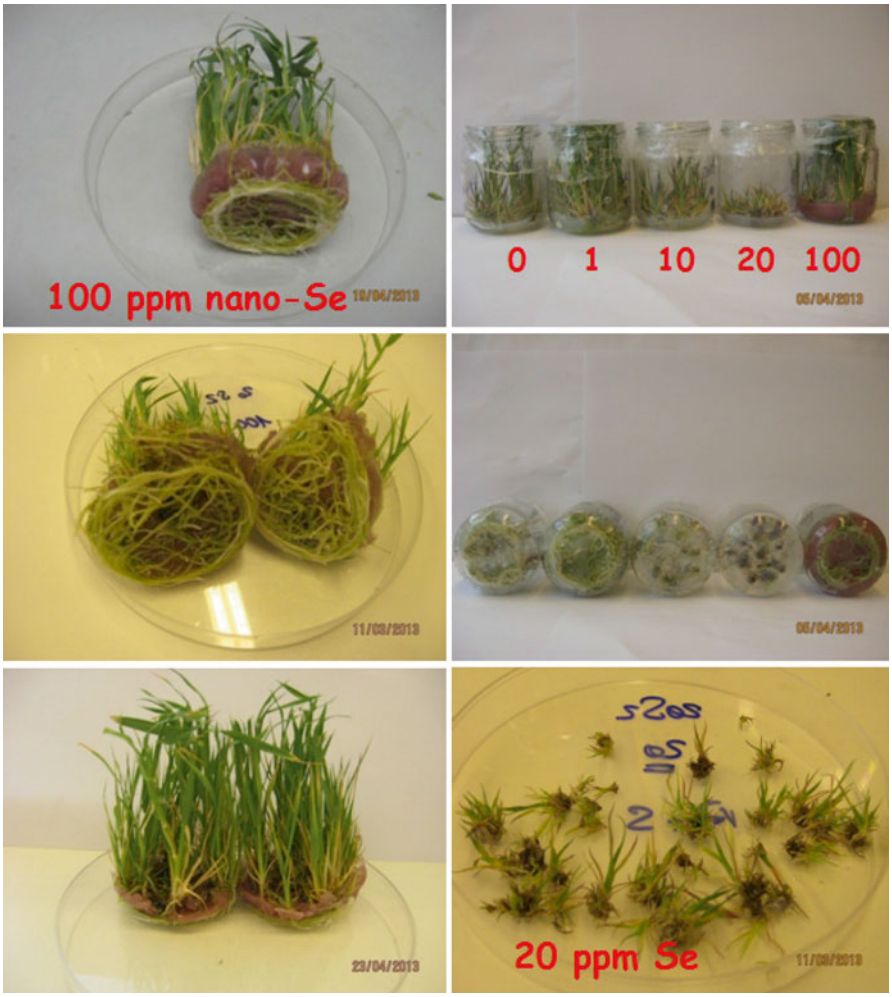

Fig. 6.5 The comparison between the effects of different concentration of Se forms as selenate and Se-nano (from 0, 1, 10, and 20 mg Se kg^{-1}, and 100 ppm nano-Se, from *left* to *right* respectively), where the distinguished effect of nano-Se on rooting of giant reed (Hungarian ecotype 20SZ) in solid media can be noticed (Photos by H. El-Ramady; Domokos-Szabolcsy et al. 2014)

providing steady but very low concentrations, and generate specific physiological effects such as stimulation of organogenesis, shoot and root growth in tobacco cultures. The nanoSe in 265–532 μM concentration stimulated not only the root initiation but also root elongation and biomass production. Therefore, SeO_4^{2-} uptake is too efficient, the steady but very low concentration cannot be generated in the plant tissues, consequently the long-term physiological effect of nanoSe cannot be reproduced. Like the Se, other beneficial elements could show differences in plant biological effects depending on their ionic and/or elemental forms (Domokos-Szabolcsy et al. 2012).

Therefore, it could be concluded that, nano elemental Se has a very special characterization, where Nano-Se, which is bright red, highly stable and of nano size in the redox state of zero (Se^0), is nanoparticles manufactured for use in nutritional supplements and developed for applications in medical therapy. It has been reported that Nano-Se have a higher efficiency in upregulating selenoenzymes and exhibit less toxicity than selenite. There is still open question that, the biological effects of this form of Se in higher plants.

6.15 Conclusions

Selenium, the moon goddess 'Selene', appears to have conferred not only her name, but also her nature on this micronutrient. The facts of Se is intriguing, enigmatic and challenging (even capricious) for researchers. Seleniferous soils are potentially useful in their use, but the soils need to be better identified and field testing needs to be done before they may be considered potentially usable for an intense agricultural system of farming. World selenium resources need to be managed so that this non-renewable vulnerable resource is not squandered. Se uptake, mobilization and assimilation are quite well understood and are similar to sulphur, however there are some steps not well understood, especially enzymatic and non-enzymatic steps leading to the reduction to selenide. Se hyperaccumulating plants do have differences in uptake and sequestration of Se which require more investigations, and essentiality of Se to higher plants also needs to be resolved. Growth potential of Se plants as agricultural crops for biomass production and identification of the chemical species of Se present and their quantification in plants is necessary for any use in health supplementation. Plant species that grow solely on seleniferous soils often have extremely high tissue Se concentrations and are termed "Se accumulator plants", whereas plant species that colonize both seleniferous and non-seleniferous soils generally have lower tissue Se concentrations and are termed "Se-indicator" plants. Plant species that cannot tolerate high tissue Se concentrations are termed "Se nonaccumulator" plants. It is thought that differences in Se metabolism, in particular the ability to exclude SeCys from proteins, could account for the contrasting abilities of plant species to tolerate and accumulate high tissue Se concentrations. Plant roots take up Se from the soil solution as selenate, selenite, and organoselenium compounds. Sulfate and selenate compete for uptake through sulfate transporters in the plasma membrane of root cells and, following uptake, Se is distributed and assimilated within the plant through the S transport and assimilation pathways. The preferential uptake and sequestration of Se by hyperaccumulating plant species suggests that there are several aspects relating to Se essentiality and toxicity in plants that remain unresolved. It is proposed that future research should continue to be dedicated to the search of essential Se-containing proteins and to elucidate how hyperaccumulating plants are able to tolerate high levels of Se in their tissues while circumventing toxicity. The complete understanding of Se and S metabolism in plants requires that more detailed biochemical studies and Se/S flux analyses be conducted. Molecular studies and the overexpression of genes encoding proteins involved in the uptake, transport and

assimilation of both S and Se will expand our understanding of the close relationship between these two elements and may elucidate key biochemical differences between Se hyperaccumulators and non-accumulators. In addition, such research will facilitate future strategies for genetic engineering of Se accumulating plants used for environmental restoration and human health applications. Controversy exists over the question of whether Se is an essential plant micronutrient. On a cautionary note, the appropriate concentration of exogenous Se is still a matter of intensive research. Complete elucidation of the role of Se as well as detailed protective mechanisms would be helpful for developing stress tolerance in plants.

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Chapter 7
Aromatic Amines Sources, Environmental Impact and Remediation

Luciana Pereira, Pijush Kanti Mondal, and Madalena Alves

Contents

Abstract Aromatic amines are widely used industrial chemicals as their major sources in the environment include several chemical industry sectors such as oil refining, synthetic polymers, dyes, adhesives, rubbers, perfume, pharmaceuticals, pesticides and explosives. They result also from diesel exhaust, combustion of wood chips and rubber and tobacco smoke. Some types of aromatic amines are generated

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during cooking, special grilled meat and fish, as well. The intensive use and production of these compounds explains its occurrence in the environment such as in air, water and soil, thereby creating a potential for human exposure. Since aromatic amines are potential carcinogenic and toxic agents, they constitute an important class of environmental pollutants of enormous concern, which efficient removal is a crucial task for researchers, so several methods have been investigated and applied.

In this chapter the types and general properties of aromatic amine compounds are reviewed. As aromatic amines are continuously entering the environment from various sources and have been designated as high priority pollutants, their presence in the environment must be monitored at concentration levels lower than 30 mg L^{-1}, compatible with the limits allowed by the regulations. Consequently, most relevant analytical methods to detect the aromatic amines composition in environmental matrices, and for monitoring their degradation, are essential and will be presented. Those include Spectroscopy, namely UV/visible and Fourier Transform Infrared Spectroscopy (FTIR); Chromatography, in particular Thin Layer (TLC), High Performance Liquid (HPLC) and Gas chromatography (GC); Capillary electrophoresis (CE); Mass spectrometry (MS) and combination of different methods including GC-MS, HPLC-MS and CE-MS. Choosing the best methods depend on their availability, costs, detection limit and sample concentration, which sometimes need to be concentrate or pretreated. However, combined methods may give more complete results based on the complementary information. The environmental impact, toxicity and carcinogenicity of many aromatic amines have been reported and are emphasized in this chapter too.

Lately, the conventional aromatic amines degradation and the alternative biodegradation processes are highlighted. Parameters affecting biodegradation, role of different electron acceptors in aerobic and anaerobic biodegradation and kinetics are discussed. Conventional processes including extraction, adsorption onto activated carbon, chemical oxidation, advanced oxidation, electrochemical techniques and irradiation suffer from drawbacks including high costs, formation of hazardous by-products and low efficiency. Biological processes, taking advantage of the naturally processes occurring in environment, have been developed and tested, proved as an economic, energy efficient and environmentally feasible alternative. Aerobic biodegradation is one of the most promising techniques for aromatic amines remediation, but has the drawback of aromatic amines autooxidation once they are exposed to oxygen, instead of their degradation. Higher costs, especially due to power consumption for aeration, can also limit its application. Anaerobic degradation technology is the novel path for treatment of a wide variety of aromatic amines, including industrial wastewater, and will be discussed. However, some are difficult to degrade under anaerobic conditions and, thus, other electron acceptors such as nitrate, iron, sulphate, manganese and carbonate have, alternatively, been tested.

Keywords Aromatic amines • Biodegradation • Anaerobic oxidation • Electron acceptors • Kinetics • Toxicity

7.1 Introduction

Aromatic amines are widespread chemicals with considerable industrial and environmental importance (Fig. 7.1). Their major sources include several industrial sectors such as oil refining, dyes, cosmetics, medicines, rubber, textiles, agrochemicals, explosives and as reagent intermediates in many chemical syntheses synthetic polymers, dyes, adhesives and rubbers, pharmaceuticals, pesticides and explosives (Palmiotto et al. 2001). They are also found in environmental pollution such as diesel exhaust, combustion of wood chips and rubber, tobacco smoke, and substances in grilled meats and fish (DeBruin 1999). They are also used in the synthesis of organic colorants widely used in the textile, paper, leather, plastics, cosmetics, drugs, and food industries. Azo dye reduction produces aromatic amines that are generally higher toxic than the original dyes (Van der Zee and Villaverde 2005; Pinheiro et al. 2004). Consequently, aromatic amines appear in different environments, such as in air, water and soil thereby creating a potential for human exposure. Since these compounds are potential carcinogenic and toxic agents, they constitute an important class of environmental pollutants of enormous concern which efficient removal is a crucial task for researchers. Table 7.1 shows some examples of aromatic amines, and their major origins, known to be potential hazards to human health and to the environment.

Aromatic compounds constitute the second most abundant family of organic constituents present in the biosphere, after carbohydrates. Since the start of the industrial revolution, a wide variety of aromatic pollutants have also been introduced into the environment through anthropogenic activity (Bull et al. 2011; Carmona et al. 2009). The thermodynamic stability of the benzene ring, due to its resonance structure, has contributed to the widespread production and industrial use of natural and xenobiotic aromatic compounds, but has also contributed to the persistence of these compounds, many of which are toxic when released into the environment. Aromatic amines range from simplest aniline to highly complex molecules with conjugated aromatic or heterocyclic structures and multiple substituents. About 300 chemical products and intermediates are currently manufactured from aniline. According to the USA National Toxicology Program 2005, among the 415 chemicals recognized or suspected to be carcinogenic in humans, 12% are aromatic amines. Due to the wide use of aromatic amines together with the presence of relatively specific and industrial importance, very high exposures has determined the large toxicological experimentation and permitted the development of epidemiological knowledge unparalleled for other chemical classes. The US Environmental Protection Agency (EPA) has confirmed that, since 1970, several extremely toxic and potentially carcinogenic aromatic amines have been included in the list of priority pollutants (Sun et al. 2012a). Table 7.2 lists the aromatic amines banded in Europe (EU Directive 2002/61/EC).

Due to their high solubility in water, the aromatic amines can easily penetrate through the soil and enter into the water cycle in various forms, either in chemical effluents or as the breakdown products of herbicides. Their presence in ground

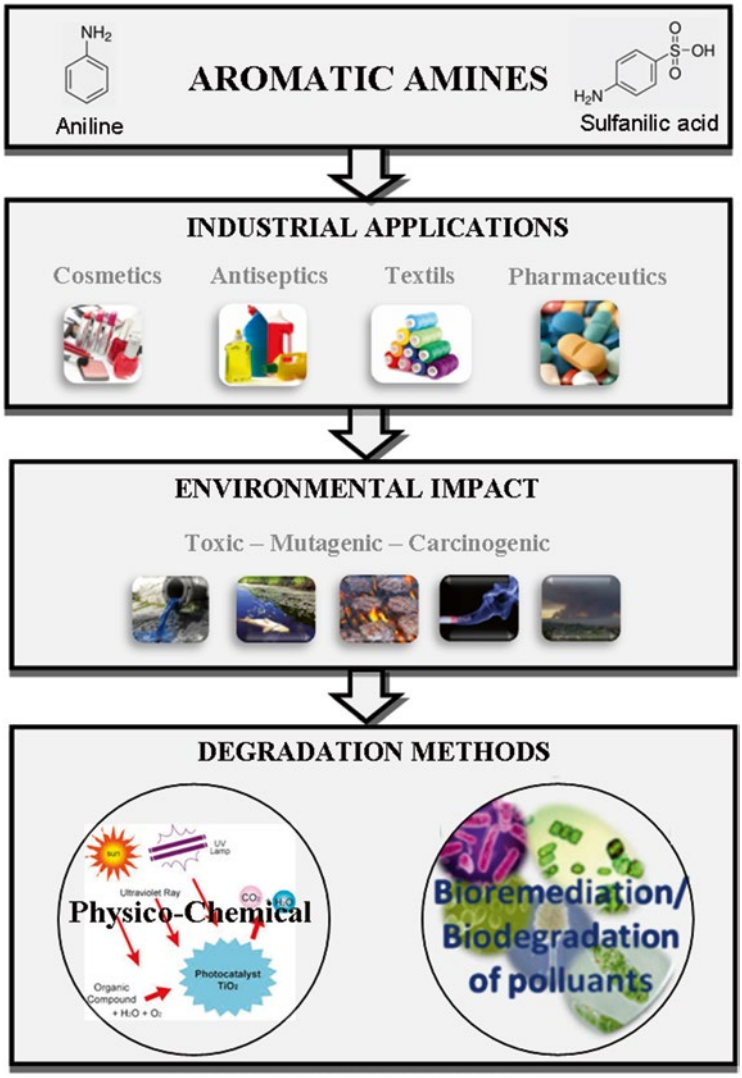
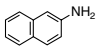
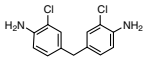
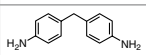
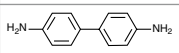
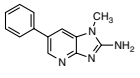


Fig. 7.1 Schematic representation of examples of aromatic amines sources, environmental and human health impact and methods of their degradation

waters or soil samples subject to industrial, agricultural or urban pollution is an increasing concern (Gan et al. 2004). Therefore, they constitute an important and diversified class of pollutants. Many of them are toxic to most living organisms due to their genotoxic or cytotoxic properties (Bull et al. 2011; Kim and Guengerich 2005).

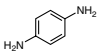
In order to protect human health and environmental safety, it is important to monitor aromatic amines in water, with sensitive and reliable methods. In recent years an extensive research activity has been directed towards developing processes to

Table 7.1 Some examples of aromatic amines and their major origins, known to be potential hazards to human health and the environment

Name	Chemical structure	Major origins	Potential impact
Aniline		Manufacture of isocyanates, rubber, dyes, explosives, pesticides, pharmaceuticals.	VOC with ozone-forming
		Oil refining	Toxic to aquatic life
		Tobacco smoke	Possibly carcinogenic and genotoxic
		Forest fires.	
4-Chloroaniline		Manufacture of dyes, pesticides, various chemicals	Toxic to humans
			Carcinogenic and genotoxic
Toluene 2,4-diamine		Manufacture of toluene diisocyanate (for elastomers), dyes, resins, fungicides	VOC
			Toxic to humans and aquatic life
			Possibly carcinogenic and genotoxic
2-Naphthylamine		Manufacture of dyes	Toxic to humans
		Tobacco smoke	Carcinogenic
4,4′-Methylenebis (2-chloroaniline)		Manufacture of polyurethanes	VOC
			Toxic to humans and aquatic life
			Possibly carcinogenic and genotoxic
4,4′-Methylenedianiline		Manufacture of polyurethanes, dyes, epoxy resins	VOC
			Recalcitrant adsorption onto particulate matter
			Toxic to humans and aquatic life
			Possibly carcinogenic and genotoxic
N-Nitrosodiphenylamine		Manufacture of dyes, pharmaceuticals, rubber	Harmful to humans
			Possibly carcinogenic
Benzidine		Manufacture of dyes	Toxic to humans
			Carcinogenic
2-Aminobiphenyl		Tobacco smoke	Genotoxic and carcinogenic
2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine		Cooking of meats	Genotoxic and carcinogenic

(continued)

Table 7.1 (continued)

Name	Chemical structure	Major origins	Potential impact
3-Trifluoromethylaniline		Intermediate for herbicides	Toxic to humans
p-Phenylenediamine		Component of engineering polymers and composites	Toxic and allergenic for humans
		Ingredient in hair dyes	
o-toluidine		Tobacco smoke	Human bladder carcinogens
4-chloro-*o*-toluidine		Tobacco smoke	Human bladder carcinogens

Adapted from Pinheiro et al. (2004)

efficiently remove highly complex structures of aromatic contaminants, including aromatic amines, from polluted water. Conventional processes for the removal of aromatic amines from industrial wastewaters include extraction, adsorption onto activated carbon, chemical oxidation, advanced oxidation, electrochemical techniques and irradiation. All of these methods suffer from drawbacks including high costs, formation of hazardous by-products and low efficiency (Franciscon et al. 2010; Mondal et al. 2010). Alternative biological methods appear to be a potentially economic, energy efficient and environmentally feasible option (Rieger et al. 2002).

Biological treatment of aromatic amines containing wastewaters, provides more specific conversions, is relatively inexpensive and usually results in complete mineralization. Microorganisms have evolved to degrade most naturally occurring organic compounds, including the persistent aromatics. Moreover, the promiscuity of the catabolic enzymes allows them to degrade, at least partially, xenobiotics that share similar structures with naturally occurring aromatic compounds (Díaz 2004). The bacterial catabolism of aromatic compounds involves several peripheral pathways that transform structurally diverse substrates into a limited number of intermediates that are further processed by a few central pathways to the central metabolism of the cell (McLeod and Eltis 2008). There are two major strategies to degrade aromatic compounds depending on the presence or absence of oxygen. In the aerobic catabolism of aromatics, oxygen is not only the final electron acceptor but also a co-substrate. In contrast, the anaerobic catabolism of aromatic compounds uses a completely different strategy, based on reductive reactions, to attack the aromatic ring. While the aerobic catabolism of aromatic compounds has been studied for several decades (Brown and Laboureur 1983; Pinheiro et al. 2004; Van der Zee and Villaverde 2005), this may not apply to all aromatic amines. Among the many

Table 7.2 The list of Aromatic amines banded in Europe according to the EU Directive 2002/61/EC

Serial no.	CAS No.	Substance
1	92-67-1	4-aminodiphenyl
2	92-87-5	benzidine
3	95-69-2	4-chloro-o-toluidine
4	91-59-8	2-naphthylamine
5	97-56-3	4-amino-2′,3-dimethylazobenzene[a]
6	99-55-8	2-amino-4-nitrotoluene[a]
7	106-47-8	4-chloroaniline
8	615-05-4	2,4-diaminoanisole
9	101-77-9	4,4′-diaminodiphenylmethane
10	91-94-1	3,3′-dichlorobenzidine
11	119-90-4	3,3′-dimethoxybenzidine
12	119-93-7	3,3′-dimethylbenzidine
13	838-88-0	3,3′-dimethyl-4,4′diaminodiphenylmethane
14	120-71-8	4-cresidine
15	101-14-4	4,4′-methylene-bis-(2-chloroaniline)
16	101-80-4	4,4′-oxydianiline
17	139-65-1	4,4′-thiodianiline
18	95-53-4	2-aminotoluene
19	95-80-7	2,4-diaminotoluene
20	137-17-7	2,4,5-trimethylaniline
21	90-04-0	2-methoxyaniline
22	60-09-3	4-aminoazobenzene[b]

[a]Amines 5 and 6 are analysed indirectly via reduction to the amines 18 and 19 respectively
[b]No analytical procedure is currently available for 4-aminoazobenzene

different aromatic amines tested, only a few were degraded. Some of them, substituted with hydroxyl or carboxyl group, were degraded under methanogenic and sulphate reducing conditions (Kalyuzhnyi et al. 2000; Razo-Flores et al. 1999). It has been demonstrated that especially sulfonated aromatic amines are often difficult to degrade (Razo-Flores et al. 1996, 1997a; Tan et al. 2005). A drawback of using aerobic treatment, with the aim of degrading aromatic amines from azo dye cleavage, is that many of them are prone to autoxidation once they are exposed to oxygen. Since autoxidation often involves enlargement of the molecules, their biodegradability may consequently decrease. Alternatively, nitrate, instead of oxygen, can be used as electron acceptor (Pereira et al. 2011). Indeed, several ecosystems are characterized by lack of oxygen, such as aquatic sediments, stratified lakes, wetlands and some soil horizons. In those environments, microorganisms can utilize compounds like nitrate, iron, sulphate, manganese and carbonate as electron acceptors. It has been reported that at least some aromatic amines can be degraded coupled to nitrate reduction (Kahng et al. 2000; Pereira et al. 2011; Vázquez-Rodriguez et al. 2008; Wu et al. 2007).

The anaerobic degradation of aromatics is a more recently discovered microbial capacity that still awaits a deeper understanding despite the fact that microbial metabolism in the absence of oxygen is the most ancient of all life processes (Lovley 2001). In fact, many habitats containing large amounts of aromatic compounds are often anoxic, e.g., aquifers, aquatic sediments and submerged soils, sludge digesters, and intestinal contents, and at aerobic sites with high carbon concentrations, molecular oxygen is more rapidly consumed than replenished (Lovley 2003). Thus, anoxic conditions dominate in many natural habitats and contaminated sites, and the anaerobic catabolism of aromatic compounds by microorganisms becomes crucial for the biogeochemical cycles and for the sustainable development of the biosphere. The mineralization of aromatic compounds by facultative or obligate anaerobic bacteria (and some archaea) can be coupled to anaerobic respiration with a variety of electron acceptors, e.g., nitrate, sulfate, iron(III), manganese(II), and selenate, with each one conserving different yields of energy. The greatest energy conservation is reached when nitrate is the final electron acceptor, followed by ferric ion.

In this chapter, aromatic amines, sources and their environmental impact is outlined. The available methods for aromatic amines monitoring are described and degradation methods are reviewed, with special emphasis on biodegradation. Various parameters affecting the biodegradation, such as the type of electron acceptors, and some kinetic considerations are revised.

7.2 Types of Aromatic Amines and Structure

Aromatic amines are generally identified as those chemical compounds having in their molecular structure one or more aromatic rings, with one or more amino substituents. They range from the simplest aniline to highly complex molecules with conjugated aromatic or heterocyclic structures (Fig. 7.2). Therefore, they can be classified in three types: monocylic, polycyclic and heterocyclic. They contain single or multiple aromatic rings bonded to nitrogen aryl groups. Heterocyclic aromatic amines are also bearing one or more amino substituents with different functional group. The common denominator is an amino group bound to an aromatic system. The activity of aromatic amine depends upon the position and structure of amine group and aromatic ring, respectively. They are the second most abundant family of organic constituents present in the biosphere after carbohydrates. Since the start of the industrial revolution, a wide variety of aromatic pollutants have also been introduced into the environment through anthropogenic activity (Fekete et al 2010). Some examples or aromatic amines, their origin and impact are listed in Table 7.1.

Aniline, which is essentially phenylamine, is the simplest aromatic amine. Commercial aniline can be chemically synthesized from nitrobenzene which is prepared from benzene with nitric acid by electrophilic substitution reaction, as shown in Fig. 7.3, or from chlorobenzene by heating with ammonia in the presence of a copper catalyst. Some aromatic amines are natural, such as 2- and 4-aminobenzoic

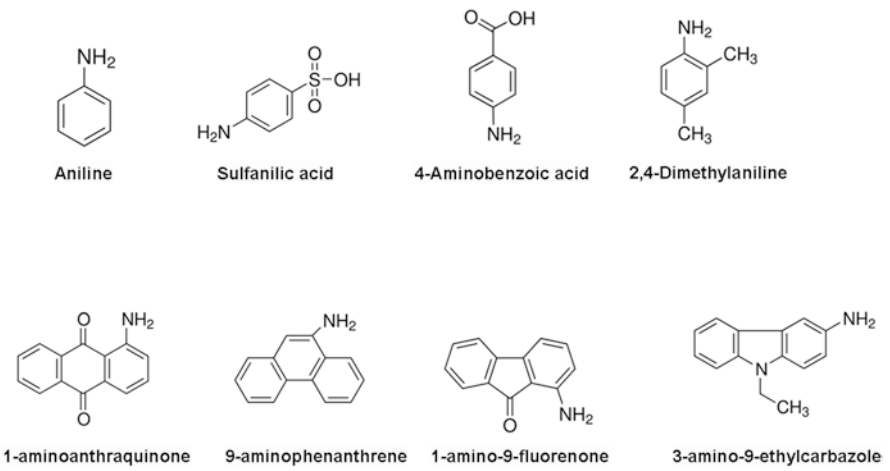


Fig. 7.2 Examples of monocyclic aromatic amines: aniline, sulfanilic acid, 4-aminobenzoic acid, 2, 4-Dimethylaniline; and heterocyclic aromatic amines: 1-aminoanthraquinone, 9-amino-phenanthrene, 3-amino-9-ethylcarbazole

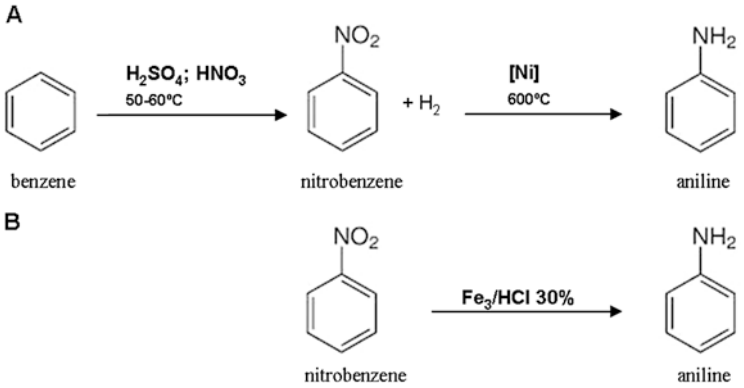


Fig. 7.3 Aniline chemical synthesis. (**a**) nitrobenzene, prepared from benzene with nitric acid by electrophilic substitution reaction and further treatment with niquel at 600 °C or (**b**) from chloro-benzene by heating with ammonia in the presence of a copper catalyst

acids, others are xenobiotics, like 3-aminobenzoic acid, aminosalicylates, aniline and aminophenols. In addition, they can result from chemical or bio transformation of other organic compounds. Several substituted phenylenediamines, the benzenediamines, are intermediates in the synthesis of polyurethanes, and others are used in the dyestuff industry (Chung 2000). Certain substituted-benzenediamines are important commercial ingredients in semipermanent and permanent hair dyes (Garrigue et al. 2006; Nohynek et al. 2010). Non-industrial sources of aromatic amines are the combustion of tobacco, automobile exhaust fumes, the burning/pyrolysis of protein-rich vegetable matter, cooking and subsequent consumption of

meats and they are also present in road tars (Lewtas 2007). Heterocyclic aromatic amines are formed, along with Polycyclic aromatic amines, when meats or fish are grilled or otherwise cooked at high temperatures (Combes and Haveland-Smith 1982). Heterocyclic aromatic amines are the major mutagenic compounds isolated from broiled and grilled meats and fish and have been shown to induce tumours in multiple organs, including the colon and mammary gland, in rodent bioassays (Melo et al. 2008). Depending on their chemical structure and their mechanism of formation, these xenobiotic genotoxic substances can be grouped into two main families. The first named 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) type or aminoimidazoazaarenes, includes amines containing a 2-aminoimidazole group generated from the reaction of free amino acids (especially creatine and creatinine) and hexoses at ordinary cooking temperatures. The other amines, called non-IQ type or pyrolytic, are formed through the pyrolytic reaction of amino acids and proteins at temperatures between 200 and 300 °C (Szterk et al. 2012; Toribio et al. 2002).

Primary aromatic amines are used as a starting material for the manufacture of azo dyes. This class of dyes is often used in the colouring process of textiles and leather. Once these compounds hold the functional azo group -N=N-, they have the capacity to release certain aromatic amines by the reduction of this azo bond. Because many different types of sulfonated azo dyes are currently being utilized, a wide variety of sulfonated aromatic amines will be formed under anaerobic conditions that will not easily be biodegraded and will constitute an important part of untreated Chemical Oxygen Demand fraction in azo dyes containing wastewater treatment. A significant fraction of the alkylanilines is used in the synthesis of dyes and may be release after dye reduction. Examples of alkylanilines include 2-methylaniline, 3-methylaniline, 4-methylaniline, 2,3-dimethylaniline, 2,4-dimethylaniline, 2,5-dimethylaniline, 2,6-dimethylaniline, 3,4-dimethylaniline, 3,5-dimethylaniline, 2-ethylaniline, 3-ethylaniline and 4-ethylaniline. This group of aromatic amines are present in the environment as a result of other sources as well: a fraction of them is used as intermediate in the synthesis of pharmaceuticals, agrichemicals, that are example 2, 6-diethylamine and 2-methyl-6-ethylamine involved in the synthesis of chloroacetanilide herbicides, and photographic chemicals. A subclass alkylanilines, arylamines, has been documented in cigarette smokers (Skipper et al. 2010).

7.3 Analysis of Aromatic Amines

As aromatic amines are continuously entering the environment from various sources and have been designated as high priority pollutants, their presence in the environment must be monitored at concentration levels lower than 30 mg L^{-1}, compatible with the limits allowed by the regulations. Efforts towards the development of accurate, reproducible and low detection limit methods for the quantification or aromatic amines in the environment have been made. Determination and monitoring of aromatic amines during their treatment and of the intermediates and final reaction

products is also necessary. Methods based on voltammetry (Chey and Adams 1977), potentiometry with specific electrodes (Vytras et al. 1982) and spectrophotometric quantification after a specific colour-generating reaction or derivatisation to a chromophore (Verma et al. 1988; Zatar et al. 1998) were firstly proposed. However, despite the fact that the earliest simpler methods are not being discharged and are sometimes very useful, due to their poor sensitivity and selectivity they have been gradually replaced by modern advanced methods. Those include gas chromatography (GC) and high-performance liquid chromatography (HPLC) coupled with different detectors. Mass spectrometry (MS) and capillary electrophoresis (CE) are nowadays also common methods for determining aromatic amines (Table 7.3). Combined methods such as GC-MS, HPLC-MS and CE-MS have been also applied and will be discussed in this chapter. The most common disadvantages of the methods are the detection limits and need long pre-concentration processes for a good sensitivity. The costs involved, particularly in instrumentation and skilled staff requirements are also factors in consideration. Additionally, most of the times samples cannot be analyzed directly by instrumental methods and pretreatment is often considered as an indispensable step prior to determination and quantification methods (Moradi et al. 2010). Many methods have been reported for the extraction of aromatic amines from environmental water samples and will be described below.

Standard methods for aromatic amines arising from the reductive cleavage of azo dyes have been established in Europe, such as the French norm AFNOR XP G08-014 for dyed textiles or the German method DIN 53316 for dyed leather (Pinheiro et al. 2004).

7.3.1 Spectroscopy

7.3.1.1 UV/Visible

UV/visible spectroscopy is a very useful method for the routine monitoring of industrial effluent discharges. In addition, direct UV/visible spectrophotometry can be an ideal technique for the monitoring of treatment processes such as biodegradation, chemical oxidation and reduction, photo-oxidation, photolysis and adsorption, operated for the removal of residual amines and other aromatics.

Spectrophotometry in the ultraviolet (UV) range has repeatedly proven to be a fast, inexpensive and reliable method for the monitoring of many compounds in urban and industrial wastewaters (Narayana and Sunil 2009; Pinheiro et al. 2004). Through the application of spectral analysis, quantitative and qualitative wastewater parameters can be estimated on direct samples in just a few minutes, using portable or online field instrumentation. Perez (2001) has successfully applied UV spectral deconvolution on wastewater monitoring in a chemical industry, for the estimation of aniline derivative concentrations. In the case of textile effluents, the use of the UV range of the spectra (200–350 nm) for aromatic amine determination is particularly useful to avoid interference by visible colour of dyes. The characteristic

Table 7.3 Some examples of methods for detection and quantification of aromatic amines (main advantages and disadvantages)

Method	Advantage	Disadvantage	Reference
HPLC/ED	Fast and simple	Costs of equipment	Lizier and Zanoni (2012)
	Simple pre-treatment step	The separation of amine compounds at HPLC/ED condition still remains problematic due to the interactions with silanol groups in the chromatography columns	
	Higher sensitivity than other conventional methods		
	Lack of baseline stabilization		
	High accuracy and sensitivity		
PLC–ESI-MS–MS	Rapid	Costs of equipment	Moriwakia et al. (2003)
	High accuracy and sensitivity	Complexity	
	Pre-treatment is much simpler than that of the conventional method	High maintenance requirements	
		High skilled labour requirements	
LPT-TD-GC-MS	High sensitivity	An additional device is needed to perform thermal desorption that would increase the cost of instrument	Zhang et al. 2009
	Good reproducibility	Some aromatic amines with high boiling point have severe peak tailing since it is difficult to desorbs them quickly	
	Low organic solvents consumption	Costs of equipment and maintenance	
		Complexity	
		High skilled labour requirements	
TLC with cinnamaldehyde as a reagent	Use of cheap, commercially available and nontoxic reagent	Only aromatic primary amines with electron-donating groups give positive results	Guo and Chen (2010)
	Simple, fast and colorimetric detection	No detection using aromatic primary amines with electron-acceptor groups was possible	
	Detection in the nanomolar range		

(continued)

Table 7.3 (continued)

Method	Advantage	Disadvantage	Reference
HPLC/DAD	Detection at a very low levels	Need of extraction and purification	Melo et al. (2008)
	High accuracy		
	Detection at various wavelengths		
Spectrophotometry and HPLC	Spectrophotometry is less expensive and simple	The spectrophotometric method may be used for preliminary analysis and detecting	Pielesz et al. (2002)
	HPLC gives higher accuracy	In the spectrophotometric method the accuracy of amine determination is limited	
		HPLC is more expensive	
Detection in a resin bound aromatic amines using *p*-nitrophenylester	Simple and sensitive test for the monitoring of coupling reactions to free aromatic amines during solid phase synthesis		Van der Plas et al. (2007)
HPLC/FD-MS	Simpleness for the preparation of aromatic amine derivatives	Costs of equipment and chemicals	Zhao and Suo (2008)
	Repeatability, precision, and recovery were excellent for the efficient HPLC analysis	MS maintenance requirements	
	High sensitivity and ease-of-handling of the PPIA method		
UV–visible and FTIR	Simple and fast	In the FT-IR analysis, interference by the yeast extract added to the medium restricted data interpretation, showing very similar spectra	Franciscon et al. (2009)
TLC with orcinol as reagent	Simplicity	Only qualitative detection	Janghel et al. (2005)
	Accuracy		
SPME-GC-MS	Rapid, one-step, solvent-free extraction and concentration system	Costs of equipment and chemicals	DeBruin et al. (1999)
	High specificity and reproducibility	Complexity	
		Maintenance requirements	

(continued)

Table 7.3 (continued)

Method	Advantage	Disadvantage	Reference
GC Bromination of the aromatic ring system to yield the corresponding bromo derivatives	The derivatization method is very easy to perform	Certain analytes are not detected as intact brominated molecules because of the reported cracking they undergo under the bromination conditions and only qualitative conclusions can be drawn	Dados et al. (2004)
	Detection limits achieved are extremely low and superior to those acquired by the GC-MS method		
	High reproducibility		
HPLC-MS-MS	No need of preliminary derivatisation or pre-concentration steps	Cost of equipment	Mortensen et al. (2005)
	Excellent accuracy	MS maintenance requirements and killed labour requirements	
GC-SPME	The novel fibres exhibit high thermal stability (to 340 °C) and solvent stability and high affinity for the aromatic amines	Cost of equipment	Zeng et al. (2001)
	Fibres could be re-used	Need of pre-treatment	

HPLC High liquid chromatography, *ED* electrochemical detector, *ESI* electrospray ionization, *MS* tandem mass spectrometry, *LPT* Liquid-phase sorbent trapping, *TD* Thermal desorption, *GC* Gas chromatography, *TLC* Thin layer chromatography, *DAD* Diode Array, *FD* Fluorescence detection, *FTIR* Fourier transform infrared spectroscopy, *SPME* Solid phase microextraction

absorption of dyes in the visible region (400–700 nm), where most of the aromatic amines show little absorption, provides a way to monitor azo dye reduction with aromatic amines formation and independently assess their residual concentration level. Many researchers have followed the degradation of dyes by UV-visible and observed that the decrease of absorbance at the visible range, characteristic of the dyes, was followed by the increase and formation of new peaks at the UV range, indicating that a reaction occurred. For example, azo dyes reduction to the correspondent aromatic amines has been reported and the increase of absorbance on the UV region is a first sign of aromatic amines formation (Brás et al. 2001; Franciscon et al. 2009, 2012) (Fig. 7.4). However, dyes and other organic molecules also absorb in the UV region and, therefore, UV/visible spectroscopy may be very advantageous when complemented with other techniques. The UV spectrum of aniline in a basic solution presents two well defined maxima bands at 230 and 281 nm, while in acidic conditions, does bands are less defined and under streme acidity the spectrum shape does not show any specific absorption band and is not reliable (Fig. 7.5) (Gonzalez et al. 2007). The aromatic amine spectra show extensive peaks in the all UV range and identification only by using this technique may not be adequate. The UV/visible absorbance regions of some aromatic amines are listed in Table 7.4.

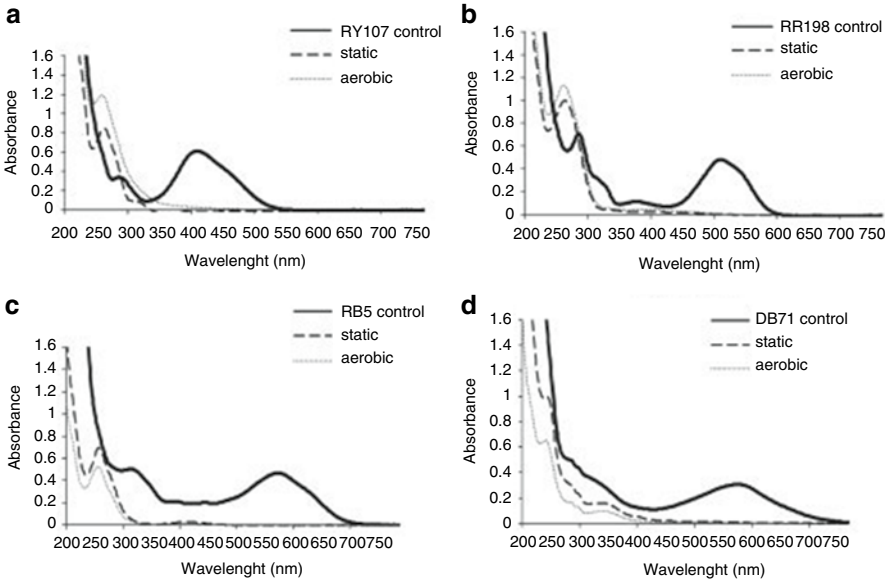


Fig. 7.4 UV–vis spectra of the azo dyes before (*straight line*) and after decolourisation under microaerophilic (*dashed line*) and aerobic (*dotted line*) conditions, by the *Brevibacterium* sp. strain VN-15, isolated from an activated sludge process of a textile company. A: Reactive Yellow 107; B: Reactive Red 198; C: Reactive Black 5; D: Direct Blue 71

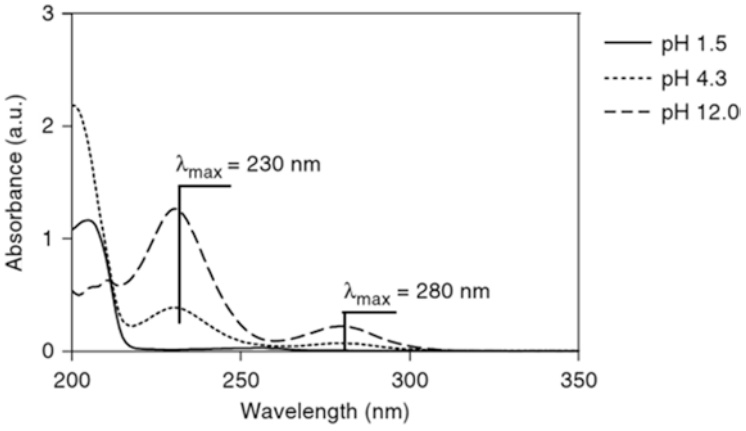


Fig. 7.5 UV spectrum of aniline (15 mg L^{-1}) under different pH aqueous solutions (Gonzalez et al. 2007)

7.3.1.2 Fourier Transform Infrared Spectroscopy

FTIR has also been applied to detect many compounds (Pielesz 1999). The infrared sensing is a powerful tool in the detection of organic species in aqueous solutions due to the detection speed and the abundant chemical information obtained. This

Table 7.4 UV absorbance range for some aromatic amines (Adapted from Pinheiro et al. 2004)

Aromatic amine	Main absorbance regions
Aniline	196, 230, 281
o-phenylenediamine	210, 239, 295
m-phenylenediamine	211, 238, 289
p-phenylenediamine	197, 241, 305
p-nitroaniline	229, 297–310
Sulfanilic acid	191, 215, 258–269
Metanilic acid	235, 260–270, 290
2,4-diaminophenol	207, 301
2,6-diaminophenol	213, 280
2-aminoresorcinol	205, 234, 271
o-methoxyaniline	238, 288
p-methoxyaniline	234, 297
p-chloroaniline	242, 295
2,3-dichloroaniline	236, 292
m-methylaniline	234, 284
5-aminosalicylic acid	325
m-trifluoromethylaniline	235, 290
4-aminobiphenyl	270-280
benzidine	280-290
1-naphthylamine	211, 242, 320–327
2-naphthylamine	213, 237, 272–291
4-nitro-1-naphthylamine	247-259

spectroscopy technique deals with the infrared region of the electromagnetic spectrum, that is light with a longer wavelength and lower frequency than visible light. Spectral data is collected in a FTIR spectrometer in a wide spectral range (1000–4000 cm^{-1}). This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time. Bond lengths and bond angles are continuously changing due to this vibration. A molecule absorbs infrared radiation when the vibration of the atoms in the molecule produces an oscillating electric field with the same frequency as the frequency of incident IR light. Infrared spectroscopy exploits the fact that molecules absorb specific frequencies that are characteristic of their structure; the frequency of the absorbed radiation matches the frequency of the bond or group that vibrates.

As example, the N–H stretches of amines are in the region 3300–3000 cm^{-1}. These bands are weaker and sharper than those of the alcohol O–H stretches which appear in the same region. In primary amines (RNH_2), there are two bands in this region, the asymmetrical N–H stretch and the symmetrical N–H stretch (Fig. 7.6). Secondary amines (R_2NH) show only a single weak band in the 3300–3000 cm^{-1} region, since they have only one N–H bond. Tertiary amines (R_3N) do not show any band in this region since they do not have an N–H bond. The N–H bending vibration of primary amines is observed in the region 1650–1580 cm^{-1}. Usually, secondary amines do not show a band in this region and tertiary amines never show a band in

Fig. 7.6 Asymmetrical and symmetrical N-H stretch in primary amines. The N–H stretches of amines are in the region 3300–3000 cm^{-1}. In primary amines (RNH_2), there are two bands in this region, the asymmetrical N–H stretch and the symmetrical N–H stretch

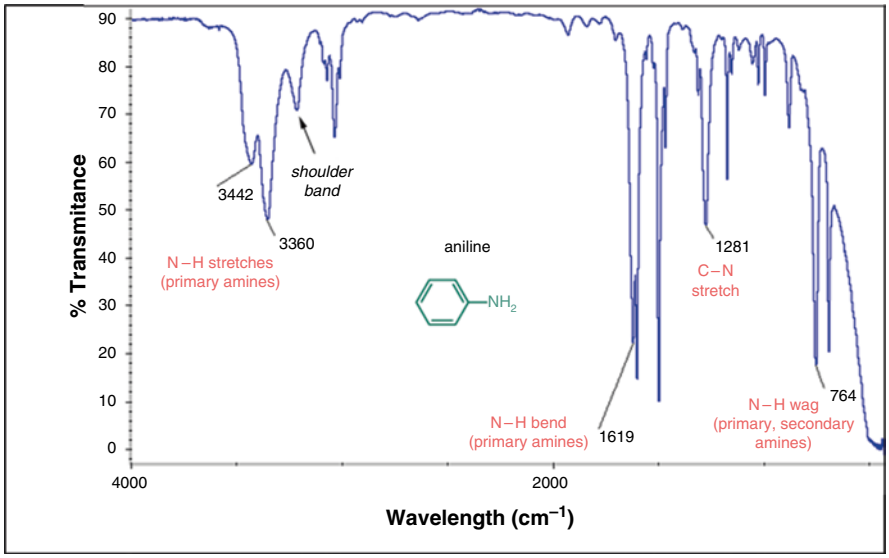


Fig. 7.7 FTIR spectra of aniline. Aniline shows two N–H stretches (3442, 3360 cm^{-1}). The shoulder band corresponds to an N–H bending vibration. As aniline is an aromatic compound, the C–N stretch appears at 1281 cm^{-1} rather than at lower wavenumbers

this region. Another band attributed to amines is observed in the region 910–665 cm^{-1}. This strong, broad band is due to N–H wag and observed only for primary and secondary amines. The C–N stretching vibration of aliphatic amines is observed as medium or weak bands in the region 1250–1020 cm^{-1}. In aromatic amines, the band is usually strong and in the region 1335–1250 cm^{-1}. The FTIR spectrum of aniline is shown in Fig. 7.7. This primary amine shows two N–H stretches (3442, 3360 cm^{-1}). The shoulder band corresponds to an N–H bending vibration. The C–N stretch appears at 1281 cm^{-1} rather than at lower wavenumbers because aniline is an aromatic compound.

Some aromatic amines resulting from the reduction of azo dyes have been identified by FTIR (Franciscon et al. 2009; Pielesz 1999). However, FTIR by itself is not always sufficient, so Mass spectroscopy, Gas and/or Liquid chromatography are techniques currently being used as complementary ones for compounds identification.

7.3.1.3 Mass Spectroscopy

MS is an analytical technique that measures the mass-to-charge ratio (*m/z*) of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules. Charged molecules or molecule fragments are generated by ionization of the chemical compounds. The technique has both qualitative and quantitative applications. In a complex mixture, where interference compounds and also in mixtures containing unknown molecules, fragmentation of the formed ion MS/MS is necessary to confirm the molecular structure for a certain mass. Indeed, the fragmentation pattern helps on determination of the structural information an unknown compound with a certain molar weight, once different compounds have the same molar weight.

An important enhancement to the mass resolving and mass determining capabilities of mass spectrometry is using it in sequence with chromatographic separation techniques. Indeed, most aromatic compounds identification when MS is applied result from the combination with chromatographic methods. This allows the sample separation prior to ionization in the mass spectrometer and we can even refer this as a requirement for complex mixtures. Combined methods will be described below in this chapter.

7.3.2 Chromatography

Chromatography can be used to monitor the progress of a reaction, identify and/or separate compounds present in a given mixture and determine the purity of a substance. The mixture is dissolved in a fluid called the *mobile phase,* which carries it through a structure holding another material called the *stationary phase.* The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention, namely retention time (Rt), on the stationary phase and thus changing the separation. The separation and identification of compounds by chromatography depends on their chromatographic behaviour and that is related with many factors that are related with the chemical structure. Therefore, chromatographic parameters need to be determined, independently of the method used. However, for the same compound, different conditions have been successfully applied. Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for more advanced use, being thus a form of purification. Analytical chromatography is done normally with smaller amounts of material and is used for measuring the relative proportions of analytes in a mixture.

7.3.2.1 Thin Layer Chromatography

TLC is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose (blotter paper). This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture, known as the mobile phase, is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility.

Thin-layer and paper chromatography for aromatic amines identification were earlier reported in the 1970s-1980s (Franc and Koudelková 1979; Ghafoor and Bark 1982; Srivastava and Dua 1975). In their report, Ghafoor and Bark (1982) have tested halogen, alkyl and nitro aniline derivatives and showed how the substituents on the aromatic structure may affect the chromatographic behavior of aromatic amines. More recently, Janghel et al. (2005) have developed a new TLC technique based on the reaction of the aromatic amines *o*-nitroaniline, *p*-nitroaniline, *m*-nitroaniline, *p*-phenylenediamine and *m*-phenylenediamine, with orcinol on a TLC plate, leading to colour derivatives. The colour derivatives migrated by the action of temperature gradient and the intensity of the spots compared with those of the standards. Primary aromatic amines such as aniline, *p*-toluidine, *o*-toluidine, *m*-toluidine, *p*-anisidine, *o*-anisidine, *m*-anisidine, 2-aminophenol, 4-aminophenol, 3-aminophenol, *p*-phenylenediamine, *o*-phenylenediamine, *m*-phenylenediamine, *p*-(4-chlorobenzyl)aniline, 4,4-diaminobiphenyl, 2-aminonaphthalene have been identified on a TLC plate (Guo and Chen 2010). The colorimetric method is based on the reaction mechanism of cinnamaldehyde with the amines on a TLC plate. A yellow spot appears immediately from the mixing of a colorless solution of amines with a colorless solution of cinnamaldehyde on a TLC plate, meaning the presence of the aromatic amine (Fig. 7.8). The method is advantageous, as the reagent is inexpensive, commercially available and non-toxic, the detection is sensitive and selective, and the procedure is simple and fast.

7.3.2.2 High Performance Liquid Chromatography

Separation of aromatic amines by HPLC is presently a common practice for the analysis of these substances in water, avoiding the need for pre-derivatisation and the risk of thermal degradation in Gas Chromatography. HPLC is often used for direct analysis of aromatic amines. As example, Melo et al. (2008) have analysed by HPLC the presence of heterocyclic aromatic amines in Portuguese bovine meat dishes prepared by three different cooking methods. Other examples are given in Table 7.2.

Different columns such as C18, C8 and cyano, of various internal diameters, as example 2.1 and 4 mm, and different mobile phase compositions have been used. Both reversed-phase, using octyl or octadecyl silica derivatives, and ion chromatog-

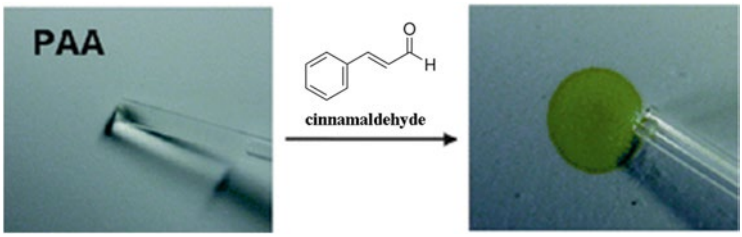


Fig. 7.8 Photo of TLC identification of aromatic amines by the colorimetric method based on the reaction mechanism with cinnamaldehyde (Guo and Chen 2010). When an aromatic amine is present, am yellow spot appears immediately from the mixing of a colorless solution of amines with a colorless solution of cinnamaldehyde on a TLC plate

raphy, using cation exchange resins, methodologies have been developed, with fine tuning of separations being achieved by temperature, mobile phase composition and gradient adjustments. For reversed phase separations, mixtures of aqueous buffers with acetonitrile or methanol are widely used, though, for the separation of sulfonated amines, the addition of ion-pair reagents such as quaternary ammonium salts or tertiary amines has been reported (Ramalho et al. 2004). The most common used detection method is diode array detection (DAD) which allows on-line identification of the analytes in the whole spectra and has a low cost. However, the generally applicable UV detector sometimes lacks high sensitivity, especially when analysing real samples for which wavelengths below 230 nm often cannot be used for quantitation due to matrix interferences Fluorescence detection is sometimes used as a complement to DAD, because unavoidable interferences are frequently produced when using UV detection. Monitorization of aromatic amines degradation by HPLC has been done by many authors (Carvalho et al. 2010; Khalid et al. 2009). Aromatic amines resulted from azo dye reduction have also been commonly identified by HPLC, using standard compounds for comparison (Carvalho et al. 2008; Mendes et al. 2011; Ramalho et al. 2004) (Fig. 7.9).

Pre or post-column derivatisation are sometimes applied to improve peak shapes and to increase detection sensitivity. Many types of fluorescent derivatisation reagents have been developed, although there are still many reports describing various shortcomings in applications (Greaves et al. 2001; Kudlich et al. 1999). Zhao and Suo (2008), have synthesized two novel fluorescent labelling reagents, 2-(2- phenyl-1H-phenanthro-[9,10-d]imidazole-1-yl)-acetic acid (PPIA) and 2-(9-acridone)-acetic acid (AAA), which are easily accessible and very stable in solution or their crystal states. Effects of derivatisation conditions were investigated for the separation of six monocyclic aromatic amines, aniline, 2-methylaniline, 2-methoxyaniline, 4-methylaniline, 4-chloroaniline and 4-bromoaniline. The PPIA and AAA were compared through whole procedures and the results indicated that the PPIA was better than the AAA. The fluorescent detection sensitivity and peak shapes of PPIA-labelled aromatic amines were improved greatly, compared with

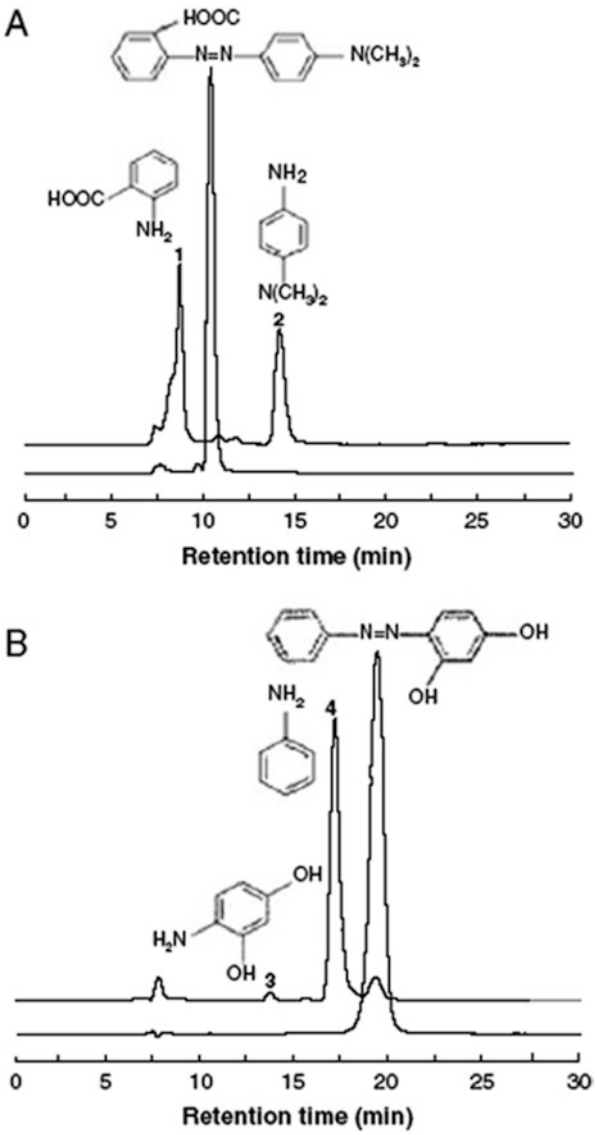


Fig. 7.9 HPLC chromatograms of the dyes (*thin line*) and 24 h of their enzymatic degradation (*thick line*): methyl red (**a**); Sudan orange G (**b**). Products of the reaction were identified, in comparison to the standards: 2-aminobenzoic acid (1), N,N′-dimethyl-*p*-phenylenediamine (2), 4-aminoresorcinol (3) and aniline (4) (Mendes et al. 2011)

direct UV detection without labelling. Furthermore, the mass spectra of PPIA labelled derivatives were more specific for their characterization than those of unlabelled ones. In addition, linearity, limits of detection, recovery, reproducibility and precision of the whole procedure were also determined.

7.3.2.3 Gas Chromatography

GC is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. In gas chromatography, the mobile phase is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal column. Devices reported for simple quantification or aromatic amine peaks in GC include flame ionisation, nitrogen-selective, flame photometric and electron capture detectors.

GC has been widely used for amine analysis because of its inherent advantages of simplicity, high resolving power, high sensitivity and short analysis time. In very complex mixtures, common in samples for aromatic amine analysis, GC offers better peak carrying capacity in chromatograms than liquid chromatography techniques. However, GC is sometimes unsatisfactory owing to the adsorption and decomposition of the solutes on the column. In addition, some of the amines cannot be separated at all by GC or exhibit problems of peak tailing. Due to the polar nature of aromatic amines, GC methodologies generally require derivatisation into apolar, volatile, thermally stable products prior to injection into the chromatographic column, though recent columns and equipments can allow separation of some aromatic amines without derivatisation. Another reason for derivatisation is to protect the compounds from chemical reactions prior to analysis. Sample concentration before injection is also often required. This can be achieved by liofilization of the aqueous solution and further dissolution in organic solvent or direct extraction with organic solvents. Derivatisation reactions for the determination of amines by GC with respect to reactivity, selectivity and sensitivity have been reviewed by Kataoka (1996). Applications to the determination of individual amines, ammonia and N-nitrosamines in various environmental samples are also described.

Skarping et al. (1983), have tested a method for the trace analysis of aromatic amines identification by capillary GC. It involved conversion of the amines into the corresponding amides by reaction with a perfluoro fatty acid anhydride prior to separation and quantification. Detection limits were in the low picogram range. Haas et al. (1997), have developed an analytical method for the determination of aromatic amines in water is introduced that uses iodination with a Sandmeyer-like reaction to replace the amino group by iodine in aqueous solution. The non-polar derivatives are extracted with pentane or toluene, separated with gas chromatography and sensitively detected with an ECD. Thirteen major metabolites of nitroaromatic explosives were investigated. Latter, a method of derivatisation based on the bromination of the aromatic ring in an acetic medium was experienced by the same group in order to improve determination of aromatic amines by GC and detection with an electron capture detector (Schmidt et al. 1998). From the 56 aromatic amines tested, only for 6 of them derivatives were not obtained. This derivatisation method can be easily and quickly performed and offers the possibility of separation of positional isomer derivatives. The authors have compared both derivatisation

methods and concluded that the derivatisation of aromatic amines with iodination and bromination supplement each other in terms of sensitivity and selectivity. More recently, Dados et al. (2004), have tested a GC method whereby the aromatic amines originating from azo dyes in their derivatised form by bromination could be detected at ultra-low concentration levels by gas chromatography. Bromination of aromatic ring of the amines allowed their detection by GC with electron capture detection (ECD) exploiting the presence of the electron withdrawing groups in the analyte. Each derivative produced an easily interpretable mass spectrum with specific and prominent fragment ions. Out of the twenty aromatic amines tested only the bromination of 2,4-diaminoanisole was not feasible.

7.3.3 Capillary Electrophoresis

In traditional electrophoresis, electrically charged analytes move in a conductive liquid medium under the influence of an electric field. Introduced in the 1960s, the technique of CE was designed to separate species based on their size to charge ratio in the interior of a small capillary filled with an electrolyte. The technique uses fused silica capillaries under an electrical field, and separation occurs either solely on the basis of molecular weight and charge, electrophoretic mobility, or associated with differential solubilisation in surfactant micelles. Associated with pre-concentration, pre-derivatisation or incapillary derivatisation techniques in order to overcome the problem of inherent low sensitivity, this method has shown potential for excellent separation efficiency (Asthana et al. 2000; Cavallaro et al. 1995; Sun et al. 2009).

Aromatic amines are well known to show good electrochemical behaviour. Taking advantage of the polar, ionisable nature of aromatic amines, capillary electrophoresis methods, both in the capillary zone electrophoresis and the micellar electrokinetic chromatography modes, have also been proposed for their fractionation in environmental samples (Martínez et al. 2000; Oguri 2000; Ye and Huang 2007). A wide range of techniques have been applied for post-separation identification and quantification of CE peaks in aromatic amine analysis such as UV spectrophotometric, electrochemical (amperometric) and fluorescence detection (Asthana et al. 2000; Sun et al. 2009). Signal enhancement can be obtained through pre- or post-column derivatisation reactions with a chromophore or fluorophore (Asthana et al. 2000). In CE, indirect detection using a background electrolyte bearing also a chromophore or a fluorophore offers potentially higher sensitivity levels (Oguri 2000). Two novel pre-column fluorescent derivatisation reagents, 2-(2-Phenyl-1H-phenanthro-[9,10-d]imidazole-1-yl)-acetic acid (PPIA) and 2-(9-acridone)-acetic acid (AAA), have been developed and compared for analysis of primary aromatic amines by high performance liquid chromatographic fluorescence detection coupled with online mass spectrometric identification (Zhao and Suo 2008).

7.3.4 Combined Methods

The challenge of aromatic compounds identification and quantification has been to develop rapid analytical methods that unequivocally identify them in complex matrices and at low level. In combined methods, usually a purification step is carried out, followed by a separation technique such as HPLC, GC or CE, followed by MS. Peak identification is often a challenge in chromatographic aromatic amine analysis, due to the occurrence of multiple amine structures, interfering substances such as other aromatics and products of amine condensation. So, when chromatographic methods are used, the confirmation of the chromatographic peaks using selective techniques is common, since numerous co-elutions can occur leading to false peak identification. Commonly, for compounds identification by spectroscopy and chromatography, standard compounds are needed and this and this presupposes already having an idea of the products formed and their commercial availability.

The high basicity, reactivity and polar nature of aromatic amines are responsible for different problems involved on the extraction and detection by chromatographic analysis (Akyüz and Ata 2004). Though sample clean-up and specific derivatisation reactions at pre- or post-column level can minimise interferences, the coupling to spectral analysis methods, MS or diode array spectrophotometry, has emerged as a powerful aid. GC-MS has been widely used for the analysis of chemicals in environment because of the high selectivity and resolution of GC columns and mass spectrometry. This technique is suitable for the analysis of low polarity compounds; however, it is difficult to detect polar compounds directly by this technique. HPLC-MS, commonly referred as LC-MS, on the other hand, is an excellent and powerful tool for detection of polar compounds. There are several advantages in the use of LC-MS for the analysis of environmental samples, such as direct detection of highly polar compounds, no need of derivatisation, direct injection of polar solutions, and high selectivity (Zhao and Suo 2008). This combined technique has been frequently used in the last few years for environmental analysis of heterocyclic aromatic amines (Kataoka 1996).

Several reports on applications of combined methods such as GC–MS (DeBruin et al. 1999; Razo-Flores 1997), LC-MS (Fay et al. 1997; Moriwakia et al. 2003; Mortensen et al. 2005; Zhao and Suo 2008), HPLC coupled to an electrochemical detector, HPLC-ED, (Lizier and Zanoni 2012) for aromatic amine analysis have been published. As example, Fig. 7.10 shows the LC-MS analysis of the azo dye, Acid Red 37, reduction by an azoreductase isolated from *Acinetobacter radioresistens* bacteria, to the correspondent aromatic amine 7,8-diamino-3[(aminoxy)sulfonyl] naphthalene-1-ol (Ramya et al. 2010). Capillary electrophoresis, either with mass spectrometry (CE-MS) ultra-violet (CE-UV) or electrochemical (CE-ED) and florescent detection (CE-FD) has also been proposed (Asthana et al. 2000; Sentellas et al. 2003, 2004). The coupling of electrochemical detectors (ED) in HPLC systems has shown great potential in the quantification of trace organic compounds in various matrices (Lizier and Zanoni 2012). A new method for the determination of four aromatic amines in water samples was developed by Wang et al. (2008) using

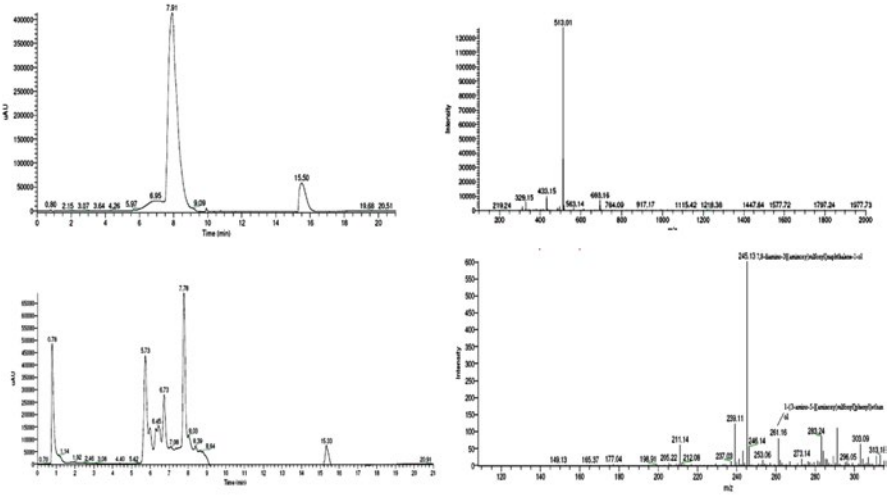


Fig. 7.10 LC-MS analysis of Acid Red 37 reduction to the correspondent aromatic amines, 1-{3-amino-5-[(aminoxy)sulfonyl]phenyl}ethanol and 7,8-diamino-3[(aminoxy)sulfonyl] naphthalene-1-ol, catalyzed by an azoreductase isolated from *Acinetobacter radioresistens* bacteria (Ramya et al. 2010). (**a**) LC chromatogram and (**b**) MS spectra of Acid Red solution before bioreduction; (**c**) chromatogram and (**d**) MS spectra of Acid Red solution after 24 h of bioreduction

dispersive liquid–liquid microextraction (DLLME) technique combined with HPLC-variable wavelength detection (HPLC-VWD). An IR spectroscopy method combined with solid-phase-micro-extraction (SPME) was proposed by Yang and Tsai (2001) to detect chlorinated aromatic amines in aqueous solutions. The sensitivity of SPME-IR was enhanced as compared to common IR, because SPME films exclude water molecules interference. The method was greatly accurate and sensible, linear coefficients were around 0.995 and the detection could be lower than 100 ppb.

7.3.5 Sample Pre-treatment

Several sample pre-treatment methods have been proposed, aiming both at sample clean-up to reduce interferences and pre-concentration of which some have been duly validated and integrated in standardised analysis procedures and automated devices (Pinheiro et al. 2004). Those pre-treatments will increase the accuracy and sensitivity of the detection and lead to faster analyses and better reproducibility. Besides, these procedures may attain minimal sample volume requirements and avoid of analyte losses through evaporation. Liquid-phase extraction, LPE, (Alaejos et al. 2008; Sun et al. 2012b) solid-phase extraction, SPE, (Alaejos et al. 2008; Martínez et al. 2000), solid-phase microextraction, SPME, (Alaejos et al. 2008; DeBruin et al. 1999; Sharma et al. 2011), liquid–liquid–liquid microextraction,

LLLME, (Hou and Lee 2003), liquid phase microextraction, LPME, (Tao et al. 2009), LLE–SPE tandem extraction (Alaejos et al. 2008) and headspace single-drop microextraction, HS-SDME, (Zhou and Ye 2008), have been reported for the extraction of aromatic amines from environmental water samples.

Due to the high solubility of aromatic amines in water, the extraction step is of great difficulty. Also, it is of large importance to achieve high performance in the following separation and detection. Liquid-liquid extraction and Solid-phase extraction are the two most frequently used methods for the extraction of these amines from aqueous samples. Solid-phase microextraction and liquid-phase microextraction can also be applied to reduce the requirements of sample and organic solvent, but are less common. For practical applications of these methods in routine inspection work, there are still some problems to resolve such as special interface, limited adsorption capacity, and flavoursome procedures. Zhang et al. (2009) have proposed an alternative approach based on sorbent trapping followed by thermal desorption (TD). Aromatic amines from azo dye reduction in liquid-phase could be directly entrapped on Tenax-TA, which then was subjected to TD for analyte recovery and the subsequent determination by GC-MS. Using this approach, the authors could detect 21 aromatic amines with high sensitivity and good reproducibility. The method was applied to determine aromatic amines in textile samples and the results showed good agreement with conventional solid extraction method. The main advantages of this method were the low organic solvents consumption and convenient procedure. On the other hand, this method was more durable than other miniaturized approaches such as solid-phase microextraction and liquid-phase microextraction, since the sorbent tube is durable enough to be reused many times. An additional device is needed to perform TD that would increase the cost of instrument. In addition, some aromatic amines with high boiling point have severe peak tailing since it is difficult to desorb them quickly.

Another important point to be considered is related with sample storage before analysis. Considering the analytical aspects, the knowledge of how stable a compound is in the particular environmental compartments, as well as of the degradation products, has great value for the validity and reliability of analytical results. It is important to ensure that the obtained measurements reflect the compound concentration in the investigated matrix at the moment of sample collection. Degradation during sample storage step, which frequently takes place, should be minimized as significantly as unlikely.

7.4 Toxicity of Aromatic Amines

Recent progress in cancer research has revealed the complexity of the interaction between exogenous exposures and the physiology of an organism. The existing knowledge favours the idea that most, if not all aromatic amines, have a carcinogenic potential. The epidemiological literature leaves little doubt that a specific few aromatic amines are the cause of bladder cancer in occupationally exposed persons and there is a convincing argument to be made that exposure to aromatic amines via

tobacco smoke is a major, if not predominant, factor in causing bladder cancer in smokers (Yu and Ross 1998; Hecht 2003). The biochemical mechanisms by which aromatic amines might induce cancer have been investigated extensively and are now thought to be reasonably well understood (Skipper et al. 2010; Bull et al. 2011). Human population studies that have incorporated measures of metabolic genotype and phenotype tend to support the biochemical mechanisms inferred from experimental studies (Yu et al. 2002). It appears that, for this class of chemical carcinogens, the linkage between the experimental setting and the human condition is as strong as any. Non-occupational exposure to arylamines, a subclass of which are the alkylanilines, has been well documented in cigarette smokers (Skipper et al. 2010) and are believed to be the constituents of tobacco smoke that lead to the development of bladder cancer. Exposure to these alkylanilines potentially occurs through breathing ambient air containing combustion products or through the use of hair dyes. This class of aromatic amines is of particular interest because of demonstrated carcinogenicity in animals and humans and the broad exposure to many of these compounds (Kim and Guengerich 2005).

The investigators from the National Institute for Occupational Safety and Health have reported a correlation between *o*-toluidine and aniline exposure, noting the increased incidence of bladder cancer (Tannenbaum 1991; Robinson et al. 2001). Diphenylamine, which often contained *p*-aminobiphenyl, is reported as a human bladder carcinogenic agent as well (Tannenbaum 1991; Acquawell et al. 1991). The aromatic amine *p*-phenylenediamine, also listed as 1,4-benzenediamine; *p-phenyldiamine* and 4-phenylenediamine, is commonly found in hair dyes and is reported as a chemical that can damage the nervous system, cause lung irritation and cause severe allergic reactions (Chung et al. 1995).

The first concern with human exposure to carcinogenic aromatic amines arose in the dye manufacturing industry as early as the late nineteenth century (Weisburger 1997). It is worth noting that the many of the studies on the toxicity and carcinogenity of aromatic amines is due to the fact that they are the product of azo dye reduction. Only a few dyes have been found to be inhibitory to the microbial population in the aquatic environments (Pinheiro et al. 2004). However, there is ample evidence indicating that ingested azo dyes are reductively cleaved into aromatic amines. N,N-dimethylaminoazobenzene, commonly known as Methyl Yellow, had been used as a food colorant to enhance the colour of butter, but soon was discovered to be carcinogenic and toxic and was banned in the United States (Combes and Haveland-Smith 1982). Brown and De Vito (1993), in their extensive literature review, pointed out that there are three principal modes of activation for azo dyes: (i) azo dyes that are toxic only after reduction and cleavage of the azo linkage to give aromatic amines, (ii) azo dyes with a structure containing free aromatic amine groups that can be metabolically oxidized without azo reduction, and (iii) azo dyes that may be activated via direct oxidation of the azo linkage to highly reactive electrophilic diazonium salts. Azo reduction is, therefore, one of the most important metabolic activation steps in relation to the mutagenicity, carcinogenicity, and other possible biotoxicities of many azo dyes. Chung (2000) have reviewed the mutagenicity and carcinogenicity of aromatic amines metabolically produced from azo dyes reduction. They pointed out that azo reduction is an important step for the

genotoxicity of many azo dyes and the mutagenic moieties of most of these compounds are phenylenediamine and benzidine and their derivatives. A minor difference in the type and position of substituents in the molecular structure of phenylenediamine and benzidine can cause major differences both in mutagenic and carcinogenic activities (Chung and Cerniglia 1992; Chung et al. 1995). For the phenylenediamine moiety, they have observed that methylation or substitution of a nitro group for an amino group did not decrease the mutagenicity. However, sulfonation, carboxylation, deamination, or substitution of an ethyl alcohol for the hydrogen in the amino groups leads to a decrease in mutagenic activity. Experiments conducted by Chung et al. (1995) using Ames *Salmonella* strains TA98 and TA100, proved that 2-methyl-p-phenylenediamine and 2-nitro-*p*-phenylenediamine were more mutagenic than *p*-phenylenediamine, whereas 2-sulfo-*p*-phenylenediamine was not mutagenic. For the benzidine moiety, Chung and Cerniglia (1992) also observed that methylation, methoxylation, halogenation or substitution of an acetyl group for hydrogen in the amino group did not affect or, in some cases, even increased the mutagenicity, but complexation with copper ions diminished the mutagenicity. The analysis of relationships between chemical structures and genotoxicity such as mutagenicity and carcinogenicity can therefore help the identification and synthesis of useful compounds that are neither mutagenic nor carcinogenic.

For a long time, it was believed that only the polycyclic aromatic amines, but not the monocyclic amines have carcinogenic potential. This conviction was abandoned when occupational exposure to 4-chloro-*o*-toluidine was shown to produce bladder tumours in workers. *o*-toluidine had also to be classified as a carcinogen and the experimental results with aniline eventually put an end to this hypothesis. With each of a vast variety of monocyclic aromatic amines, *N*-hydroxylamines are metabolically formed under suitable conditions, and reactions with DNA and mutagenic activity can be demonstrated (Marques et al. 1997). No criterion can be defined at present that would allow to separate genotoxic from non-genotoxic, or carcinogenic from non-carcinogenic monocyclic arylamines (Kim and Guengerich 2005). This is primarily due to results indicating that the role of genotoxicity was overestimated. This became particularly apparent with the recent developments concerning aniline and structurally related amines. The discussion focused for a long time on the question: is Tests for mutagenicity gave contradictory results and because of the low genotoxic potency these data were considered not to be sufficient to explain the spleen tumours observed in rats (Wilmer et al. 1984; Bomhard and Herbold 2005). The studies with the most basic aromatic amine, aniline, show how intimately genotoxic and non-genotoxic effects are connected and that genotoxicity alone will not answer the question (Zwirner-Baier et al. 2003).

7.5 Non-biological Removal of Aromatic Amines

Aromatic amines have been known to be carcinogenic in humans, therefore their is an huge task for the researchers.

Several degradation methods of aromatic amines have been investigated and proposed, namely biological, chemical, physical, photocatalytic, electrocatalytic and advanced oxidation processes (AOPs). All of those methods have their own advantages and limitations and their application will be dependent on the real scenery.

The chemical oxidation of aniline and substituted primary aromatic amines yields a variety of products depending on the particular oxidant, structure of the aromatic amine and reaction conditions and sometimes polymerization can occur. The most common oxidants for waste water treatment include chlorine, chromate and permanganate (Casero et al. 1997). Oxidation with permanganate has been commonly used and it results in ring-cleavage and subsequent complete breakdown of the molecule, but, unfortunately, it is unsuitable for wastewater containing oxidable solvents such as methanol or ethanol, or large amounts of other oxidable substances. Decontamination of large volumes of aqueous solution is also inconvenient, as it requires large amounts of permanganate. Since iron is ubiquitous in the environment and is largely tolerated in living systems, there is growing interest in the use of high-valent iron as an alternative to common oxidants (Huang et al. 2001).

Among physical processes, adsorption produces good-quality effluents and is one of the most effective processes for removing aromatic amines or other pollutants. Activated carbon, fly ash, serpentine, activated alumina, bauxite, clays, zeolites, bentonite and other ecofriendly adsorbent exhibits a good capacity for removing aromatic amine from wastewater (Hocine et al. 2004; Kostelníková et al. 2008; Oda and Yokokawa 1983; Yadav et al. 2011). Recently, other advanced absorbents such as the chemically synthesized polymeric adsorbents and modified with functional groups (Jianguo et al. 2005), modified activated carbon (Han et al. 2006) and Carbon Nanotubes (CNT) (Yang et al. 2008; Al-Johani and Salam 2011) have been proposed. Adsorption processes merely transfer aromatic amines from one phase to another and, therefore, invariably generate sludge that must be disposed off, or regenerated, by some other process. Moreover, an adsorption process removes the aromatic amines from the wastewater by concentrating them on the surface, retaining their structure practically unchanged. When the support needs to be regenerated, the fate of the resulting concentrated sludge of aromatic amines presents a problem of correct disposal.

AOPs are alternative methods for the complete degradation of pollutants. Such methods have been reported to be effective for the near ambient degradation of soluble organic contaminants from waters and soils, because they can provide an almost total degradation. These methods are based on the generation of a very powerful oxidizing agent such as hydroxyl radical (•OH) which serve as an oxidizing agent for pollutants (Oturan and Brillas 2007). AOPs include photocatalysis systems such as combination of a semiconductor such as TiO_2, ZnO, WO_3, SnO_2, ZrO_2, CeO_2, CdS and ZnS, and UV light. TiO_2 has been widely used because of its various merits such as the large surface area (7–50 $m^2\ g^{-1}$), low cost, high photocatalytic activity, chemical activity and nontoxicity (Pereira et al. 2013). However, its applications have been limited for several reasons such as low photon utilization efficiency and need for a high power UV excitation source. One way to solve these

problems is the modification of catalysts by doping them with various metals such as Ag, Pt, Fe, Au, *etc*. (Ganesh et al. 2007; Osterloh 2008). The metals deposited or doped on TiO_2 act as electron traps, facilitating electron-hole separation and promoting the interfacial electron transfer process. In photocatalytic degradation of organic pollutants, the substrate molecules react with hole or, more probably, with hydroxyl radicals, to give a number of hydroxylated reaction intermediates. As example, Sánchez et al. (1998) have combined these TiO_2-assisted photocatalysis and ozonation for the removal of aniline from water and found that the decomposition rate of aniline is larger than when individually treated by either of the two methods. The combination of ozonation and photocatalysis with TiO_2 gives high yields of aniline degradation in aqueous solutions. Particularly, an ozonation pretreatment followed by photocatalysis significantly increases the yield of TOC removal in comparison to either ozonation or photocatalysis acting separately.

Fenton's reagent has also been used for oxidation of aromatic amines This reagent is a mixture of hydrogen peroxide and ferrous iron that produces •OH radicals. Such radicals have proved to effectively react with a variety of compounds such as alcohols, ethers, dyes, chlorinated phenols, pesticides, polycyclic aromatics, etc., in aqueous solutions and waste waters degradation (Casero et al. 1997). The advantage of Fenton's processes is the total mineralization of the organic compound treated to harmless compounds, for instance, carbon dioxide and water (Neyens and Baeyens 2003). Notwithstanding, it produces some unwanted compounds which are also not benign for the environment, such as ferric hydroxide sludge that requires additional separation and consequently Fenton's processes appear combined with electrochemical others. As example, the kinetics of 2,6-dimethylaniline degradation by Fenton process, electro-Fenton process and photoelectro-Fenton process was investigated by Masomboon et al. (2011). 2,6-dimethylaniline degradation in the photoelectro-Fenton process was superior to the ordinary Fenton and electro-Fenton processes. Actually, electrochemical advanced oxidation processes (EAOPs), based on the in situ electrogeneration of the highly reactive hydroxyl radical (•OH) have being developed as a promising environmental friendly technique. Torres et al. (2003) have studied the electrochemical oxidation, on Pt anodes, of industrial wastewaters containing 5-amino-6-methyl-2-benzimidazolone. At the best conditions, the compound was 100 % degraded in 45 min. However, because the reaction intermediates exhibited high toxicity and non-biodegradability, the electrolysis had to continue for 3 more hours in order to obtain a biocompatible solution that could further be mineralized in a fixed bed biological reactor. In the anodic oxidation using boron doped diamond (BDD), as it is a high O_2-overvoltage anode, degradation of the pollutants is mainly mediated by hydroxyl radicals formed at its surface from water oxidation (Panizza et al. 2008; Santos et al. 2010). These electrochemical processes using BDD are able to perform chemical conversion/combustion with high current efficiency, without the additional use of reactants and, consequently, in most cases without the formation of by-products. This electrode material presents also the advantages of chemical inertness and extended lifetime. BDD anodes have been used in the anodic oxidation of aromatic amines, such as aniline, metanilic and sulfanilic acids (Santos et al. 2010; Carvalho et al. 2006), p-aminophenol (Ratiu

et al. 2010) and naphthalenesulfonates (Panizza et al. 2006). Electrodegradation of aniline and its three monosulfonated forms (ortanilic, metanilic and sulfanilic acids) was study by Santos et al. (2010) in order to understand the influence of introducing in the structure of the aniline a sulfonic group in different relative positions to the amino group. Efficiency of degradation followed the order aniline > metanilic acid > sulfanilic acid > ortanilic acid. More recently, the influence of the different groups in the structure of the aniline on electrodegradation using a BDD anode was assessed by Pacheco et al. (2011) by testing 4 aromatic amines with different substituent groups, 3-amino-4-hydroxy-5-nitrobenzenesulfonic acid (A1), 5-amino-2-methoxybenzenesulfonic acid (A2), 2,4-dihydroxyaniline hydrochloride (A3) and benzene-1,4-diamine (A3). Results have shown a good electrodegradation of all the amines tested and the efficiency was A4 > A3 > A2 > A1.

7.6 Biodegradation of Aromatic Amines

According to literature and industrial expertise, biodegradation have been seen as the most effective, economical and environmentally ecological processes to remove organic pollutants from water as well as soil. Microorganisms play an essential role in recycling carbon and maintaining the health of the biosphere (Aust et al. 1994). Biodegradation takes advantage of this capacity. Bacteria have evolved to degrade most naturally occurring organic compounds, including the persistent aromatics. Moreover, the promiscuity of the catabolic enzymes allows bacteria to degrade, at least partially, xenobiotics that share similar structures with naturally occurring aromatic compounds (Díaz 2004). The bacterial catabolism of aromatic compounds involves a wide variety of peripheral pathways that activate structurally diverse substrates into a limited number of common intermediates that are further cleaved and processed by a few central pathways to the central metabolism of the cell (McLeod and Eltis 2008). Electron acceptors play a vital role for aromatic amine degradation in aerobic/anaerobic environments. In this context, degradation of aromatic compounds can occur under aerobic or anaerobic conditions, depending on the presence or absence of oxygen. Under aerobic degradation of aromatics, oxygen is always the final electron acceptor, but is also a cosubstrate for the two key processes, hydroxylation and oxygenolytic ring cleavage of the aromatic ring, carried out by oxygenases (Parales and Resnick 2006; Vaillancourt et al. 2006). On the contrary, under anaerobic conditions, degradation of aromatic compounds uses a completely different strategy, based on reductive reactions, to attack the aromatic ring. Many habitats containing large amounts of aromatic compounds are often anoxic, e.g., aquifers, aquatic sediments and submerged soils, sludge digesters, and intestinal contents, and at aerobic sites with high carbon concentrations, molecular oxygen is more rapidly consumed than replenished. Local microbial communities are capable of using locally available electron donors and acceptors to perform biodegradation of those aromatics (Carmona et al. 2009). This anaerobic mineralization can be coupled to anaerobic respiration with a variety of electron acceptors,

e.g., nitrate, sulfate, iron(III), manganese(II), and selenate, with each one conserving different yields of energy. The greatest energy conservation is reached when nitrate is the final electron acceptor, followed by ferric ion. The energy conservation when sulfate is the electron acceptor is much more limited (Carmona et al. 2009).

Since the redox potential of the electron-accepting system in the anaerobic breakdown of aromatic compounds dictates the biochemical strategy that is applied for the degradation of such compounds, there is wide biochemical diversity among anaerobic aromatic degraders. On the other hand, aromatic compounds can participate in anaerobic metabolism by serving as electron acceptors rather than electron donors, generally with accompanying modifications of ring substituents that do not perturb the benzene nucleus itself (Gibson and Harwood 2002).

7.6.1 Aerobic Biodegradation

Aniline is the simplest aromatic amine and extensive studies have been carried out on aerobic aniline degradation. Bacterial species of *Pseudomonas* (Hinteregger et al. 1992), *Comamonas* (Parales et al. 1997), *Acinetobacter* (Kim et al. 1997), *Rhodococcus* (Aoki et al. 1983), *Frateuria* (Murakumi et al. 1999), *Moraxella* (Zeyer et al. 1985) and *Nocardia* (Bachofer et al. 1975) have been shown to be able of degrading aniline and/or its derivatives. Highly aniline tolerant bacteria are desirable for environmental applications as well as for the biotransformation of aniline and its analogues into useful chemical products. *Pseudomonas* sp. are considered as the most aniline tolerant bacterial strains, utilizing concentrations of up to 32 mM (Konopka et al. 1989). Nevertheless, Liu et al. (2002) have isolated a novel bacterial strain, *Delftia* sp. AN3, that efficiently utilizes up to 53.8 mM (5000 mg L^{-1}) of aniline. *Delftia* sp. AN3 uses a *meta* cleavage pathway for aniline degradation. The authors have proposed the pathway of aniline degradation (Fig. 7.11). Enzymes involved in aniline degradation were analyzed and their catalytic parameters were determined. Börnick et al. (2001) and Worch et al. (2002) both measured degradation rates of aromatic amines in river water fed to biofilm water treatment systems. The 20 tested substances included aniline and several of its chloro, bromo, methyl, nitro and mixed derivatives, N,N-dimethylaniline, N-ethylaniline and 1-naphthylamine. Half-degradation times from 0.5 h for 1-naphthylamine to 35 h for 2,4,5-trichloroaniline were reported. For the latter compound, a biofilm adaptation period of 6 weeks resulted in a 20-fold increase in the biodegradation rate. Six amines, including aniline and *o*-toluidine, showed half-degradation times under 1 h. Toräng et al. (2002) compared the degradation rates of aniline in laboratory shake flask simulation tests with field rates in the river Rhine. The estimated half-life of aniline in the Rhine at 15 and 22 °C was 9 h and was consistent with laboratory batch test performed with concentrations below 25 μg L^{-1}. The results indicate that laboratory shake flask batch tests with low concentrations of test substance can be good predictors of degradation rates in natural water bodies. Wang et al. (2007) pointed out that the strain PN1001, isolated from the activated sludge of an oil refinery wastewater treatment plant, is a member of the *Pseudomonas species* capable of

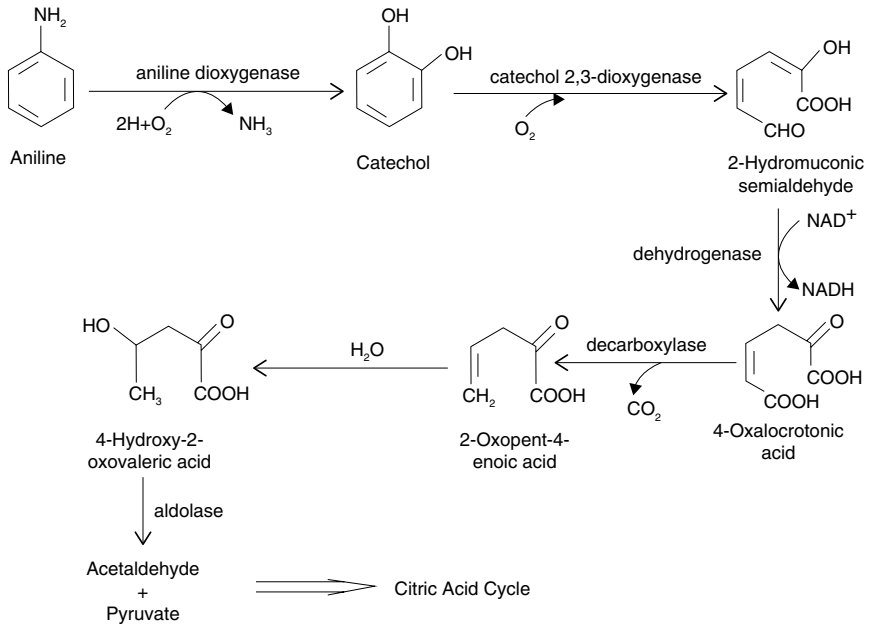


Fig. 7.11 Proposed pathway of aniline degradation by *Delfia* sp. AN3 (Liu et al. 2002)

degrading aniline. A group of aromatic amines that are more difficult to degrade even under aerobic conditions are represented by aryl sulfonates. The presence of sulfonated group on aromatic ring not only confers a xenobiotic character, but also recalcitrant nature to these compounds (Barsing et al. 2011). Though, the aerobic degradation of sulfonic acid (SA) by isolated cultures and by microbial communities samples from sites previously contaminated with aromatic amines has been reported (Tan et al. 1999). Some authors found, however, that SA is not degraded under aerobic conditions with activated sludge from a plant treating domestic effluent (Tan et al. 2005; Yemashova and Kalyuzhnyi 2006). Of all the ten sulfonated aromatic amines tested by Tan et al. (2005) for their aerobic and anaerobic biodegradability, and toxicity potential in a variety of environmental inocula compounds, only two aminobenzenesulfonic acid (ABS) isomers (2- and 4-ABS) were degraded. The observed degradation occurred only under aerobic conditions with inocula sources that were historically polluted with sulfonated aromatic amines. These results indicate that cultures adapted to the compounds to be degraded, can easily undergo biodegradation and also that there is some specificity relatively to the type of species involved in biodegradation. Carvalho et al. (2008) investigated that sulfanilic acid and aniline are easily degraded by three different types of aerobic inocula: domestic wastewater and activated sludge from municipal and industrial treatment plants. The idea that an easier degradation of SA would occur with biomass from an industrial treatment plant was not confirmed in this results. On the contrary, a longer lag phase was required, corroborating the idea that degradation of SA has a higher specificity. Also, its biodegradation was not observed with activated sludge from a lab scale reactor fed with glucose.

Nitroanilines are another important type of aromatic amines which formed from reduction of azo dyes and anthropogenic activities. Khalid and coworkers (2009) have reported on the degradation of nitroanilines by isolated species and a mixed microbial culture. They found that most of the nitroanilines are biodegradable within 48 h of incubation and that the mixed microbial culture is more efficient than isolated species. 2-Aminobenzene sulfonic acid (2-ABS)/4-ABS was easily biodegradable if proper enrichment culture was available. Aminobenzene sulfonic acids are degraded through ring cleavage pathway to release ammonia and methane in stoichiometric amounts. More recently studies on aromatic amines biodegradation are those of Barsing et al. (2011), Jin et al. (2012) and Zhang et al. (2011).

A few bacterial cultures, utilizing napthyl amines as the sole organic carbon source, have been also reported. *Sphingomonas sp*. Starin ICX could decolorise AO7 and degrade 1-amino-2-napthol (Coughlin et al. 2002). Sulfonated naphthylamines are among the most common products of bacterial decolorisation of azo dyes. The most intensively studied bacterial strain with the ability to degrade naphthalenesulfonates is *Sphingomonas xenophaga BN6,* which degrades various amino and hydroxy naphthalenesulfonates to the corresponding amino or hydroxysalicylic acids (Stolz (1999). A complete mineralization requires a co-culture consisting of *Sphingomonas xenophaga BN6* as well as *Pseudomonas Sp.* BN6 or other bacterial strains, which degrade substituted salicylic acids.

Recently, a Yeat strain, *Candida methanosorbosa* BP-6 was isolated from the wastewater pool of the old factory "Boruta" in Zgierz and tested, for the first time, on aniline biodegradation (Mucha et al. 2010). The strain growth well in the presence of aniline and could degrade it. Biodegradation intermediates and final products were identified by HPLC and the authors proposed the intradiolic pathway for aniline biodegradation.

Aerobic biodegradation of aromatic amines, however, can lead to the autooxidation of the aromatic amine with formation of larger and difficult to degradade molecules. Tan et al. (1999), have pointed out that 4-aminophenol rapidly suffered autoxidation to oligomeric or polymeric humic like substances, phenomena likely to happen with aromatic amines bearing hydroxyl substituents. The same phenomenon was mentioned for 1-amino-2-naphtol and 5-aminosalicylic acid, an effect which could effectively compete with biodegradation, increasing aromatic amine recalcitrance. Sulfonated *o*-amino hydroxybenzenes and *o-amino-hydoxynaphthalenes* were easily decomposed upon exposure to oxygen (Kudlich et al. 1999), Dimers and quinone derivatives were found among the oxidation products, some of which could be biodegraded by activated sludge.

7.6.2 Anaerobic Biodegradation

Aromatic amines are the reduced salt of nitro aromatics or azo compounds. The electron donating amino groups formed from the reduction of nitro and azo groups are estimated to carriage a serious problem to further reductive bio transformations

by anaerobic granules. Aromatic amines with carboxy, hydroxyl and methoxy substituents are potentially mineralizable in methanogenic consortia (Razo-Flores et al. 1997a). Aromatic amine reduction is a tough task for biodegradation in methanogenic environment. In anaerobic condition, nitrate (NO_3^-), sulfate (SO_4^{2-}), metals such as iron (Fe^{3+}) and manganese (Mn^{4+}), or even CO_2 can play the role of oxygen, accepting electrons from the degraded contaminant. In addition to new cell matter, the byproducts of anaerobic degradation may include nitrogen gas (N_2), hydrogen sulfide (H_2S), reduced forms of metals, and methane (CH_4), depending on the electron acceptor.

Razo-Flores et al. (1997b) have published the first work on the completely biodegradation of an azo dye in the absence of oxygen. The electrons required for the reductive cleavage of azo dyes by anaerobic microorganisms are known to be derived from co-substrates. 5-aminosalicylic acid (5-ASA) was incubated with the methanogenic consortia in the presence of the specific methanogenic inhibitor 2-bromoethane-sulfonate. Acetate was identified as the major intermediate formed, indicating that the degradation of 5-ASA occurs via acetogenic fermentation. However, when the biodegradability of aniline, amino phenols, amino benzoates and 5-aminosalicylate by methanogenic sludge was tested, all the compounds, except aniline, were at least partially biodegraded, though with lag-phases between 25 and 110 days and in some cases requiring pre-adaptation of the culture to 2-nitrophenol. Batch methanogenic toxicity and biodegradability of 2-, 3- and 4-aminobenzoic acids (ABA) as well as 4- and 5-aminosalicylic acids (ASA) have been studied in the presence of two mesophilic (Shell and cattle) and one thermophilic sludges. 5-aminosalicylic acid (5-ASA) could be completely mineralized by all the sludges tested, but 4-aminosalicylic acid (4-ASA) could not be degraded at all by any of the sludges. All three aminobenzoic acid are principally biodegradable, but sludge source and adaptation are essential. Both mesophilic sludges were able to perform a complete mineralization of 2- aminobenzoic acid but this was not a case for the thermophilic sludge. 3-aminobenzoic acid was not biodegraded only in the presence of the Shell sludge. On the contrary, 4-aminobenzoic acid was quantitatively mineralized only by the Shell sludge. All the adapted sludges were able to mineralize the corresponding amino-aromatics in N-deprived media. Cross-acclimatization trials showed that 2-aminobenzoic acid, 5-aminosalicylic acid and salicylic acid adapted sludges were unable to degrade any other amino-aromatics tested that manifest about a different nature of key bacteria responsible for primary decomposition of these substrates. All the aromatic amines tested practically did not have any toxic effect on methanogenosis up to their concentration 3–7 g L^{-1}, moreover some of them even exert a stimulating effect on acetoclastic activity, especially when not very active sludges are used.

Citrobacter freundii strain, WA1, was isolated from a 5-aminosalicylate degrading methanogenic consortium (Savelieva et al. 2004) The methanogenic enrichment culture degraded 5-aminosalicylate completely to CH_4, CO_2 and NH_4^+, while *C. freundii* strain WA1 reduced 5-amino salicylate with simultaneous deamination to 2-hydroxy-benzyl alcohol during anaerobic growth with electron donors such as pyruvate, glucose or serine. When grown on pyruvate, *C. freundii* WA1 converted

3-aminobenzoate to benzyl alcohol and also reduced benzaldehyde to benzylalcohol. Pyruvate was fermented to acetate, CO_2, H_2 and small amounts of lactate, succinate and formate. Less lactate (30 %) was produced from pyruvate when *C. freundii* WA1 grew with 5-aminosalicylate as co-substrate. Chen et al. (2009) have demonstrated that an integrated anaerobic and aerobic biofilm reactor system, with a high level of aerobic effluent recirculation to the anaerobic reactor, can effectively treat highly concentrated and toxic nitrogenous aniline wastewater, with the simultaneous removal of carbon and nitrogen. In this system, ammonification, methanogenesis, and denitrification reactions occur simultaneously in a single anaerobic biofilm reactor, and the aerobic reactor is used for further COD reduction and autotrophic nitrification. Complete degradation of aniline by the facultative fraction of methanogenic granular sludge exposed to oxygen was described by Tan et al. (1999). Conversely, sulfanilic acid and 5-aminosalicylic acid mineralization required the addition of an aerobic enrichment culture obtained from river sediments exposed to sulfonated aromatic compounds. More recently, Pereira et al. (2011) investigated the fate of the aromatic amines aniline and sulfanilic acid, under denitrifying conditions in up flow anaerobic sludge blanket reactors (UASB). The results demonstrated that, in reactor 1 containing nitrate as electron acceptor, aniline could be degraded under denitrifying conditions while sulfanilic acid remained. In the reactor 2, containing nitrite as electron acceptor, a chemical reaction led to immediate disappearance of both aromatic amines and the formation of an intense yellow coloured solution. The reason to test nitrite was based on the fact that the first step of denitrification yields nitrite, a compound that has been found to react with aromatic amines, resulting in deamination, thereby yielding aromatics with a higher biodegradation potential (Seymour et al. 2002). Investigation on the degradation of aminoaromatic compounds by anaerobic microbial communities from lake sediments promotes apprehension of mechanisms of self-clarification of natural habitats (Lin'kova et al. 2011). Understanding of anaerobic biodegradation of (amino) aromatic substances allows predicting the destiny of these xenobiotics in nature and treatment facilities and is a fundamental basis for creation of specific biotechnologies for abatement with such pollutions.

7.6.3 Kinetics of Aromatic Amine Biodegradation

In order to determine the lifetimes of organic chemicals, under the assumption that degradation mechanism is being present, kinetics studies are carried out and can be evaluated by monitoring its disappearance, at different compound concentrations, during a certain time interval until steady state. The obtained results indicate the time required for the attainment of equilibrium during the biodegradation of the organic compound. The knowledge of kinetic parameters of aromatic amine biodegradation is also a very important issue both for the prediction of their fate and for the design of wastewater treatment plants. Batch assays are typically used to measure kinetic characteristics. Various analytical techniques are applied to monitor

these studies, which were described before, however, for monitorisation over time of reaction, UV-vis in the case of coloured samples, and HPLC either for coloured and non-coloured samples, are the most common due to their simplicity, low maintenance and low labour and low cost.

Although biological degradation is very complex in nature, several investigations have shown that the overall kinetics are typically described by the pseudo-first order rate law (Mälzer et al. 1993; Worch et al. 2002). The first-order degradation kinetics may be expressed as follows (Eq. 7.1):

$$\frac{dC}{dt} = K_1 \mathrm{C} \tag{7.1}$$

Where, **C** represents the concentration of a degraded compound at the time **t** and $\boldsymbol{K_1}$ is the first-order rate constant. $\mathbf{C_0}$ **and** $\mathbf{C_t}$**,** are the initial and the residual, at certain time, concentrations, respectively. . In practice, the first-order rate constant often is replaced by a half-life, **H**, and the degradation rate is expressed as follows where H is $0.693/K_1$ (Eqs. 7.2 and 7.3):

$$\frac{dC}{dt} = -\frac{0.6933C}{H} \tag{7.2}$$

$$\ln\left(C_t\right) = \ln\left(C_0\right) - \frac{0.693t}{H} \tag{7.3}$$

If the initial rate of the reaction is measured over a range of substrate concentrations, denoted as [S], the reaction rate (v) increases as [S] increases. However, as [S] gets higher, the enzyme becomes saturated with substrate and the rate reaches the enzyme's maximum rate, $V_{\max}$.

The kinetic constants, K_{M} and $V_{\max}$, are critical to attempts to understand how enzymes work together to control metabolism and are calculated by the Michaelis–Menten equation (Eq. 7.4):

$$V0 = \frac{V_{\max}\left[S\right]}{Km + \left[S\right]} \tag{7.4}$$

where V0 is the initial rate and Km is the concentration of substrate that leads to half-maximal velocity.

In many cases, at higher substrate concentration, inhibition occurs and the rate of reaction decreases.

The measured kinetics of biodegradation of a single organic compound as sole carbon source in a batch reactor depend on the history of the culture, the identifiability of the parameters, and the manner in which the experiment to measure them is run. The initial substrate to biomass ratio used in the experiment is particularly important because it influences both parameter identifiability and the expression of the culture history.

Carvalho et al. (2008) reported that sulfanilic acid biodegradation by aerobic microorganisms appears as a sigmoid curve and the kinetic parameters followed the pseudo first order kinetics. Worch et al. (2002) demonstrated that biodegradation kinetics of aromatic amines fitted by a pseudo-first order. Taking into account the possible analytical errors, from a first approximation it was assumed that the degradation curves follow a first order rate law. The rate constants estimated in that series of experiments range from about 2 h^{-1} for aniline t about 0.03 h^{-1} for 2,4,5-trichloroaniline. The half-lives ranged from 20 min for aniline to more than 20 h for 2,4,5-trichloroaniline.

The activities of aniline dioxygenase, catechol 2,3-dioxygenase and other enzymes involved in aniline degradation were determined by Liu et al. (2002). The *Km* and *Vmax* of aniline dioxygenase were 0.29 mM and 0.043 mmol $mg_{protein}^{-1}$ min^{-1}, respectively. The *Km* and *Vmax* of catechol 2,3-dioxygenase for catechol were 0.016 mM and 0.015 mmol $mg_{protein}^{-1}$ min^{-1}, respectively.

7.6.4 Factors Effecting Biodegradation

Different biological processes for aromatic amine degradation have been discussed previously. All of them present vantages and disadvantages and their appropriate adoption depends on the special application. In this chapter, one could not fail to mention that those processes and their rates depend in turn on the nature of the environment as well as the native properties of the aromatic amine. The aromatic amine biodegradation studies on aerobic/anaerobic technology have mainly been focused on general aspects on type of electron acceptors, effects of substrate type, organic loading rate, dissolved oxygen concentration, COD:N:P ratio, effect of pH, effect of temperature, types of reactor and characteristics of the sludge. Some other special factors may equally affect the biodegradation. Among these factors, the presence of heavy metals will also affect the reactions and is supposed as the reason behind microbial aggregation. Liu et al. (2002) have earlier described that the heavy metal ions Hg^{2+} and Ag^{+} are toxic to growing cells, and completely inhibition of growth occurred at 0.02 mM Hg^{2+} and 0.1 mM Ag^{+}. In their study, a bacterial strain, AN3, was able to use aniline as sole carbon, nitrogen and energy sources. The optimal temperature and pH for growth and degradation of aniline were 30 °C and 7.0, respectively. The efficiency of aniline degradation increased gradually when the temperature was increased from 10 to 30 °C. Complete degradation occurred when aniline concentration was below 22 mM, but above this, the degree of degradation decrease and only 20 % was obtained at the maximal concentration tested. Cell growth simultaneous with aniline degradation and inhibition was observed at aniline concentrations above 32 mM, explaining the decrease on the process effectiveness.

Khalid and co-authors (2009) have studied the effect of many parameters on the biodegradation of 4-nitroaniline under aerobic conditions by pure and mixed bacterial cultures. Individual strains were unable to degrade 4-nitroaniline

completely even with longer incubation period (168 h) but achieved rapid degradation when grown together: 92 % degradation in 48 h, followed by complete degradation at 72 h. Authors have assumed that the combined enzyme systems of the mixed bacterial culture were more effective than the enzymes from the individual isolate. Alternatively, cooperation within microbial communities can occur through exchange of growth cofactors and removal of toxic metabolites. Degradation of 4-nitroaniline by the bacterial consortium was inhibited when the substrate concentrations increased above 0.1 mmol L^{-1}, and there was an inverse linear relationship between the rate of degradation reaction and 4-nitroaniline concentrations over the range between 0.1 and 1 mmol L^{-1}. The authors have also observed that the addition of yeast extract up to 5 g L^{-1} to the medium accelerated the biodegradation of 4-nitroaniline by several orders of magnitude, possibly by acting as a bacterial growth-promoting factor. In contrast, addition of glucose, sucrose, mannitol or NH_4NO_3 had strong inhibitory effects on the degradation of 4-nitroaniline by the mixed bacterial culture, what was attributed to preferential use of these sources of carbon and nitrogen by the bacteria for their growth. The degradation of 4-nitroaniline by the bacterial consortium was optimal at slightly alkaline pH (7.2) with incubation at 35 °C under aerobic conditions. The degradation of 4-nitroaniline by the bacterial consortium was optimal at slightly alkaline pH, 7.2, with incubation at 35 °C under aerobic conditions. Similar optimal pH and temperature conditions were also found by Lin'kova et al. (2011) for the biodegradation of 2- and 4-aminobenzoic acid and 5-aminosalicylic acid by anaerobic microbial communities (30 °C and pH of 6.5–7.5).

Kalyuzhnyi et al. (2000) have studied the biodegradability of 2-, 3- and 4-aminobenzoic acids (ABA) as well as 4- and 5-aminosalicylic acids (ASA) in the presence of two mesophilic (Shell and cattle) and one thermophilic sludges. 5-ASA was completely mineralised by all the sludges used. On the other hand, 4-ASA was not degraded at all by any of the sludges used. Both mesophilic sludges were able to perform a complete mineralization of 2-ABA but this was not a case for the thermophilic sludge. 3-ABA was not biodegraded only in the presence of the Shell sludge. This finding emphasizes an extreme importance of substituent position in xenobiotic compounds to be biodegraded. At the same time, it is notice that the process is affected by the tip of biomass use. In addition, when previously adapted, sludges were able to mineralize, but only, the corresponding aminoaromatics.

The presence of salts, special in textile wastewaters, can also affect the process. O'Neill and co-authors (2000) have investigated the degradation of aniline by bacterial consortia under aerobic, fermentative, nitrate-reducing and sulphate-reducing conditions and a variety of salt concentrations, 0.2, 4 and 7 % NaCl w/v, and pH values, 5 and 7. Degradation of aniline was achieved under all combinations of salinity, however, the rate of bacterial growth decreased with increasing salinity. Cultures were able to adapt to changes in pH and to most changes in salinity, suggesting that in treatment plants the microflora may be able to cope with fluctuations in salinity and pH. Growth rates were unaffected by aniline concentration in the range of 0.25–1 g L^{-1} and an additional nitrogen source, two parameters of wastewater which are also likely to fluctuate.

7.7 Conclusions

Aromatic amines are important industrial compounds and their use is widespread. They range from the simple structure of aniline to most complex ones. Furthermore, aromatic amines are used in the synthesis of many compounds and are part of their integral structure, such as the azo dyes. Those compounds result also from common daily human activities such as cooking, smoking, diesel exhaust, combustion of wood chips and rubber, etc. The high impact of those compounds as pollutants and the increasing knowledge on their carcinogenic properties, leads to the rise interest on their detection, their fate, monitorization and degradation. Progress in the detection and treatment methods has, thus, being made. Combined methods such as HPLC-MS, GC-MS, CE-MS seem to be very effective, special for the monitorization of degrading reactions and when quantification rigor is need, once the results obtained from the complementary analyses give a more confident and complete information. Although, the complexity and costs of those methods make UV-visible spectroscopy and/or HPLC very common when only punctual monitorization is required.

Among the various methods on aromatic amines degradation, biodegradation seems to be promising. Researchers have been testing different biodegradation conditions and, with regard to electron acceptor, both aerobic and anaerobic conditions have been reported. However, scarce information has been stated to elucidate the mechanisms. Aromatic amines are commonly difficult to degrade under anaerobic conditions. Some of them, substituted with hydroxyl or carboxyl group, were degraded under methanogenic and sulphate reducing conditions. A drawback of using aerobic treatments is that many of them are prone to autoxidation once they are exposed to oxygen, resulting in bigger molecules with lower biodegradability. Other electron acceptors such as nitrate, iron, sulphate, manganese and carbonate have, alternatively, been tested and successful results achieved.

7.8 Future Perspectives

While the aerobic catabolism of aromatic compounds has been extensively studied, the anaerobic degradation of aromatics is a more recently discovered microbial capacity that still awaits a deeper understanding despite the fact that microbial metabolism in the absence of oxygen is the most ancient of all life processes. The natural attenuation of pollution carried by different microorganisms although may be improved by engineered techniques, either by bioaugmentation (addition of selected microorganisms) or by biostimulation (addition of nutrients) and by genetic engineer. However, the biochemical and genetic bases of the anaerobic degradation of aromatic compounds are not very well established yet due mainly to the difficulties in routinely growing and genetically manipulating the aromatic-degrading microorganisms. The reaction mechanisms are not yet clear, accordingly further

studies are needed. On the other hand, the effect of reaction conditions still need to be better elucidated. The increased use of high-throughput techniques such as genomics, metagenomics, proteomics, metabolomics, as well as systems biology approaches for addressing biological complexity from a holistic perspective and the application of the network theory to biology will certainly contribute significantly to unraveling the intricate regulatory and metabolic networks that govern the anaerobic biodegradation of aromatic compounds as well as their distribution and ecophysiological relevance to carbon flux in the environment.

The knowledge of the environmental impact of aromatic amines is fundamental and, though the many toxicological studies, effect of this compounds at very little amount has to be continuously evaluated. In this way, the wide analytical techniques available are fundamental and studies may integrate combined methods to complement the information given by each. It is worth noting that also the analytical techniques are constantly evolving.

Researchers do not stop testing different methods of pollutants remediation. In view of biodegradation, different microorganisms and conditions will be tested. As pointed in this chapter, electron acceptors play a very important role and, therefore, other chemicals and/or novel materials need to be prepared and experienced. The advances in materials sciences, special on nanomaterials, may open a new world on the biotechnological processes and a big way has yet to be traveled.

A very important aspect of organic compound degradation in the environment is the possibility of applying these processes in natural and industrial environment, and at large scale. Accordingly, it is necessary to know the processes in full detail, so that their effectiveness can be controlled.

Concerning the chemical manufactures, the progress is to avoid the use of the most dangerous compounds and the substitution by similar innocuous ones. Also the technology has to be developed in order to release as less as possible chemicals to the environment.

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