Bioindicators and biomonitors
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Chapter 17
Molluscs as bioindicators

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Abstract

The ecological role and importance of molluscs as one of the most species-rich phyla of the animal kingdom are briefly summarised with special emphasis on certain characteristics, which make them especially suited for monitoring programmes in the field. The advantages, perspectives and limitations for the use of terrestrial and aquatic molluscs for the monitoring of chemical stressors in their specific environment are compared. Furthermore, examples of bioaccumulation and biological effect monitoring surveys are given with a differentiation of sub-organism, organism and community level effects for the latter. Finally, the possibilities for monitoring of tributyltin compounds in coastal and freshwater ecosystem are demonstrated as a case study.

Keywords: molluscs, accumulation, biological effects, biomarker, monitoring, indication, endocrine disrupters, tributyltin

1. Introduction: The "molluscan gap" – ecological relevance of molluscs and their role in ecotoxicology

The molluscs represent one of the most diverse and species-rich phyla of the animal kingdom. With more than 130,000 known recent species they are only second to the arthropods (Gruner, 1993; Gruner et al., 1993). Of the seven molluscan classes, gastropods make up more than 80% of the species with bivalves constituting the major part of the rest (15%). The other five classes, in decreasing species numbers, are the Polyplacophora, the highly evolved cephalopods and finally the scaphopods, Aplacophora and Monoplacophora (Table 1). Especially the cephalopods exhibit a comparative degree of physiological and neuronal complexity and organisation as vertebrates. Cephalopods from the genus *Architeuthis* represent with an overall length of more than 20 m the largest living invertebrates, but also the smallest metazoans can be found within the molluscs, some Aplacophora with a size of less than 1 mm.

Although molluscs are basically a marine group of animals, gastropods and bivalves have also expanded their distribution to various freshwater environments. Gastropods have additionally penetrated into a huge variety of terrestrial habitats so that molluscs can be found today from the abysses of the sea to mudflats, from lakes and rivers and their banks to forests, alpine mountains, but also in steppes and desserts; they occur on nearly all latitudes of the planet from polar to tropical temperatures (Hyman, 1967; Purchon, 1968).

Table 1. Overview of the different classes and subclasses within the phylum Mollusca indicating their species numbers and geographical distribution.

Class Subclass	Number of species	Distribution
Aplacophora	250	Exclusively marine benthic organisms; from low tide level to depths > 6000 m
Polyplacophora	1000	Exclusively marine benthic organisms; mainly in the eulittoral zone, but also in depths up to 4000 m
Monoplacophora	20	Exclusively marine benthic organisms in depths between 170 and 6500 m; reported from the Pacific, south Atlantic, the Antarctic region and the Indian Ocean (Gulf of Aden)
Gastropoda	110,000	Cosmopolitan in all terrestrial, freshwater and marine environments, including steppes, desserts, alpine mountains, polar regions, the deep sea and the pelagic zone
Prosobranchia	60,000	
Pulmonata	44,000	
Opisthobranchia	6000	
Bivalvia	20,500	Cosmopolitan in all freshwater and marine environments from the eulittoral to the abyssal zone and from tropical to polar regions
Protobranchia	550	
Filibranchia	2200	
Eulamellibranchia	17,500	
Septibranchia	250	
Scaphopoda	350	Exclusively marine cosmopolitans; in sediments from the eulittoral zone to depths of 7000 m
Cephalopoda	760	Exclusively marine cosmopolitans, as benthic or pelagic organisms from surface waters to the deep sea

Due to their ubiquitous distribution and enormous species number, molluscs play important ecological roles in the different aquatic and terrestrial ecosystems of the world. They provide key species for ecosystem functioning, e.g. for litter decomposition but also because they contribute to huge amounts of the biomass on the different trophic levels in ecosystems (from primary consumers to top predators). Many other groups feed on molluscs, like echinoderms, fish, birds and mammals. Molluscs act as vectors for a number of human-relevant parasites and diseases, e.g. as intermediary hosts of trematodes, and a number of mollusc species live as endoparasites themselves (Purchon, 1968; Götting, 1996).

In contrast to their ecological importance, the contribution of molluscs to ecotoxicological research and routine measurements in the laboratory is much smaller. This is especially true for the standard testing of chemicals, where species from other invertebrate groups, like arthropods, mainly insects and crustaceans, but also nematodes and annelids are much more considered. This is particularly due to the fact that life cycles of molluscs are normally longer than of most other invertebrate test species and that the maintenance of healthy mollusc brood stocks requires more technical and thus financial efforts, namely for those aquatic species which have a planktonic larval phase. The exception from this general rule is the field of bioindication and biomonitoring where molluscs have been successfully used to obtain information on the quality of terrestrial, marine and freshwater ecosystems and to quantify the exposure to and effects of contaminants in their environment (Markert et al., 1999). This is particularly the case for the two most diverse classes of molluscs, gastropods and bivalves, while cephalopods play a more secondary role, and representatives from the remaining four classes have not been used as bioindicators according to the published literature.

As canaries already warned miners in the European coal mines of the 19th century of "bad air", methane occurrence and the danger of firedamp explosions, it has long been known that molluscs are indicators of poor water quality. More than 90 years ago, Ortmann (1909) described for streams in North America and Kolkwitz and Marsson (1909) for German surface waters that the lack or decline of freshwater mussel populations is an effect of pollutants. In 1976, the "Mussel Watch" was initiated in the United States of America as one of the first geographical large scale environmental surveillance programmes which made use of living organisms (Goldberg, 1975; Goldberg et al., 1978). The original scheme covered a coordinated and standardised sampling and measurement of pollutants in four bivalve species (two *Mytilus* species, *Crassostrea virginica* and *Ostrea equestris*) at more than 100 sample sites on the coast of North America. The mollusc tissues were analysed for heavy metals, radionuclids, halogenated hydrocarbons, and petroleum hydrocarbons and provided useful data on baseline levels of these substances. The mussel watch programme was adopted by further countries in the following years and was employed almost on a global scale so that information on coastal ecosystem pollution with certain substances is available today worldwide (cf. 3.1).

Molluscs are for a number of reasons well suited as bioindicators or biomonitors. Although these aspects are also shared with other systematic groups of the animal kingdom, it is the unique combination of these different features which characterises molluscs as ideal bioindicators. The most important characteristics are:

- Gastropods and bivalves are widespread and abundant in all marine and freshwater ecosystems worldwide. Additionally, gastropods can also be found in almost all terrestrial environments.
- Although some terrestrial gastropods are endemic with a rather limited distribution, most molluscs, especially those living in the aquatic environment, exhibit a broad distribution within and even between continents, facilitating their use in geographical large scale surveys. Furthermore, a number of species and genera are even cosmopolitans (e.g. mussels of the genus *Mytilus* with the two species *M. edulis* and *M. galloprovincialis* being the most widespread).
- Many molluscs are key species for the functioning of marine, freshwater and terrestrial ecosystems so that it is likely that a pollutant that affects such a mollusc population will also exhibit a negative impact for the entire ecosystem. Examples of pollution effects on the ecosystem level, caused by an interference with mollusc populations, will be provided in the Section 3.3.
- The majority of gastropod and bivalve species exhibit an extremely limited mobility or are completely sessile as adults. Therefore, these molluscs represent

the contamination of their habitat ideally. The only exceptions are pelagic snails from the prosobranch genus *Janthina*, the Heteropoda (*Atlanta*, *Carinaria*, *Pterotrachea*) and Pteropoda (*Hyalela*, *Creseis*, *Styliola*) (Fioroni, 1981). Most of the aquatic mollusc species, especially in the temperate, subtropical and tropical region, have a planktonic larval stage which guarantees a high dispersal potential and allows a recruitment of populations even in those habitats where sexually mature adults might have become extinct due to the high level of contamination.

- Molluscs represent a broad variety of reproductive modes, like simultaneous and consecutive hermaphroditism, gonochory and parthenogenesis, each of them combined with semelparity or iteroparity so that effects of contaminants affecting specifically these types of reproduction can be monitored. Furthermore, molluscs exhibit an extraordinary variation of life-cycle-strategies, especially with respect to their longevity. While the majority of the cephalopods, the marine opisthobranch snails and most of the freshwater and terrestrial gastropods are short living species with a maximum life span of one year, the marine prosobranch snails and many bivalves are long-living so that they can integrate contaminations of their environment over long time periods. Approximately 40% of the marine bivalve species and more than 20% of the marine prosobranches attain maximum ages of more than 14 years according to Heller (1990). For single species even longer life spans have been reported, like for example more than 50 years for the abalone *Haliotis cracherodii* by Powell and Cummins (1985) and more than 100 years for some marine (120 years for *Panope generosa*, 150 years for *Crenomytilus grayanus* and 220 years for *Arctica islandica* according to Jones, 1983) and freshwater bivalves (116 years for *Margaritifera margaritifera* according to Bauer, 1987).
- Most gastropod and bivalve species used for biomonitoring and bioindication purposes are relatively large and therefore easy to handle and to culture. Consequently, they can be used both under laboratory and field conditions, for active and passive biomonitoring.
- Due to the lack of an exoskeleton, as it is present in arthropods, molluscs are in direct contact with the ambient medium (water or soil). Therefore, chemicals can be taken up not only from the diet (via the gastro-intestinal tract) but also additionally from ambient water or soil via the integument, including the respiratory organs in aquatic species, resulting in a greater accumulation potency for contaminants.
- Compared with other invertebrate groups like arthropods and especially vertebrates, molluscs exhibit only a limited ability to excrete pollutants directly via their kidneys or other excretory organs and tissues, to metabolise organic chemicals, and to physiologically inactivate toxic heavy metals, e.g. by the formation of and binding to metallothioneins (Lee, 1985; Berger et al., 1995a; Legierse et al., 1998). As a consequence, molluscs attain higher bioaccumulation or bioconcentration factors for many toxicants than other systematic groups. Therefore, pollutants might exhibit negative impacts on molluscs at lower environmental concentrations than on other invertebrates or vertebrates, facilitating their use as a kind of ecological early warning system.
- The high sensitivity of molluscs to environmental chemicals is also represented by the fact that they contribute in a disproportionate extent to the "red lists" of

endangered wildlife species worldwide. Recent data from Germany indicate that not less than 204 (= 61%) of the 333 occurring freshwater and terrestrial mollusc species are rated as threatened or already extinct (Jungbluth and von Knorre, 1995). Similar data are only available for Sweden and Madeira, where 25 of 133 and 72 of 190 terrestrial gastropods are endangered or already exterminated (Waldén, 1986). Unfortunately, for the marine and coastal environment no comparable surveys have been made in the past but there are numerous reports of population declines for a number of marine molluscan species which have attained a specific economic or scientific interest. For some of these reports it has been shown that environmental chemicals are the causative agent (cf. 3.4).

- The internal organisation, especially the normal morphological and histological structure of the different organs and tissues, and the physiology of the most abundant gastropod and bivalve species used for biomonitoring is characterised quite well. Our knowledge on the biology and ecology of these species has improved considerably in recent decades.
- Consequently, biological effects of environmental stress in general and of contaminant exposure in particular are measurable at various levels of biological organisation (from molecules to communities).
- Molluscs are non-controversial as organisms for ecotoxicological research, especially as test animals and for environmental monitoring.

2. Terrestrial bioindication and biomonitoring with molluscs

Gastropods represent the only molluscan class in terrestrial ecosystems and consequently, snails are the only molluscs which can be used for bioindication and biomonitoring purposes in these environments. Most terrestrial gastropods belong to the class pulmonates (order Stylommatophora), but also the members of the two prosobranch subtropical and tropical families Hydrocenidae and Helicinidae and the highly endangered European littorinid snail *Pomatias elegans* are living in terrestrial environments (Gruner, 1993).

For the time being, molluscs play clearly a secondary role in the surveillance of terrestrial ecosystems when compared with lichens, bryophytes, vascular plants and other invertebrate groups like nematodes, annelids and arthropods. Nevertheless, biomonitoring attempts with snails have found an increasing interest during the last decade and a number of promising projects have already been conducted. Most of them made use of the snails' bioaccumulation potential for metals and organic contaminants, but there are also examples for surveys which assessed the biological effects of soil contaminants on different levels of biological organisation.

2.1. Bioaccumulation

A number of invertebrate species are known to be efficient accumulators of trace elements (Dallinger, 1994). Generally, metal accumulation by such organisms is favoured by their limited ability to excrete these contaminants directly after their uptake and also by efficient physiological inactivation mechanisms, such as intracellular compartmentalisation, or metal inactivation by binding to metallothioneins (cf. 2.2). Such biological accumulators have often been used as accumulation indicators of environmental metal pollution. Ideally, metal concentrations in the animal's body reflect environmental pollution levels quantitatively. In reality, however, many factors like the nutritional, physiological and reproductive status, the sex and age of the animals influence such quantitative relationships. Therefore, these factors have to be considered carefully before invertebrates in general and particularly terrestrial snails can be utilised as accumulation indicator for metal pollution. Coughtrey and Martin (1977) compared the concentrations of Cd, Cu, Pb and Zn in the garden snail *Helix aspersa* collected from sites of varying degree of metal contamination. Already in this early study the authors found a positive linear relationship between metal uptake and body weight and concluded that molluscs of similar weight and/or size should be used for monitoring purposes. The different patterns of metal uptake by different organs were interpreted as a result of the organ-specific physiological activity. Even today with our more detailed knowledge on the physiology of pollutant accumulation and the role of detoxification enzymes and metallothioneins (cf. 2.2), the demand of Coughtrey and Martin (1977) for a comprehensive study of the physiological aspects of heavy metal uptake in molluscs is still valid.

There are numerous reports on the use of pulmonate snails as accumulation bioindicators so that only a limited number of examples can be presented here. A common characteristic of all these programmes is that their geographical focus is local or regional at its best, but that no attempts have been made so far to perform larger geographical surveys. One of the few exceptions is the use of slugs (*Arion rufus*, *A. ater*, *Limax cinereoniger*, *L. maximus*) within the "Ecological Effect Cadaster Baden-Württemberg" in Germany, which is designed as a long range monitoring programme and now running since several years (Spang, 1995). Adult snails of comparable size are sampled in autumn each year at an extended number of permanent surveillance sites all over the country with a sample size of 5 to 10 specimens. After a defecation period of three days in the laboratory the entire snails are frozen, the tissues homogenised, freeze-dried and analysed for a number of metals and organic pollutants.

Gomot de Vaufleury and Pihan (2000) used young garden snails (*Helix aspersa*) for an active biomonitoring of metals in France. About two months old snails from the laboratory were caged and exposed for four weeks on the soil in different areas, including a forest remote from human activities as a field control, urban and industrial sites, including waste disposal dumps. Additionally, laboratory controls were analysed in parallel. At the end of the experiment, mortality, growth (cf. 2.3) and accumulation of Cd, Cu, Pb and Zn in the foot and the visceral complex were measured. While the Cu concentrations where almost identical in the foot and visceral complex at all analysed sites indicating a strong homeostasis ability of the snails for this essential element, the tissue concentrations of Cd, Pb and Zn in the visceral complex where 2- to 50-fold higher compared to the foot in the different areas. Furthermore, the snail accumulated significantly higher amounts of Pb and Zn at the contaminated sites when compared with the laboratory and field controls (Fig. 1).

The main advantage of such an active biomonitoring approach is that it can be easily modified to consider also many other groups of contaminants, like pesticides, polycyclic aromatic hydrocarbons (PAH), etc. (e.g. Coeurdassier et al., 2001), although

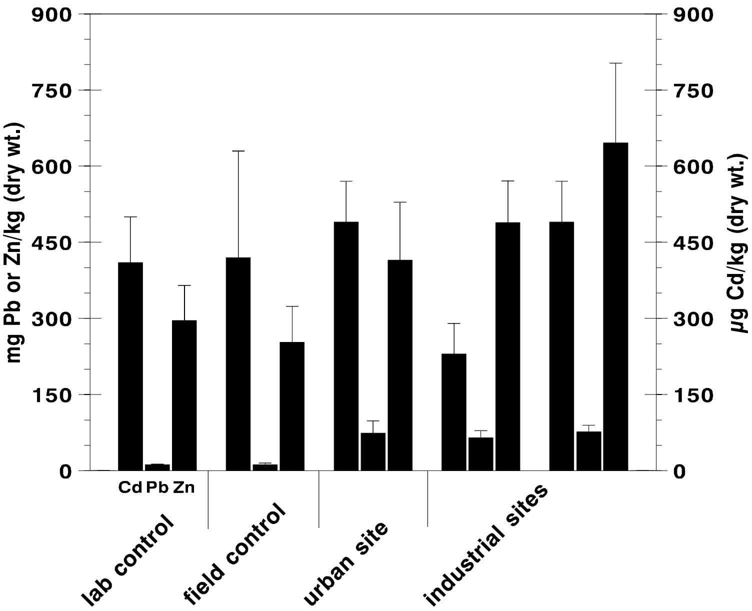

Figure 1. Concentrations of cadmium, lead and zinc in the visceral complex of young garden snails (*Helix aspersa*) transplanted from the laboratory to different exposure sites in France (values from Gomot de Vaufleury & Pihan, 2000).

heavy metals and other trace elements have gained much more interest than organic pollutants in the past. Bertani et al. (1994) used the land snail *Eobania vermiculata* to monitor the mercury exposure in Italy in an area affected by a chlor-alkali complex. Additionally, Hg residues were also assessed in plants, soils and air from the same region. The results indicate a low bioavailability of Hg for the snails as the mercury levels within the gut exceeded with values between 0.2 and 5.97 mg/kg (dry wt.) those in the soft tissues of the snails with 0.03 to 0.72 mg/kg (dry wt.). The latter concentrations were in the same range as measured in plants at the same sampling sites indicating a low bioaccumulation of Hg in this snail species. Gaso et al. (1995) conducted a study of radionuclide pollution with ^{226}Ra, ^{137}Cs and ^{40}K in the surroundings of a storage centre for radioactive wastes in Mexico. The parallel measurements of radionuclide residues in *Helix aspersa* and in soil samples from the same stations allowed a calculation of bioaccumulation factors, which were generally lower than 1 (5.5×10^{-2} and 2.0×10^{-3} for ^{226}Ra and ^{137}Cs in soft tissues, respectively). Nevertheless, the ^{226}Ra levels in these snails exceeded those measured at a reference site 100 km away by a factor of 9.

A further advantage of these types of active surveys is that they can also be used to evaluate the contamination of industrial waste dump sites and to monitor the efficiency of in situ-remediation techniques. An example is provided by Pihan and Gomot de Vaufleury (2000) who utilised two terrestrial snail species, *Helix pomatia* and *H. aspersa*, to assess the intensity of heavy metal pollution around the waste dump site before and after the remediation process. Active biomonitoring approaches with specimens taken from the laboratory avoid additionally the natural variability in autochthonous land snail populations. Due to the live-long exposure of wild snails to pollutants present in their natural habitat, they might have produced specific detoxification enzymes (e.g. MFO system) or metal-binding molecules (e.g. metallothioneins) resulting in a marked modification of pollutant accumulation. Consequently, the measured contaminant concentrations in the tissues of the indigenous bioindicators will not necessarily reflect the concentrations of organic compounds or trace elements in their environment. This might have contributed to the results reported by Richmond and Beeby (1992), who found lower lead body burdens in a *Helix aspersa* population from a heavily polluted area than in low contaminated reference regions of England.

One of the main problems with accumulation monitoring studies is the evaluation of the results and the development of assessment criteria. Berger and Dallinger (1993) have established a classification system for heavy metal contamination in the pulmonate snail *Arianta arbustorum*. They distinguish three levels of contamination, named as classes. Class 1 represents the background or control level, class 2 represents slightly contaminated areas with heavy metal body burdens in snails, which are typically found near major traffic routes and in cities, while the highest concentrations (class 3) are attained in the vicinity of smelters and mines.

Land snails have been successfully used as bioaccumulation indicators or monitors in the past and will also play a prominent role in this area of environmental surveillance in the future. Nevertheless, it has to be considered that such studies can only offer rather limited insights into the ecological and ecotoxicological relevance of the actual pollutant exposure in the environment. Although they provide information on the bioavailability of contaminants in ecosystems, it is hardly possible to derive any predictions of biological effects of these pollutants at the given level of exposure; such predictions are the main perspectives of biological effect monitoring.

2.2. Biological effects on the sub-organism level

Terrestrial snails offer the possibility to assess the effects of environmental pollutants on the sub-organism level using a wide range of structural and physiological endpoints, which are generally referred to as biomarkers or biological markers. In most studies a differentiation between biomarkers of exposure and biomarkers of effect is made (e.g. Handy and Depledge, 1999). The advantages and limitations of the application of biomarkers in terrestrial invertebrates with respect to the ecotoxicological risk assessment in soils and the monitoring of soil contamination were recently reviewed by Kammenga et al. (2000). Such studies raise in general a problem, which has been already addressed by Markert and Oehlmann (1998), the ecological relevance for higher biological integration levels (populations, ecosystems, etc.). Organisms, populations, biocenoses and ultimately entire ecosystems are naturally subject to a number of biotic and abiotic stress factors (e.g. climatic fluctuations, varying radiation, food availability, predation, parasites, diseases, competition), including pollutants. This stress situation is of existential importance at every level of biological organisation. The ability to react to stressors is therefore a vital attribute of all living systems. Conversely, no evolutive development of individual species and thus of the ecosystem as a whole is possible without natural stressors. Many of the biomarker studies raise the question, whether the investigated sub-organismic reactions of pollutants are really indicating an adverse biological effect in the sense that they are damaging individuals or even the population or if they are just indicating that the individual is coping with this stress. Workers in this field may have been too interested in expanding the merits of their various techniques without giving full regard to the question "What does it mean, how relevant and applicable is it?". Thus, the present situation is that a number of scientifically sound techniques are available which, whilst being very sensitive, yield little information on the impact of pollution on the health of the community. It has to be pointed out that the ultimate objective of ecotoxicology is not the protection of the individual (as it is for human toxicology), but of the population. From a biological point of view it can therefore be argued that unless an effect has consequences at the population level it is insignificant. An alternative view, which may be related to the precautionary principle, is that preventive action should be taken when effects on the biomarker level are detected in individual animals. But nevertheless, it is this relationship between observed changes on the sub-organismic level and the consequences for the population which has been and is still lacking in most studies, but is required for biological effects in indicator species to be of value protecting the health of the environment.

2.2.1. Ultrastructural alterations

The assessment of cytotoxic effects of environmental pollutants by ultrastructural analyses of target cells and tissues is extremely time consuming and expensive. Therefore, such studies play clearly a secondary role for biomonitoring purposes in general. This is also due to the fact that alternative approaches, like the measurement of membrane stability (e.g. neutral red retention time assay) are available today. Köhler and Triebskorn (1998) have investigated in a comparative study the effects of heavy metal exposure on a number of soil invertebrates, including the slug *Deroceras reticulatum*. Ultrastructural alterations in the cells of the midgut gland epithelium were recorded qualitatively and quantitatively by transmission electron microscopy. The authors propose an "impact index" which itself is easy to handle for the assessment of ultrastructural damage by heavy metals in the indicator species, but also in this study the problem is the data collection with enormous technical and time efforts.

2.2.2. Metallothioneins (MTs)

Metallothioneins (MTs) are low-molecular-weight, cytosolic proteins with a high cysteine content, showing a strong affinity toward certain essential and nonessential trace elements, such as Cd, Cu and Zn (Kägi and Schäffer, 1988). So far, MTs have been identified in a large number of tissues and species throughout the animal kingdom, including a number of terrestrial snails (Dallinger, 1994; Dallinger et al., 2000). Although a variety of biochemical data prove MTs to be structurally well defined and highly conserved proteins, their biological function is still under discussion (Cherian and Chan, 1993). A number of studies demonstrated that the synthesis of MTs can be induced by certain trace elements, but also by organic chemicals and other non-chemical stress factors, like infections, starvation and injuries (Cherian and Chan, 1993, Berger et al., 1995a). Nevertheless, it has been shown that Cd, Cu, Hg and other trace elements are the most potent inducers of MT synthesis and it has therefore been speculated that the detoxification of metals is the primary biological function of these proteins, although further physiological roles such as the involvement in the cellular homeostasis of essential trace elements, gene regulation and protection against oxidant stress are evident in invertebrates (Engel and Brouwer, 1987; Sato and Bremner, 1993).

The involvement of MTs in cadmium detoxification of terrestrial gastropods has been proven in detail for a number of species, e.g. the Roman snail *Helix pomatia* (Dallinger, 1993; Berger et al., 1995a; Dallinger et al., 2000), slugs from the genus *Arion* (Dallinger et al., 1989) and *Arianta arbustorum* (Berger et al., 1995b). Compared to other invertebrate taxa such as earthworms, land snails have the advantage that their MTs are by far less unstable, particularly under conventional conditions of preparation, facilitating their use in biomonitoring surveys (Dallinger et al., 2000).

A promising perspective is the finding that MTs in metal-loaded organisms can be present in different isoforms that are specifically synthesised in response to different metals. The latter fact may have implications for the use of specific MT isoforms as potential element-specific exposure biomarkers for metal stress in invertebrates as proposed by Dallinger (1994). One possible strategy to achieve this objective may be to assess parameters of MT synthesis at the molecular or biochemical level. In any case care must be taken to consider intrinsic physiological parameters, such as nutritional or developmental factors, which could also interfere with MT synthesis (Dallinger, 1994).

2.2.3. Heat shock proteins (HSPs)

Heat shock proteins (HSPs) represent a second class of cellular stress molecules next to the MTs described previously, which both offer some protection from cellular damage. The term "heat shock protein" was coined by Ritossa (1962), who first described these proteins in *Drosophila melanogaster* following an exposure to high temperatures. In the following years, a range of environmental stressors have been shown to induce HSPs (compare Table 2), including trace metals, organic chemicals, temperature variations, changes in osmolarity, oxygen deficiency and UV radiation (Sanders, 1990; Schlesinger, 1990; Bauman et al., 1993; Myrmel et al., 1994). A number of HSP families are distinguished, classified by their molecular weight: HSP90, HSP70, chaperonin (= HSP60) and the so-called LMWs (with low molecular weights). The primary biological function they are involved in is the ensuring of the correct spatial arrangement and folding of cellular proteins (Hartl, 1996). Although all HSPs seem to be highly conserved in evolutionary terms and allow therefore the investigation of stress effects in the broadest sense in a variety of species, independent of their systematic status, the HSP70 family plays the most prominent role in this respect. HSP70 has been identified in archaeobacteria, several species of algae, vascular plants, many terrestrial and aquatic invertebrate taxa, including molluscs, and all chordate classes (Lewis et al., 1999). This protein family exhibits the largest specific activity compared to other HSPs and is thus easier to detect. An increase in the total specific

Table 2. Selection of chemicals and non-chemical environmental stressors that induce heat shock proteins in various biological taxa (modified from Lewis et al., 1999).

Environmental stress	Taxa studied
Cadmium	Nematodes, molluscs, crustaceans, fish, mammals
Copper	Algae, nematodes, molluscs, crustaceans
Lead	Nematodes, molluscs, crustaceans, mammals
Mercury	Nematodes
Silver	Molluscs, crustaceans
Zinc	Nematodes, molluscs
Arsenite	Protozoa, nematodes, fish, amphibians, mammals
Mixtures of metals	Molluscs, crustaceans, fish
Lead	Nematodes, molluscs, crustaceans, mammals
Tributyltin (TBT)	Molluscs
Fluorothane	Molluscs, crustaceans, fish
Organophosphates and carbamates	Crustaceans, fish
Thermal stress	Nematodes, molluscs, crustaceans, fish, amphibians, reptiles, mammals
UV radiation	Bacteria

activity of HSP70 within an organism can be used as a non-specific indicator of stress. The various detection methods for HSPs with their advantages and limitations have recently been reviewed by Lewis et al. (1999).

The induction of HSP is generally slower, but persists longer, during exposure of organisms to chemicals compared to thermal stress, probably due to the uptake and clearance kinetics of the particular trace elements or compounds. A number of authors have found no relationship between tissue concentrations of contaminants and the HSP response, e.g. Lundebye et al. (1997). This can be at least partly explained by the fact that HSP induction may not always be involved in the cellular detoxification of pollutants: Paraquat is one of the few herbicides that induce HSPs in animals, while MTs seem to be more important and effective at low heavy metal exposure levels.

HSPs have been used as biomarkers also in terrestrial snails for biomonitoring purposes. Köhler et al. (1992) conducted a comparative evaluation of HSP70 induction in three diplopod, one isopod and two slug species (*Deroceras reticulatum* and *Arion ater*) both, in the field and in the laboratory. They tested the effects of elevated temperatures and the exposure to heavy metals and organic compounds (molluscicides) on the gastropods, but were unable to find any differences in the HSP70 levels in slugs exposed to the molluscicide cloethocarb and the control groups. Furthermore, Köhler et al. (1996) reported that *Deroceras reticulatum* exposed to sublethal concentrations of Cd, Pb or Zn for three weeks, showed an increased expression of HSP70.

With respect to HSPs, there are many contradictory studies available, as also pointed out by De Pomerai (1996) and Lewis et al. (1999) in their recent reviews. They identified a number of areas of so-far neglected research, like spatial and seasonal variability in baseline specific activities and inducibility of HSPs. Furthermore, it should be recognised that normal or background protein quality and content of the tissues may vary considerably between tissues, specimens and sampling sites and consequently, specific HSP activities in bioindicators should be interpreted with caution.

2.2.4. Lysosomal stability and membrane integrity

The attraction of using cytological techniques are that they allow pollution effects to be detected at the earliest stage where the functioning of the cell may be impaired. They have gained wide interest and acceptance in many invertebrate studies, although to a lesser extent for terrestrial gastropods. Especially, parameters such as lysosomal stability (or integrity) can distinguish between specimens from polluted and "clean" sites though not always reflecting a dose response relationship. The sensitivity of these parameters to general environmental stressors such as temperature and nutrition of the animals and the apparent over-riding effect of the reproductive status restricts the periods and conditions, under which they can be applied. The assessment of lysosomal stability as a general measure of membrane stability may require the transplantation of caged animals for monitoring purposes to control at least some of the intrinsic biological parameters, which affect this biomarker.

Lysosomes have the ability to concentrate a wide range of environmental pollutants, both organic and inorganic, resulting in an increase of membrane permeability and loss of acid hydrolases into the cytosol, eventually causing cellular damage (Moore, 1990). The assessment of lysosomal stability is therefore a parameter that correlates significantly with the environmental conditions. First experimental evidence for molluscs was provided by Bayne et al. (1979) using marine bivalves. On a routine basis, the damage to biological cell membranes with a resulting efflux of hydrolases from the lysosomes into the cytosol is measured by the neutral red retention (NRR) assay. The vital cell stain neutral red is readily adsorbed by cells and then actively accumulated by the lysosomes resulting in an intense red colouring of these organelles. Any damage to the membranes impedes the ability of the lysosomes to accumulate and/or retain the stain.

The NRR assay has been used extensively in a number of surveys with haemocytic and digestive gland cell lysosomes from a number of marine bivalves, including *Mytilus edulis*, *M. galloprovincialis*, *Ostrea edulis* and *Crassostrea virginica* (e.g. Lowe and Pipe, 1994). Like already stated for MTs and HSPs before, the NRR assay responses not only to pollutants but also to natural stressors, like temperature and osmolarity changes (Ringwood et al., 1998). So far, only few attempts have been made to apply this biomarker also for terrestrial biomonitoring with snails. Recently, Snyman et al. (2000) have utilised this biomarker with haemocytes in the garden snail *Helix aspersa* in South Africa to monitor the effects of the agrochemical copper oxychloride, which is commonly used as a broad-range fungicide on a variety of fruits and vegetables, especially in vineyards. They found a significant and concentration dependent decrease of the NRR time by up to 80% compared to the control.

2.2.5. Oxidative metabolism

The occurrence of oxidative enzymes is a general characteristic of all living systems. One of the most important enzyme systems is the cytochrome P-450-dependent monooxygenase system, which is also named the mixed- or multi-function oxidase (MFO) system. The ancestral gene for cytochrome P-450, the terminal component of the MFO system, is thought to have originated over 2 billion years ago, and subsequent divergent evolution has produced many different forms or isoenzymes (Nebert et al., 1989). In the animal kingdom, the MFO system has been detected in almost all systematic taxa, including the molluscs (Livingstone et al., 1990). It functions in the transformation of endogenous and exogenous compounds and serves as an important bio-catalyst in numerous and diverse biochemical pathways. In the animal kingdom, the roles played by cytochrome P-450 in endogenous pathways include the synthesis and degradation of steroids, prostaglandins, fatty acids and a broad spectrum of further biological molecules (Schenkman and Kupfer, 1982). In metabolism of xenobiotics, cytochrome P-450 plays key roles in the transformation of natural toxins and anthropogenic pollutants (e.g. aflatoxins, aldrin, biphenyls, nitroanisole, benzo[a]pyrene and other PAHs, hydrocarbons mixtures as diesel oil and crude oil), but also in the metabolic activation and inactivation of many chemical carcinogens. The nature and significance of these functions in an organism will depend largely on the complement of particular cytochrome P-450 proteins present, their catalytic function and regulation.

In molluscs, the MFO is localised mainly in the microsomes of the digestive gland, although it was also found in other tissues like gills and haemocytes. The levels of microsomal MFO components and activities are similar in different species and populations of the same species from around the world, but are generally up to two orders of magnitude lower than in most vertebrate species and a number of other invertebrate taxa such as arthropods (Lee, 1985; Livingstone et al., 1990; Dauberschmidt et al., 1997).

The activity of cytochrome P-450 and of the entire MFO system may be increased by exposure to a wide range of organic xenobiotics. This induction of the MFO system has been widely used as a biomarker of exposure in aquatic biomonitoring programmes with molluscs (cf. 3.2), but surprisingly, no attempts have been made to use this sensitive response to organic pollutant exposure also in terrestrial snails so far. Increases of the MFO activity by a factor of 6 to 10 in areas contaminated with PAHs and other hydrocarbons are reported in aquatic studies (e.g. Baumard et al., 1998), although the inducibility of the molluscan MFO system seems to be apparently more limited compared to vertebrates and insects (Livingstone et al., 1990).

A general problem limiting the applicability of this biomarker for monitoring studies is the marked seasonal variation of the MFO activities in most species analysed yet. In *Mytilus edulis* for example, the MFO activity and the cytochrome P-450 specific content of the digestive gland decline with the approach of spawning and the eventual release of gametes with a consequent increase during autumn when food reserves are built-up again (Livingstone, 1988; Fig. 2). For mussels and other bivalve species also sex-related differences were described with females showing generally higher activities than males (Livingstone et al., 1989).

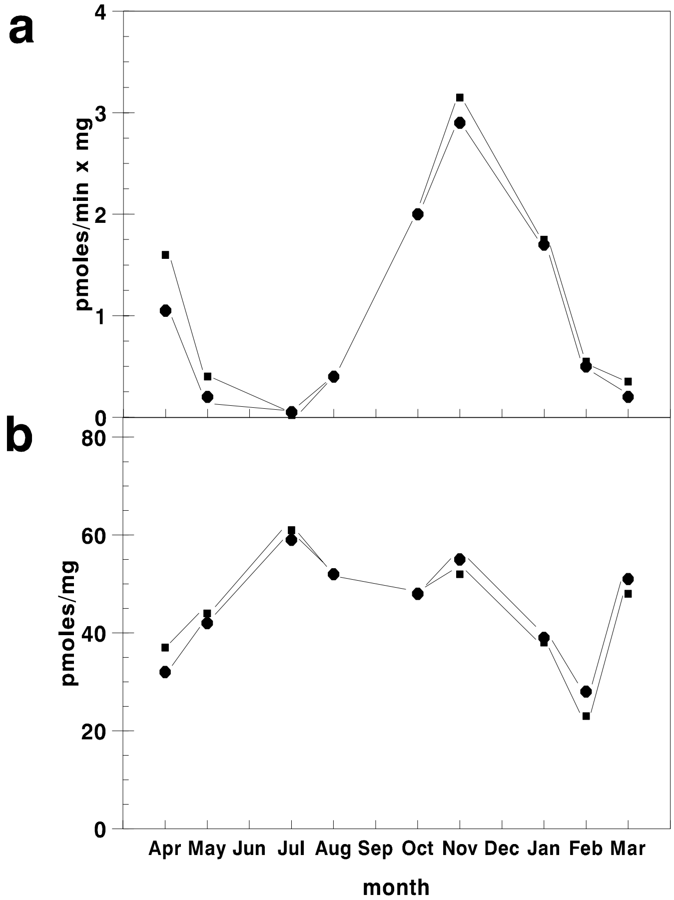

Figure 2. Seasonal variations in NADPH-independent 7-ethoxycoumarin O-deethylase (ECOD) specific activity as a measure of MFO activity (a) and cytochrome P-450 specific content in digestive gland microsomes of *Mytilus edulis* (b). Mean values of six samples, each consisting of the pooled tissue of six mussels, are presented: ■, males; ●, females (values from Livingstone et al., 1989).

2.2.6. Acetylcholinesterase (AChE) activity

A special group of enzymes, the esterases, have been widely utilised in the past two decades to assess the exposure of wildlife populations to two important classes of agricultural pesticides, the organophosphorus and carbamate insecticides. The class "B esterases" (according to Aldridge, 1953) represent serine hydrolases, which are inhibited by organophosphates such as paraoxon and includes the acetylcholinesterase (AChE), the target molecule for the majority of these compounds. The natural substrate for AChE is the neurotransmitter acetylcholine (ACh), which is used by almost all taxa of the animal kingdom to transmit neuronal impulses across the synapse. An incoming (afferent) impulse results in an ACh release into the synaptic cleft, is followed by the diffusion of the transmitter through the cleft and a binding to postsynaptic ACh receptors, which finally leads to a depolarisation of the postsynaptic membrane causing a further conduction of the impulse. An inhibition of the AChE induces a permanent depolarisation of the postsynaptic membrane, resulting in a prolonged transmission of impulses with tetani and finally the death of the exposed specimen as ultimate effects (Oehlmann and Markert, 1997).

Many organophosphorus pesticides are applied in an inactive form and have to be activated by monooxygenases (e.g. the MFO system) in vivo to the AChE-inhibiting form. An example is the (more or less inactive) pesticide parathion, which is metabolised to the active agent paraoxon. The organophosphate inhibits the esterase through reaction with the serine at the active site of the enzyme at very low concentrations; thus the insecticide acts as a suicide substrate. Carbamates exhibit a comparable mode of action with a carbamylation of the serine moiety of the enzyme, but this interaction is less strong and carbamylated esterase can spontaneously reactivate while a reactivation of organophosphate-inhibited AChE is very slow (Thompson, 1999).

There are a number of factors apart from inhibition by organophosphates or carbamates, which affect the esterase activity of animal specimens and need to be taken into account when monitoring programmes are designed or their results are interpreted. A reduced AChE activity can be due to an exposure to other xenobiotics and even metals, it can be caused by starvation, parasite infections and other diseases. Additionally, there are also a number of natural sources of variation which can affect AChE activities (Rattner and Fairbrother, 1991). The most important are species-specific and even interindividual differences within a species, age related, diurnal and even seasonal changes.

In contrast to the broad use of AChE activity measurements as a biological marker of organophosphate and carbamate exposure in aquatic molluscs (cf. 3.2), only few attempts have been made with this assay for terrestrial monitoring. Two of the rare examples are the studies of Schuytema et al. (1994) and Coeurdassier et al. (2001), who investigated the effects of a dietary uptake of various organophosphorus insecticides and the carbamate carbaryl in the garden snail *Helix aspersa*. The inhibition of AChE activity was next to clinical effects the most sensitive parameter with a decrease of 80% in activity at an insecticide concentration of 250 µg/g in the food of the gastropods (Fig. 3a).

In land molluscs, Rorke and Gardner (1974) reported a strong inhibition of AChE activity in the haemolymph of *Helix aspersa*, caused by a carbamate or by the active oxon of fenitrothion. Young and Wilkins (1989) did not observe any inhibition of this activity in the slug *Deroceras reticulatum* when exposed to methiocarb. They explained this resistance with a difference in the sensitivity of the species' five AChE isoenzymes.

2.3. Biological effects on the organism and community level

Biological effects of pollutants or other environmental stressors on the organism or community level have been used only in a very limited number of monitoring studies with terrestrial snails. Honek (1993) investigated the value of shell banding in the

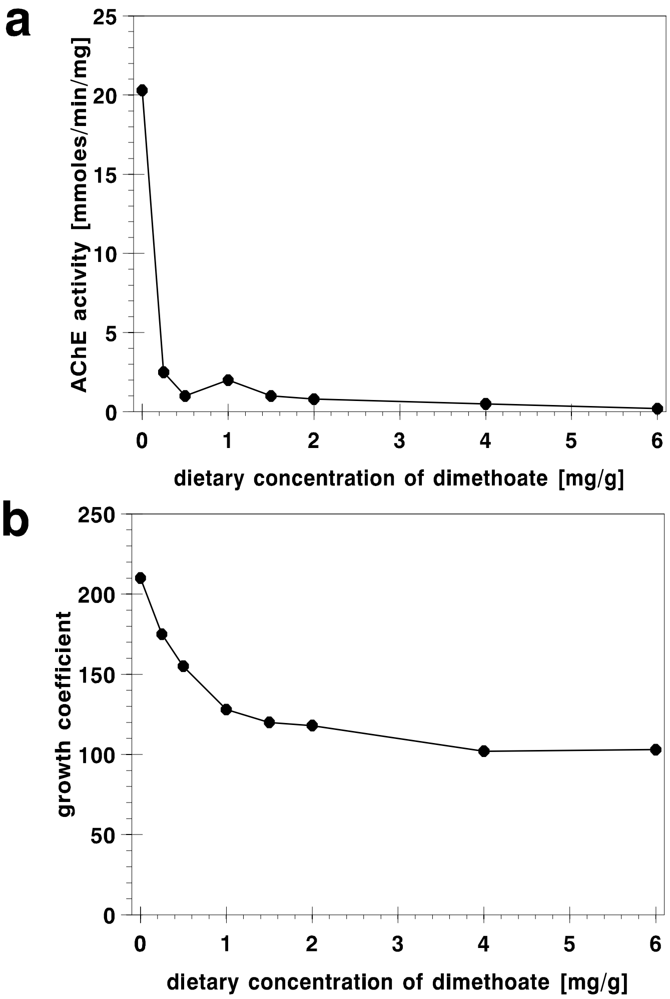

Figure 3. Effects of the organophosphorus insecticide dimethoate on acetylcholinesterase (AChE) activity (a) and growth in the garden snail *Helix aspersa* (b) after four weeks of dietary exposure (values from Coeurdassier et al., 2001).

pulmonate *Helicella candicans* in the Czech Republic as an indicator of industrial air pollution. He found a significant positive correlation between the incidence of melanistic shell phenotypes in more than 180 analysed populations and a decrease of incident sun exposure. Although some of these areas with a high proportion of dark-shelled snails are characterised by a high degree of industrial air pollution, in other regions natural environmental conditions seemed to be the underlying cause for the observed melanism, like meteorological aspects (fog and clouds) or high and dense vegetation covers. The author concluded on the basis of his field investigations and parallel experiments in the laboratory that in areas of reduced sunshine duration and/or intensity, dark snail specimens may be at an advantage, especially during the breeding season in autumn. Because the above mentioned natural factors affect the incidence of melanistic forms in the populations, the value of this biological marker as an indicator of air pollution is rather limited.

The structure of terrestrial snail biocenoses is analysed as part of the "Ecological Effect Cadaster Baden-Württemberg" in Germany. The objective for the assessment of this synecological marker is to detect shifts in the species composition, which might be attributed to environmental pollution or non-chemical stressors (Spang, 1995). The effect cadaster is designed as a long range monitoring programme and includes an annual sampling of molluscs at approximately 200 permanent observation areas in forest, agricultural and urban ecosystems, mainly in autumn. The quantitative sampling of snail species with a body length of more than 5 mm is performed in an area of 3×2 m at these stations. Smaller species are sampled in two adjacent areas of 0.25 m^2 each from the vegetation cover, the litter layer and the upper 10 cm of the soil. The smaller gastropods are generally analysed in the laboratory under dissection microscopes because it is imperative for a characterisation of the recent biocenosis to distinguish living snails from dead shells.

Pollutant effects on the level of single organisms such as survival (or mortality), growth, weight gain, reproduction, morphological and histological alterations have been assessed in a number of terrestrial biomonitoring surveys with terrestrial gastropods. Gomot de Vaufleury and Pihan (2000) used the garden snail *Helix aspersa* as an active effect biomonitor to detect the biological impact of metals in soils on growth and mortality. During the four weeks of their study the mortality was 10.7% in the field control and 0% in the laboratory control, but up to 47.6% in those groups transplanted to industrial and urban sites. Snails transplanted from the laboratory to metal-polluted sites in the field exhibited statically significant lower shell and body weight at the end of the experiment. The latter effect, which was assessed separately for the foot and the visceral complex, was due to dry weight differences and not to the moisture differences of the tissues. A comparable approach has been made with the same bioindicator species by Coeurdassier et al. (2001), but with a focus on organophosphorus insecticides as a model class of organic contaminants. A statistically significant increase of the mortality was observed in snails exposed to dietary dimethoate concentrations of 250 μg/g after four weeks. Regardless of the criterion used to assess growth, like total fresh or dry weight gain, growth coefficients of soft body or shell diameter (Fig. 3b), a concentration dependent growth inhibition was always detectable at $\geqslant$250 μg dimethoate/g. Additionally, the authors found a number of typical morphological alterations in the snails following an exposure to the pesticide like a dropping of the eyestalks and a characteristic extension of the body with a swelling of the foot and the mantle edge forming a fold, which protrudes from the shell. Comparable results were also reported by Schuytema et al. (1994) for *Helix aspersa* in experiments with a number of further organophosphorus insecticides and the carbamate carbaryl (Table 3). These observations indicate that in situ encagement of land snails can be applied as a valuable active biomonitoring approach to assess the degree of soil pollution with metals and organic pollutants.

A very interesting example of the potential of terrestrial gastropods as biomonitoring tools to assess anthropogenic effects on ecosystems was published by Graveland

Table 3. Comparison of lethal and sublethal effects of different organophosphorus insecticides and the carbamate carbaryl in the garden snail *Helix aspersa* following a dietary exposure (values from Schuytema et al., 1994; Coeurdassier et al., 2001).

Compound	Exposure time (d)	Effects	LC_{50} (μg/g)	NOEC (μg/g)	LOEC (μg/g)
Azinphosmethyl	10	Mor; Es, Sb	188	34	155
Aminocarb	10	Mor; Es, Sb	313	40	154
Trichlorfon	10	Mor; Es, Sb	664	127	501
Dimethoate	10	Mor; Es, Sb	>6,000	100	250
	28	Mor; Es, Sb	3,700	100	250
Fenitrothion	10	Mor; Es, Sb	7,060	138	797
Methylparathion	10	Mor; Es, Sb	>10,000	39	156
Carbaryl	10	Es, Sb	>10,000	626	2,500

Es = eyestalk dropped; LC_{50} = lethal concentration for 50% of tested specimens; LOEC = lowest observed effect concentration; Mor = mortality; NOEC = no observed effect concentration; Sb = swollen body.

et al. (1994) and Graveland and van der Wal (1996). They report that on poor soils in the Netherlands, an increasing number of great tits, *Parus major*, and other forest passerines produce eggs with defective shells, resulting in low reproductive success. This effect was attributed to calcium deficiency and has been observed in Germany and Sweden, too. Snail shells were the main calcium source for birds in forests where defective eggshells did not occur, but were very rare in areas where tits often had eggshell defects. The authors investigated whether a decrease in snail abundance on poor soils could be responsible for the decline in eggshell quality and the underlying reasons for this decline. There was a marked and highly significant negative correlation of the snail abundance and the incidence of eggshell defects in the birds. Furthermore, the gastropod density correlated with the calcium content and also with pH of the litter layer. The liming of a calcium-poor forest soil with a low snail abundance resulted in a marked recovery of gastropod densities and also in an improved reproductive success of *Parus* populations within the next four years. An extended survey indicated that snail densities had declined on calcium-poor soils over last two decades, but not on calcium-rich soils. The authors concluded that acid deposition was responsible for the reduced calcium content on poor soils. This anthropogenic acidification caused a decline of terrestrial mollusc populations depriving the bird populations of their most important calcium source.

3. Aquatic bioindication and biomonitoring with molluscs

Although six of the seven classes within the molluscs comprise exclusively of aquatic species and even the majority of species of the remaining class, the gastropods, are non-terrestrial, it is evident, that snails and bivalves are the only two molluscan groups which have been widely used for bioindication and biomonitoring purposes in the past. Representatives from the remaining classes found some interest for ecotoxicological testing of chemicals in the laboratory but no attempts have been made so far to consider them for field surveys.

It is virtually impossible to give a complete overview of the use of molluscs in aquatic bioindication and biomonitoring programmes within the scope of this book chapter. As an alternative approach examples will be presented showing that marine and freshwater gastropods and bivalves are widely applied as accumulation indicators and monitors of effects of contaminants on different levels of biological integration. If appropriate, we will also refer to review publications, which provide more detailed information in special areas of interest. The section will end with the group of tributyltin compounds as a special case study: Molluscs have not only played a prominent role in monitoring programmes for these organometallic pollutants in the past but were also affected by tributyltin from the lowest to the highest levels of biological integration. Consequently, the case of tributyltin effects in molluscs is one of the rare examples which show that contaminant responses can be assessed by a variety of techniques and that comparatively trivial biochemical changes induced by xenobiotics may have even consequences on the ecosystem level.

3.1. Bioaccumulation

The so-called mussel watch, initiated in the United States of America in 1976, was one of the first environmental surveillance programmes, which made use of living organisms in an extended geographical area (Goldberg, 1975; Goldberg et al., 1978). In the beginning, the mussel watch comprised of a coordinated, standardised sampling and measurement of heavy metals, radionuclids, halogenated and petroleum hydrocarbons in four marine bivalve species (*Mytilus edulis*, *M. californicus*, *Crassostrea virginica* and *Ostrea equestris*) at more than 100 sample sites on the coast of North America.

In these early years of accumulation monitoring with bivalves, a number of regional surveys were conducted to investigate the dynamics of heavy metal uptake in the indicator species and to analyse seasonal influences of environmental factors. Frazier (1975, 1976) observed in his studies that Mn and Fe concentrations, but not levels of Zn and Cu in the soft tissues of the oyster *Crassostrea virginica* correlated with shell deposition rates. The concentrations of the two latter heavy metals increased gradually during spring and early summer in the tissues, followed by a rapid loss during late summer and autumn, if ambient concentrations in sea water were low. Under high environmental exposure conditions to heavy metals, the author found a rapid and concentrations dependent uptake of Fe, Zn and Cu in summer and autumn and a delayed accumulation in early spring. At the same time, the potential of freshwater bivalves was also analysed in first investigations as by Anderson (1977), who conducted a comparative evaluation of heavy metal accumulation in the shell and soft tissues of six clam species in the Fox River in Illinois and Wisconsin.

Since 1976, bivalves have been used to assess the levels of contamination in marine ecosystems, and certain systematic groups, notably mussels and oysters, have been extensively studied worldwide (for review Phillips, 1977; Rainbow and Phillips, 1993; Boening, 1999). Their main advantage is that contamination levels in these organisms provide a time-integrated measure of pollutant bioavailability, responding essentially to that fraction of the total environmental load, which is of direct ecotoxicological relevance. Bioavailability and thus uptake of metals and other pollutants are highly dependent on chemical and biological factors. Among biological factors, there are major differences in bioaccumulation between bivalve species (Boening, 1999). Within a single species, accumulation can be a function of age, size, sex, genotype, nutritional and reproductive status. Chemical factors that influence bioaccumulation are organic carbon, water hardness, temperature, pH, dissolved oxygen, sediment grain size and hydrologic features of the system (Elder and Collins, 1991). Tissue analysis is influenced by sediments and detritus in the digestive tract at the time of collection, so that a depuration time of 24–48 h in clean water is generally necessary. However, excessive depuration times may cause inaccurate results due to a partial elimination of contaminants from body tissues. Under most circumstances, depuration is initiated with a rapid initial clearance of the pollutant, which is followed by a greatly decelerated loss after reaching a certain level (McKinney and Rogers, 1992). Uptake and accumulation in deposit-feeders would be expected to correlate to contaminant concentrations in sediments, whereas accumulation in filter-feeders would most likely reflect ambient concentrations in water (Boening, 1999).

Mussels (e.g. *Mytilus edulis*, *M. galloprovincialis*, *M. californicus*), oysters (e.g. *Crassostrea virginica*, *Ostrea edulis*) and clams (e.g. *Mercenaria mercenaria*, *Venerupis* spec., *Macoma balthica*) are the most commonly used bivalve groups for accumulation monitoring studies, while other species than those mentioned were considered only occasionally if they offer specific advantages, such as high abundance, or if habitat conditions in the study area were unsuited for the established biomonitors. Especially in tropical and subtropical regions other bivalve species have been employed as indicators. Metal accumulation in the sediment dwelling mussel *Donax trunculus* was analysed by Fishelson et al. (1999) on the Mediterranean coast of Israel, where *D. trunculus* attains densities of up to 2000 specimens/m^2. The authors found a site and age specific accumulation of Cd, Pb, Cu and Hg, with the highest values for Hg in the vicinity of a PVC producing plant and elevated Cd concentrations at a station polluted with oil and waste from the petrochemical industry. The residuals of Cd, Cu and Hg were relatively high in young and noticeably low in medium sized mussel specimens.

Gregory et al. (1999) confirmed the efficient bioaccumulation of Hg in their study with the mussel *Perna perna*. The same species was also employed by Avelar et al. (2000) as an indicator of heavy metal pollution in Brazil. Although the main objective of this study was the analysis of seasonal variations in the concentrations of Cd, Cu, Cr, Pb and Zn in the bivalves, the authors detected higher values of Cd (up to 4.57 μg/g dry wt.), Cr (up to 48.5 μg/g dry wt.) and Pb (up to 60.0 μg/g dry wt.) compared to other biomonitoring studies in tropical areas. The results showed furthermore an elevated metal accumulation during July compared to spring and autumn. Sarkar et al. (1994) suggested the two bivalve species *Crassostrea cucullata* and *Anadara granosa* as suitable bioindicators for heavy metal pollution in India and other Asian countries and Szefer et al. (1998) proposed the mussel *Mytella strigata* for the same purpose in central and south America. Further potential accumulation indicators for tropical regions are presented in the literature review of Avelar et al. (2000).

Especially if new species are introduced as accumulation monitors, the question of species- and contaminant-specific time integration capacities is crucial for the interpretation of assessed concentrations in molluscs. This objective was already addressed for the well established bivalve monitors, for example by Boisson et al. (1998) and Regoli and Orlando (1999) for *Mytilus galloprovincialis*. Boisson et al. (1998) examined lead accumulation from Pb-labelled seawater. An equilibrium was reached after 21 days. The elimination of Pb after transfer into clean sea water was biphasic with a rapid loss in the initial phase (half life: 1.4 ± 0.3 days) and a very slow excretion in the second phase (half life: 2.5 ± 0.7 months). Regoli and Orlando (1999) transplanted *Mytilus galloprovincialis* from a clean to a heavy metal polluted site. The authors describe that metal concentrations reached a steady state after only two weeks, indicating that mussels can rapidly equilibrate with enhanced environmental levels of these pollutants. After a transfer to clean sea water in the laboratory, the authors found a contaminant specific excretion rate, resulting in extreme diverse biological half life times. Due to the relatively fast uptake and long depuration half life times of the various metals, the ability of mussels to accurately record short term variations in trace element concentrations in the surrounding waters is limited, a fact which should be taken into consideration in order to define the appropriate sampling frequency for mussels used in biomonitoring programmes. Far less is known with regard to the toxicokinetics of organic contaminants in these well established monitor species and for the various non-classical mussels, which are used worldwide as surrogates.

The original idea of the mussel watch programme, which primarily aimed on the site-integrated assessment of coastal marine pollution was further developed in the past. Special emphasis was laid on the analysis of temporal contamination trends, but also on the use of mussels as sentinels after the occurrence of accidents with a release of larger amounts of pollutants. Zatta et al. (1992) conducted a survey of heavy metal and As contamination with *Mytilus galloprovincialis* in the Venetian lagoon in 1988. A comparison with data from studies, which were carried out in the same area a decade earlier revealed that the degree of heavy metal pollution in the lagoon has improved. The investigations published in Hung et al. (2001) show that the copper contamination in Taiwanese oysters increased from the time period 1980–1985 (174 ± 71 μg/g dry wt., n = 142) to 1986–1993 (513 ± 369 μg/g dry wt., n = 188) with a consequent drop in the period 1994–1996 (214 ± 89 μg/g dry wt., n = 207) and 1997/1998 (185 ± 110 μg/g dry wt., n = 58). An example for the use of two bivalves, *Crassostrea angulata* and *Scrobicularia plana*, as sentinels to monitor the extent of a chemical spill was provided by Blasco et al. (1999) after the Aznalcollar accident in Spain. In April 1998, the holding pool of the Aznalcollar mine burst its banks and 5 million m^2 of sludge poured into the nearby River Guadiamar, a tributary of the River Guadalquivir. Within hours of the accident, approximately 2.5 Hm^2 acidic water with high concentrations of metals, especially Zn, had entered the river. Within a biomonitoring programme to evaluate the impact of this spill on the fauna in the two rivers and the Guadalquivir estuary, a number of aquatic species, including the two molluscs, were analysed for accumulated metals. In the oyster *Crassostrea angulata*, Cd and Cu concentrations were far above the human consumption limits for shellfish. An increase of the Zn levels was observed, as Zn concentrations in *C. angulata* were higher than those reported 30 years ago (Fig. 4).

Mussel watch surveys were also conducted with special emphasis on organic contaminants, mainly PCBs, DDT and petroleum hydrocarbons (e.g. Geyer et al., 1984;

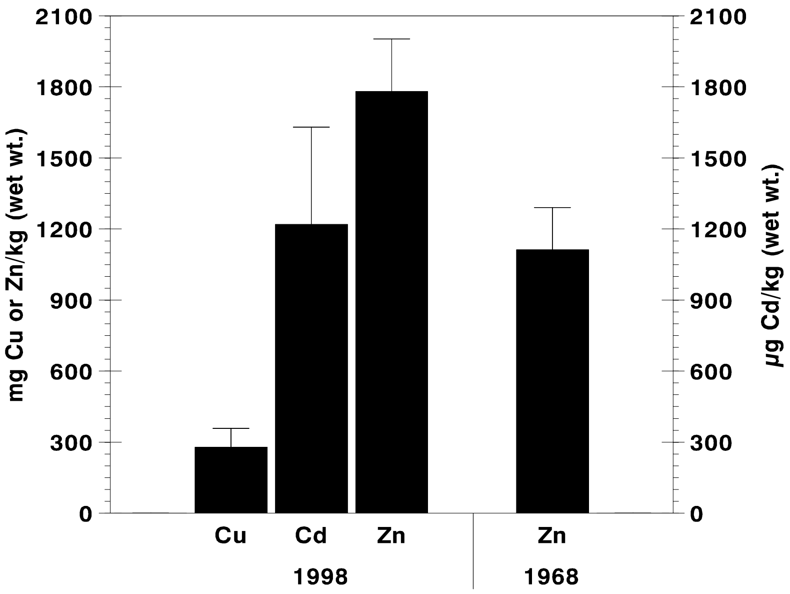

Figure 4. Concentrations of Cu, Cd and Zn (means ± standard deviation) in the soft tissues of the oyster *Crassostrea angulata* from the estuary of the River Guadalquivir, Spain, from April to September 1998 after the Aznalcollar accident in 1998 compared to Zn residues measured in oysters from the same station in 1968 (values from Blasco et al., 1999).

Stephenson et al., 1995; Villeneuve et al., 1999). In a 15 year time span, from 1977–1992, mussels were collected at 378 stations in California. From these stations, 47 were chosen to conduct statistical analyses based on the criteria that they had been sampled at least six times for total DDT, total PCBs, and total chlordanes. Declines of total DDT and chlordanes were noted at approximately half of the stations (Stephenson et al., 1995). Villeneuve et al. (1999) reported for the Mediterranean mussel watch programme that the residues of DDT and PCBs decreased by more than 80% between 1973 and 1989 in the mussels. This decrease is in agreement with the ban on DDT implemented in 1975 in western Europe and gradual cessation of PCB production in the 1970s and 1980s. Nevertheless, the residues of these compounds measured in mussels confirm the well known long persistence of DDT and PCBs, which are still present in relatively high concentrations in some regions.

In many areas, marine gastropods have been considered as additional accumulation monitors, transforming the original mussel watch into a mollusc watch. Perhaps the most extensive comparative investigation was conducted by Hung et al. (2001), who analysed interspecific differences of trace metal accumulation in 30 mollusc species in coastal waters of Taiwan. The authors showed that the highest concentrations of Cu, Zn, Cd, Pb and Cr were measured in gastropod species, while bivalves were better accumulators of Ni, As and Sn. Szefer et al. (1999) compared the metal accumulation

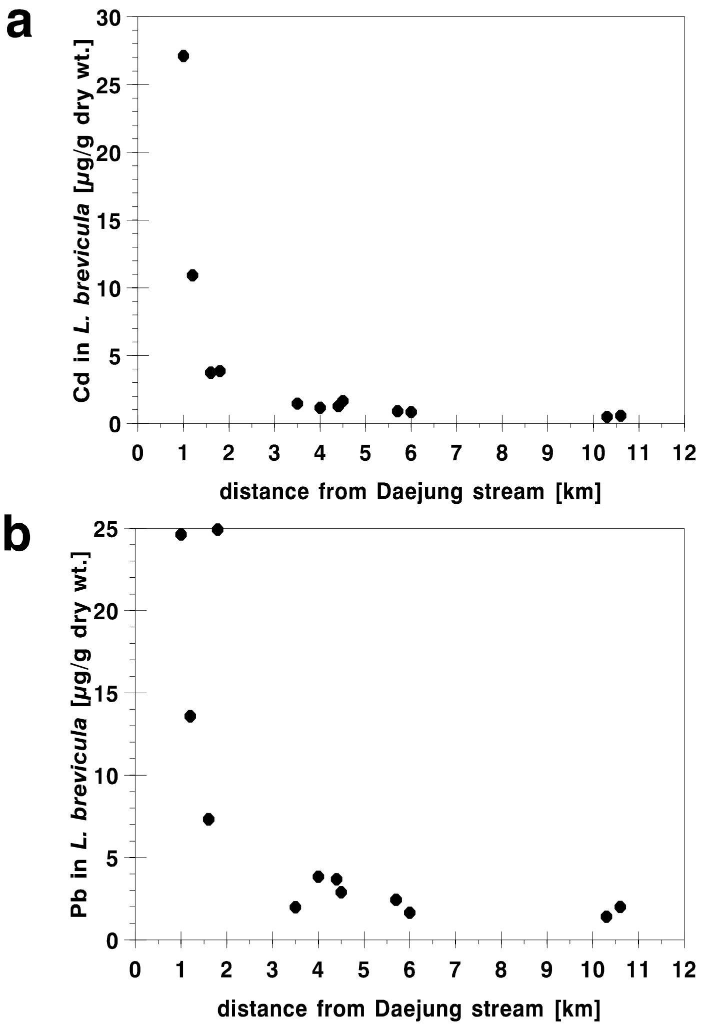

Figure 5. Decrease of Cd (a) and Pb concentrations (b) in the tissues of the winkle *Littorina brevicula* with distance from mouth of the highly contaminated Daejung stream in the southeast of Korea (values from Kang et al., 1999).

in four mollusc species, the bivalves *Ostrea cucullata* and *Pitar* spec., the gastropod *Turbo coronatus* and the polyplacophore *Acanthopleura haddoni* in the Gulf of Aden and rated the two latter species as well suited biomonitors.

The use of marine gastropods as accumulation monitors for heavy metals was reviewed by Bryan et al. (1977, 1983, 1985). Especially the edible winkle, *Littorina littorea*, has been employed as an indictor of trace metal pollution on both sides of the Atlantic and further species from the same family in other areas of the world (e.g. *Littorina brevicula* by Kang et al., 1999, 2000; compare also Fig. 5). Campanella et al. (2001) proposed two other marine gastropods, *Monodonta turbinata* and *Patella caerulea*, as cosmopolitan biomonitors. Furthermore, marine snails were successfully used as bioaccumulators of organic contaminants in the past (e.g. Walsh et al., 1994, 1995).

Freshwater molluscs have also been frequently used as bioaccumulator organisms (compare review of Elder and Collins, 1991). Two important advantages of snails and bivalves over most other freshwater organisms for biomonitoring research are their large size and limited mobility. In addition, they are abundant in many types of freshwater environments and are relatively easy to collect and identify. At concentrations of metals and organic pollutants that are within ranges common to natural waters, they are generally effective bioaccumulators. Biomonitoring studies with freshwater molluscs have covered a wide diversity of species, contaminants, and environments. The principal generalisation that can be drawn from this research is that bioaccumulation and toxicity are extremely situation dependent; hence, it is difficult to extrapolate results from any particular study to other situations where the biological species or environmental conditions are different. Under some conditions, the bioconcentration factors can be in the range of 10^3 to 10^6, relative to water. Most studies that provide comparisons among taxonomic groups indicate that bioaccumulation in molluscs is greater than that in fish (Elder and Collins, 1991).

One of the most frequently employed freshwater bivalve accumulator species is the zebra mussel *Dreissena polymorpha*, which can easily be caged and transplanted to a number of sites within a river catchment, facilitating a spatial and temporal trend monitoring of pollutant exposure (e.g. Camusso et al., 1994). For the same purpose also a number of other freshwater bivalves (e.g. Renaud et al., 1995), prosobranch gastropods, like *Bithynia tentaculata*, and pulmonates such as *Physa gyrina* and *Biomphalaria glabrata* were used (Abd Allah et al., 1999; Flessas et al., 2000). In most of these studies heavy metals were in the centre of interest, but there are also examples of biomonitoring approaches for radionuclids, organometallic compounds like methyl mercury and organic pollutants (e.g. Hameed et al. 1996, Desy et al., 2000).

Bivalve molluscs also play an important role in the environmental specimen banking (ESB) programmes in a number of countries, e.g. the zebra mussel *Dreissena polymorpha* for freshwater and the blue mussel *Mytilus edulis* in marine ecosystems in the German ESB (Klein, 1999). The ESB allows not only a monitoring of current contaminant levels in various ecosystems but offers furthermore the possibility for a retrospective detection of pollutants in stored samples given the case that a certain compound will be identified as a threat for the environment in the future (Wise et al., 1993; Rossbach and Kniewald, 1997).

Although bioaccumulation monitoring with aquatic molluscs can provide some insights into the exposure to and bioavailability of compounds in marine and freshwater ecosystems, it does not allow predictions to be derived on the biological effects of these substances on individuals or populations. This issue has been identified as a major limitation in the national ESB and mussel watch programmes. Currently, there are a number of attempts on their way, to consider also measures of biological effects in these programmes, as proposed by Klein (1999) for the German ESB and Goldberg and Bertine (2000) for the mussel watch.

3. Biological effects on the sub-organism level

The effects of pollutants on aquatic molluscs can be assessed using a variety of structural, physiological and functional endpoints on the sub-organism level. The main advantage of such an approach with respect to biological effect monitoring programmes is the capability to consider large sample numbers at comparatively low costs and in a short period of time, facilitating geographical large scale surveys. But the general problem of these so-called biomarker studies, as already stated in Section 2.2 is the ecological relevance of the assessed effects for higher biological integration levels.

3.2.1. Ultrastructural alterations

The general applicability of ultrastructural alteration assessment for biomonitoring purposes has been characterised in Section 2.2. Recently, Orbea et al. (1999) proposed to use the peroxisomal structure together with activity measurements of the peroxisomal enzyme catalase as biomarkers of environmental organic pollution in mussels from estuarine ecosystems. They sampled mussels in monthly intervals for over one year in two estuaries in the Bay of Biscay with different degrees of pollution. Stereological procedures were applied to detect changes in peroxisome structure and microspectrophotometry was used to quantify changes in catalase activity. The animals from the two studied sampling sites were characterised by different polycyclic aromatic hydrocarbon (PAH) burdens with mussels from Plentzia generally showing lower total PAH contents than mussels from Galea. The peroxisome structure in animals from the two estuaries was characterised by site-specific seasonal variations: A strong peroxisome proliferatory response was found in mussels sampled in Plentzia during the summer months, while mussels from Galea showed few variations over the year. It appeared that mussels exposed chronically to PAHs and other pollutants, such as those from Galea, lost their ability to respond to this exposure in terms of peroxisome proliferation. In contrast, mussels collected in Plentzia effectively responded to an increased bioavailability of organic pollutants during the summer by increasing peroxisome volume and surface and numerical densities in digestive epithelial cells. However, these increases were transient because elevated PAH body burdens detected in mussels sampled in Plentzia in autumn were not accompanied by a peroxisome proliferatory response.

3.2.2. Metallothioneins (MTs)

Metallothioneins (MTs) and other selectively metal binding proteins have found a comparable attention and application for aquatic studies like in terrestrial surveys (cf. 2.2). Their use as a tool in biomonitoring programmes has recently been reviewed by Viarengo et al. (1999), considering also the latest knowledge on MT gene regulation and inducibility. It appears that in fish MTs should be considered as a kind of general stress protein, which is particularly responsive to heavy metals. In aquatic molluscs and especially snails and mussels, MTs seem to be more specifically involved in responses to heavy metals and can thus be considered as a biomarker of exposure to metal contamination.

Viarengo et al. (1997) have developed a comparably easy to handle spectrophotometric method to evaluate MT concentrations in tissues of marine organisms and have shown its applicability as a biomarker of Cd, Cu and Zn exposure in the digestive gland of Mediterranean and Antarctic mussels. The authors describe a three-fold induction in exposed mussels compared to uncontaminated controls. MT levels have also been used successfully as a biomarker of heavy metal exposure in marine snails, such as the netted whelk *Nassarius reticulatus* (Andersen et al., 1989), the periwinkle *Littorina littorea* (Bebianno and Langston, 1995) and recently also for freshwater mussels. Couillard et al. (1995) employed an active biomonitoring approach using transplants of the bivalve *Pyganodon grandis*, while High et al. (1997) utilised the zebra mussel *Dreissena polymorpha*.

3.2.3. Heat shock proteins (HSPs)

The potential and limitations of the use of heat shock proteins (HSPs) as a biological marker of pollutant exposure in molluscs were already addressed in Section 2.2. HSPs have proven useful as part of a suite of biochemical markers of xenobiotic exposure in aquatic molluscs, although it has to be considered that by themselves, HSP induction is a marker of multiple stress exposure (Snyder et al., 2001). Therefore, HSPs cannot indicate exposure to any specific stressor without direct control or knowledge of almost all environmental conditions. When combined with additional physiological observations, HSPs can, however, be indicative of the severity of the stress exposure. For example, Steinert and Pickwell (1993) demonstrated that *Mytilus edulis* gill tissue showed 12-fold induction of HSP70 following an exposure to tributyltin and this induction correlated directly with a reduction in mussel filtration rates. Clayton et al. (2000) found a more stressor- and HSP family-specific effect in an investigation with the freshwater mussel *Dreissena polymorpha*. The concentration response curve for HSP60 expression in Cu exposed mussels was biphasic, with a return to control or lower levels after a maximum expression of three times control levels. In contrast, HSP60 and HSP70 levels were elevated at all tributyltin concentrations, and HSP70 concentrations increased in Cu exposed mussels beyond the induction threshold.

3.2.4. Lysosomal stability and membrane integrity

The application of parameters like lysosomal stability, measured by the neutral red retention (NRR) time assay, and size have gained widest acceptance in studies with aquatic bivalves, particularly in the blue mussel *Mytilus edulis* (e.g. Moore, 1982). Major problems of these techniques, like the general lack of a concentration response relationship and the over-riding effect of other environmental stressors next to pollutants such as temperature, salinity and nutrition, which are likely to limit the use of lysosomal stability as a biological marker for monitoring purposes (Stickle et al., 1985), have already been addressed in Section 2.2.

Matozzo et al. (2001) studied the effects of heavy metal exposure in the clam *Tapes philippinarum* on NRR capacity and found that Cd and Cu has a marked effect on this measure of membrane integrity. Fishelson et al. (1999) applied in their study with the marine bivalve *Donax trunculus* in the eastern Mediterranean an alternative to the NRR assay, the acridine orange (AO) assay. Both vital stains are accumulated in viable lysosomes so that any disturbance of the accumulation can act as an indicator of disturbed lysosomal function, a decrease of membrane stability and therefore also as a marker for cell viability. The fluorescent cationic probe AO penetrates through the plasma membrane via diffusion and is actively accumulated by lysosomes of intact cells, where it acquires a red fluorescence (Bresler and Yanko, 1995). The data of the investigation of Fishelson et al. (1999) demonstrated a site-dependent trend of decrease in the capability of the lysosomes to accumulate AO with the most prominent effect at sites of highest pollutant exposure. The decrease in the lysosomal accumulation of AO in gills and mantle epithelia correlated with a higher frequency of cytopathological processes such as lysosomal enlargement and secondary lysosome formation.

3.2.5. Phase I and phase II enzymes of pollutant metabolism

Of the wide range of biochemical measurements, which have been proposed as indicators of stress, those which appear to offer most potential are based on proteins which can be induced by specific stimuli, most notably the enzymes of xenobiotic metabolising systems. The so-called phase I enzymes catalyse a functionalisation reaction with the introduction of a polar moiety such as a hydroxyl group into the organic, often lipophilic contaminant by oxidation, epoxidation, reduction or hydrolysis. The product is then characterised by a lower (metabolic deactivation) or occasionally also by an enhanced toxicity (metabolic activation). In the following phase II, the metabolites of phase I are conjugated to various endogenous substrates such as peptides (e.g. glutathione), carbohydrates (e.g. activated glucuronic acid), sulfate or amino acids to further increase the water solubility of the metabolites (Fig. 6).

The primary oxidative enzymes involved in phase I reactions belong to a number of different enzyme families, including the cytochrome P-450-dependent monooxygenase (MFO) and the flavine-dependent monooxygenase (FMO) systems, prostaglandin synthetase (PGS) and other peroxidases, monoamine oxidase (MAO), and a number of dehydrogenases. The MFO system has found the most interest for environmental monitoring but since this system does also metabolise endogenous substrates such as steroid hormones, prostaglandines and fatty acids next to xenobiotic compounds, the interpretation of MFO induction should be made with caution (for review Livingstone, 1988; Livingstone et al., 1989; see also Section 2.2).

MFO induction as an exposure response for dioxins, PCBs, PAH and further groups of organic contaminants (Livingstone and Farrar, 1985), can be visualised by means of immunocytochemical techniques in native tissues or by electrophoretic separation of homogenates and microsomal preparations (Segner and Braunbeck, 1998). Alternatively, artificial substrates have been employed to assess MFO activities or of other enzymes of the phase I group such as 7-ethoxyresorufin-O-deethylase (EROD) or benzo(a)pyrene hydroxylase (B(a)PH). In general, it has been found that phase I enzyme levels correlate well with measured pollutant levels in the field for a number of aquatic vertebrates and invertebrates, including molluscs. EROD activities and cytochrome P-450 concentrations demonstrated the greatest sensitivity within the

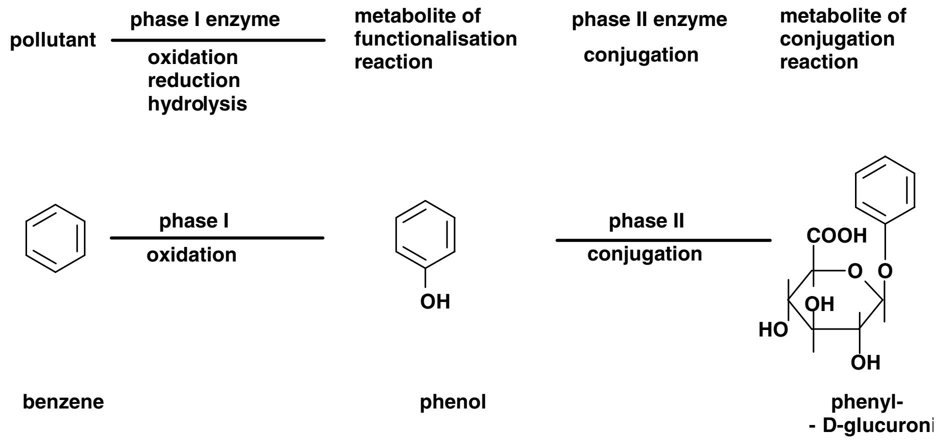

Figure 6. General metabolism scheme of xenobiotics (above) with the example of a specific metabolism of benzene (below) (modified from Oehlmann & Markert, 1997).

different parameters, which can be principally used. Further phase I enzymes like the epoxide hydrolase and phase II enzymes such as the glutathione-S-transferase have received by far less attention for biomonitoring purposes.

In contrast to the numerous studies on MFO activities and MFO induction in marine molluscs, particularly bivalves, the application of this biochemical measure of organic contaminant exposure has only occasionally been employed in freshwater environments. Wilbrink et al. (1991) provide one of the rare studies on MFO activities in a freshwater gastropod, the pond snail *Lymnaea stagnalis*, while Dauberschmidt et al. (1997) investigated the MFO system in the zebra mussel *Dreissena polymorpha*.

3.2.6. Acetylcholinesterase (AChE) activity

Although acetylcholinesterase (AChE) activities are considered in a number of biomonitoring programmes as a specific marker of organophosphorus or carbamate pesticide exposure, it has recently been shown by Labrot et al. (1996) that AChE activities can be modulated by metals in the freshwater bivalve *Corbicula fluminea* in vivo. Escartin and Porte (1997) investigated the use of cholinesterase and carboxylesterase activities in the mussel *Mytilus galloprovincialis* as a marker of pesticide exposure and effects in the Ebro Delta in Spain, an area of intense rice culture with a marked application of insecticides. They determined body burdens of organophosphates and seasonal variations of the activities of AChE and of two further esterases in the gills and digestive glands of mussels. The gill esterases were found to be more sensitive to in vitro inhibition than the same enzymes in the digestive glands. The carboxylesterase (CbE) was more sensitive than AChE and the authors concluded that CbE may play a protective role by removing a significant amount of the activated metabolite prior to reaching the target site AChE during an organophosphate intoxication. In mussels collected from the delta, gill AChE activity varied with season. The maximum activity was recorded in January and minimum values in April and May. The gill CbE exhibited its maximal activity from September to February and low activity from April to August. The low activity period coincided with the opening of paddy field irrigation channels with a consequent inflow of contaminated water into the delta. This minimum activity also coincided with maximum residues of fenitrothion and vamidothion in mussel tissues. The activity changes could be related to changes in water temperature, changes in salinity or normal seasonal variations. However, mussels collected from a less polluted reference area did not show such variations suggesting these are directly due to the effects of exposure to the pesticide in the run-off from the paddy fields.

Comparable attempts as described here for *Mytilus galloprovincialis* have been made also with freshwater mussels by Fleming et al. (1995) and Moulton et al. (1996).

3.2.7. Genetical markers

Pollutants can effectively influence the genetic stock in a population at two levels. They can select for certain characteristics in a population and thus alter the allele frequency as indicated in the study of Sultan et al. (2000) with the bivalve *Donax trunculus*. In addition, they can damage the DNA giving rise to gene mutations or chromosomal aberrations. A number of techniques exist for detecting genetic damages and these have been applied also to a number of mollusc species, primarily marine mussels. Chromosome mitotic abnormalities have been used to indicate differences in damage as a result of pollution exposure in mussel and oyster embryos and the sister chromatid exchange (SCE) system has been employed to demonstrate genetic damage in juvenile and adult *Mytilus edulis*, including early life stages. For these techniques molluscs have found wider application than for example fish, because the latter have larger numbers of small chromosomes, which give rise for a number of methodological problems. The comparatively fast rate of cell division in mussels, irrespective of their age, is of advantage compared to adult fish, as it allows shortened exposure periods to colchicine to accumulate dividing cells at metaphase and also to 5-bromodesoxyuridine (Brdu) as a marker, which itself can cause DNA damage. Recently, a simpler method, the micronucleus (MN) test, has been proposed for monitoring contamination exposure and effects in marine molluscs. This test, which seems to be predictive of the reproductive success, has the advantage of being karyotype independent and is thus less expensive to conduct on samples without extended pretreatments, although Burgeot et al. (1996) concluded on the basis of their comparative study that the MN test has to be improved before it can be applied for monitoring as a routine test. The incidence of micronuclei and other nuclear abnormalities in gill cells and haemocytes of Mediterranean mussels were investigated by Venier et al. (1997). The authors reported a concentration dependent increase for both markers with respect to benzo[a]pyrene exposure. Mersch and Beauvais (1997) transplanted caged zebra mussels, *Dreissena polymorpha*, to six monitoring sites in France, receiving industrial effluents suspected of containing genotoxic chemicals. After an exposure period of two months, the induction of MN in haemocytes was successfully determined as a criterion for genetic damage.

The SCE technique and its applicability to mussels such as *Mytilus galloprovincialis* is described in detail by Martinez-Expositio et al. (1994) and Pasantes et al. (1996).

The latter study described additionally seasonal variations and inter-population differences of SCE frequencies. Jha et al. (2000a) applied SCEs and further chromosomal aberrations as endpoints in a study of genotoxic effects of UK harbour sediments using embryos and larval stages of *Mytilus edulis*. The evaluation of the genotoxicity gave a positive response for all considered endpoints, emphasising the need for the assessment of short and long term genotoxic impacts of dredged disposal on marine biota.

One of the best studied markers of DNA damage in aquatic molluscs and particularly in marine mussels is the so-called comet assay, which is capable to determine DNA single strand breaks. Steinert et al. (1998) conducted a survey of DNA damage in San Diego Bay, which was determined in haemocytes collected from transplanted and autochthonous *Mytilus edulis* at stations in and around the Naval Station San Diego. Transplanted mussels were exposed at selected stations for approximately 30 days in plastic mesh bags. Those stations exhibiting the extremes of contaminant exposure, both highest and lowest concentrations, were easily identified by the comet assay results. The assay and in particular germ cell DNA damage determinations were found to respond rapidly to station contaminants. The robustness of the assay was investigated by Wilson et al. (1998) for *Mytilus edulis*. The authors studied baseline levels of single strand breaks in isolated gill cells and how they were affected by age or size of animals, time since collection and the applied feeding regime. Comet assay results in untreated controls were found to be highly variable over time. Fluctuations between low and very high DNA damage occurred over just 14 days post collection. No differences were observed between age or size and feeding regime of the mussels but a vitamin E supplementation in the diet of the organisms resulted in a marked reduction in the levels of DNA damage in the controls and in an increased sensitivity of the comet assay at the lower end of the concentration range.

3.2.8. Immunotoxicity

The use of immunosuppression or -stimulation as an indicator of pollution stress is complicated by the range of the different factors involved in the immune response of intact organisms. Whilst the immunological mechanisms of invertebrates are believed to be less complex than those of vertebrates, the various assays for measuring immune responses particularly in mussels have found wide application, especially the phagocytosis assay for *Mytilus edulis* (for review Pipe et al., 1995a). The immune system of molluscs depends largely on circulating haemocytes present in sinuses, which are able to migrate throughout the tissues to protect against potential pathogens and undertake immunosurveillance. These haemolymph cells can be divided into a number of subpopulations on the basis of functional and staining characteristics (Noel et al., 1994). The primary defence strategies involve phagocytosis, incorporing release of oxygen metabolites and degradative enzymes, and the secretion of agglutinating and cytotoxic compounds (Pipe et al., 1995a). The conventional methodology for assessing invertebrate phagocyte activation and phagocytosis has generally relied upon labour intensive and time consuming microscopic assessment or agarose plate assays. One of the major difficulties encountered when trying to assess overall immunocompetence is the interindividual variability, which arises due to the polygenic nature of natural mussel populations. The generation of statistically significant data requires large numbers of replicates. To overcome the logistical problems associated with performing numerous assays with sufficient replicates, a number of new techniques, based on optical density values obtained with a 96-well microtitre plate reader, have been developed and already applied for marine mussels such as *Mytilus edulis* and *Mercenaria mercenaria*, but also for freshwater clams like *Anodonta cygnaea* (Anderson and Mora, 1995).

Biomonitoring examples considering measures of immunomodulation are provided by Pipe et al. (1995b), Dyrynda et al. (1998) and more recently by Matozzo et al. (2001). Pipe et al. (1995b) sampled *Mytilus galloprovincialis* at three times of the year from various sites within the Venice Lagoon and a reference site in the north Adriatic. The immune response of the mussels was assessed using a range of assays, including total and differential cell counts, phagocytosis, degradative enzyme levels and release of reactive oxygen metabolites. Chlorinated hydrocarbons, including lindane, DDT and PCBs, together with trace metal levels were measured in digestive gland tissues from the mussels. The measurements of immune response and the contaminant levels showed seasonal fluctuations. However, the results demonstrated significant differences in a number of immunotoxicity assays, which showed some correlation with the levels of tissue-bound contaminants. Dyrynda et al. (1998) used a comparative approach with the blue mussel *Mytilus edulis* from six sites within the UK, comparing stations with histories of severe contamination problems with relatively uncontaminated reference sites. The results showed that significant differences in immunocompetence were evident between mussels from contaminated and reference sites. Haemocytes of mussels from contaminated sites showed enhanced superoxide production and reduced activity of degradative enzymes. Nevertheless, the results indicated that not all immune parameters are affected by contamination and that the type and extent of effects on immune defences vary with the nature or concentration of the contaminants.

Watermann et al. (1996) have introduced the assessment of inflammatory processes as an additional parameter of immunocompetence, measured as the incidence and/or intensities of haemocyte infiltrations and granulocytomas in the midgut gland, mucosa of the intestine and in storage tissues of the mussel *Mytilus edulis* and the winkle *Littorina littorea*. The authors found a significant positive correlation of these responses with the exposure of mollusc populations to xenobiotics in their environment.

3.3. Biological effects on the organism and community level

Due to the large number of relevant studies reported in the literature, only a limited example selection of monitoring programmes can be presented in this chapter, which employ biological effects in aquatic molluscs on the organism level, such as mortality or survival, growth, biological fitness (often termed as "condition"), structural alterations and interference with development.

3.3.1. Mortality or survival

In the 1960s and 1970s it was a standard procedure to expose caged mussels, oysters, clams or other bivalves and even snails for a certain period at different sites in coastal waters and to assess the percentage of dead specimens when the transplants were recollected at the end of the survey. These mortality data were often a kind of by-product of bioaccumulation studies. The same technique was later adopted for the monitoring of freshwater ecosystems and even for the surveillance of sewage outfalls or of other pollution point sources. The interpretation of such transplant results is not as easy as it looks at first glance because it remained under certain circumstances doubtful whether the exposed molluscs died due to the existing level of contamination or just because other environmental factors were unsuited (e.g. salinity, temperature, lack of food or hypoxia following the fouling of the exposure bags). Additionally, mortality is a rather rigid endpoint for the assessment of environmental quality in aquatic ecosystems and can therefore not compete with more sophisticated and modern biomarker techniques, speaking in terms of sensitivity. Nevertheless, even today mortality can be of some worth as an endpoint, especially in monitoring programmes with mussels, as demonstrated by Viarengo et al. (1995). The authors describe that a short exposure of *Mytilus edulis* to sublethal concentrations of pollutants such as Cu, Arochlor 1254 or other organic contaminants reduced the capacity of the animals to survive in air significantly. The effect was markedly concentration dependent and was strongly increased by pollutant mixtures. This parameter exhibits a sensitivity, which is in the same range as commonly used biomarkers, like the NRR time assay. Because this methodology is simple, inexpensive and does require only modest equipment, it can be integrated also in monitoring programmes for contaminated coastal areas.

3.3.2. Growth – scope for growth index

Chronic toxicities arising from exposure to sublethal concentrations of toxicants are often integrated and reflected in key life-history parameters (e.g., growth rates and reproductive efforts), or patterns of energy allocation of the organism. The rate of growth is one of the most sensitive measures of stress in an organism and responses also to chemical stressors in the environment. The quantification of growth is not as simple as it could be probably expected, especially in aquatic molluscs. Although molluscs offer the unique opportunity to use not only morphometric measures of their soft tissues, like weight or length, but additionally also shell parameters to assess growth, the problem remains, that many species exhibit an intermittent growth pattern. The shell is formed primarily at certain times of the year and body weight changes exhibit a marked seasonality due to the reproductive cycle with a considerable weight gain during the formation phase of the sexual products and a consequent loss of up to 80% of the individual biomass during spawning (Giese, 1959; Giese and Pearse, 1977). Part of the difficulties of quantifying and also interpreting growth measures can be overcome by the determination of the energy available for growth and reproduction in an organisms, also named scope for growth (SfG). SfG analyses have become particularly popular for monitoring studies with aquatic molluscs in the past, which might reflect the above mentioned problems of growth assessments in this special group of invertebrates. SfG is an integrative measure of the energy status of an organism at a particular time. Three critical components (food consumption, egestion and respiration) are required in order to calculate the overall energy value, represented by the index P according to Winberg (1960):

$$P = A - (R + U)$$

Where A is the energy absorbed from food, R is the equivalent energy used for respiration and U is the energy dispensed during egestion. Among those who favoured this concept for monitoring purposes with molluscs are Widdows et al. (1982) and Bayne et al. (1985). Bayne and Worral (1980) demonstrated a close relationship between this index and tissue growth in two populations of *Mytilus edulis*.

The sensitivity of SfG as a parameter for environmental monitoring, but also the limitations of such studies were recently demonstrated by Sobral and Widdows (1997). *Ruditapes decussatus* specimens from southern Portugal were exposed to a sublethal copper concentration of 10 μg/l for 20 days. The experiment showed two phases. Initially, Cu was rapidly accumulated, clearance rates declined markedly and respiration rates increased, resulting in a rapid SfG decline, which showed a negative value after five days. From day 9 on, the rate of Cu uptake declined and physiological responses were more stable with positive SfG values, but still significantly reduced when compared to the control (ca. 23% of the control values). This indicates that though animals partially recovered through detoxifying mechanisms, Cu caused sustained impairment of physiological functions. The experiment confirmed that though integrated SfG measurements are a sensitive methodology to detect deviations from normal performance and assess stress at environmental realistic pollutant concentrations, the result is also affected by the adaptation of animals to their specific environmental conditions. Cranford et al. (1999) exposed adult sea scallops, *Placopecten magellanicus*, in the laboratory under environmentally relevant conditions to different types and concentrations of drilling fluids. The authors observed a close relation between SfG values and actual growth measurements. Their results showed furthermore that chronic intermittent exposure of sea scallops to dilute concentrations of drilling wastes, which were characterised by acute lethal tests as practically nontoxic, can affect growth.

Wo et al. (1999) investigated in a comparative study the effects of sublethal Cd concentrations on different growth parameters of the intertidal marine gastropod *Nassarius festivus*. Their sensitivity based assessment suggests that SfG is the most sensitive growth biomarker, followed by the RNA/DNA ratio, and then the conventional growth measurement based on shell size and body weight. At all tested Cd concentrations, the authors determined negative SfG values (Fig. 7).

SfG measurements were performed by Widdows et al. (1995) for *Mytilus edulis* specimens, collected from 26 coastal sites from the Shetland Islands to the Thames estuary and eight offshore light vessels, to monitor changes in environmental quality along the British North Sea coastline. SFG values declined from north to south, reflecting both the major inflow of clean water from the North Atlantic via the north of Scotland, and the overall increase in environmental contamination with increasing urbanisation and industrialisation towards the south. There were coastal regions (e.g. Humber–Wash area and the Thames estuary) as well as specific sites, which showed markedly reduced SFG. The authors showed that at more than half of the sites the reduced SFG could be entirely explained by the recorded concentrations of contaminants in the tissues with polyaromatic hydrocarbons being one of the major responsible pollutant classes. At none of the stations were metals accumulated to concentrations

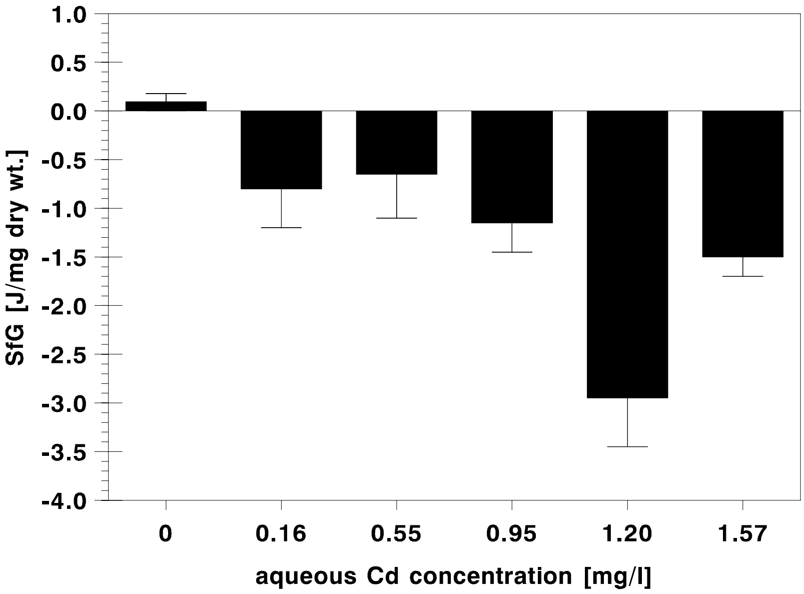

Figure 7. Effect of aqueous Cd exposure on the scope for growth (SfG) in *Nassarius festivus* (mean values ± standard deviation; values from Wo et al., 1999).

that could cause a significant reduction in SFG values. A comparative approach was performed by Widdows et al. (1997) with *Mytilus galloprovincialis* from the Venice lagoon. The authors assessed significant negative correlations between SfG values and tissue concentrations of petroleum hydrocarbons, PCBs, DDT and HCH, but found no significant correlations between SfG and metal tissue concentrations for Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn.

Din and Ahamad (1995) utilised caged blood cockles, *Anadara granosa*, to monitor the effects of a highly polluted industrial discharges along a transect with eight stations on growth rates in the Juru area, Malaysia. In this region a marked decline in fisheries was reported since the early 1970s and the authors could demonstrated a massive reduction of SfG values with the highest effects at the stations in direct vicinity of the effluent. The applicability of the SfG concept for tropical freshwater snails was recently demonstrated by Lam (1996).

3.3.3. Fitness – condition index

Integrated physiological measurements of stress can generally be estimated by determinations of the body condition index, which can be calculated in different ways: (1) ratio of dry tissue to shell weight; (2) ratio of wet tissue to shell weight; (3) ratio of wet tissue volume to internal shell volume. These responses are indicative of multiple stressors on the animal, as toxicants deplete energy reserves which were potentially destined for growth and reproduction, thus reducing also the reproductive success and the overall fitness of the organism (Lucas and Beninger, 1985). Reserves are alternatively channelled into energy consuming detoxication processes and animals from contaminated waters have retarded growth and poor tissue condition. Condition indices from mussels are, therefore, potentially very sensitive to pollution. However, condition indices are affected by a number of additional environmental stressors next to pollutants, such as salinity, temperature, infestation with parasites and food availability.

Nicholson (1999) investigated in his comparative study in Hong Kong a range of cytological and physiological biomarker responses in caged green mussels, *Perna viridis*, for their suitability to indicate pollutant exposure, including condition indices. Specimens from the contaminated locations had significantly lower condition indices than reference site individuals. Condition indices integrate stress responses on somatic growth, but are not always indicative of stress as they can be affected by seasonal changes and associated nutritional and reproductive states at spatially different sites (Roesijadi et al., 1984; Leavitt et al., 1990). Gold-Bouchot et al. (1995) reported a significant negative correlation between condition indices in *Crassostrea virginica* populations in Mexico and ambient concentrations of Cd and Zn.

Juvenile *Mytilus edulis* specimens were transplanted by Grout and Levings (2001) along a pollution gradient of acid mine drainage from an abandoned Cu mine in Canada. Cages, each containing 75 mussels, were placed at a total of 15 stations and were exposed to concentrations of dissolved Cu in surface waters ranging from 5 to 1009 μg/l for a period of 41 days. Declines in survival and condition index occurred in mussels that bioaccumulated more than 40 μg Cu/g (dry wt.). The reduced survival of transplanted mussels was supported by an absence of natural mussels in the contaminated areas.

Veldhuizen-Tsoerkan et al. (1991) collected *Mytilus edulis* specimens from a relatively unpolluted area of the Eastern Scheldt in the Netherlands and transplanted them along contaminated sites of the Western Scheldt for 2.5 and 5 months. The authors determined established stress indices, including the condition index, and found a negative effect on condition index development at polluted sites in their study area.

3.3.4. Structural alterations – histopathology

The assessment of structural alterations on the cell and tissue level has a long and successful history in environmental monitoring programmes. They provide an integrative measure for the intactness of key organs in an organism and are a powerful tool to detect a broad range of different contaminant effects. The main disadvantage of using histopathological measures for such purposes is that they are time and cost intensive and require experienced, well-trained personnel to avoid misinterpretations of the findings.

Couch (1984) proposed the atrophy of the diverticular epithelium in *Crassostrea virginica* as a non-specific pollution index, based on a correlation of this effect with the contaminant exposure level at a number of analysed stations. Weis et al. (1993) reported the same histopathological alteration in oysters transplanted from a reference site to a station where they were exposed to the wood preservative chromated copper arsenate. Bowmer et al. (1994) analysed growth, reproductive performance, structural changes and heavy metal accumulation in *Cerastoderma edule* exposed to pulverised fuel ash. Although no apparent bioaccumulation was observed, the authors found histopathological and reproductive effects, which correlated well with mortality, implying that the occurrence of relatively slight effects, particularly in the digestive gland, could be an indicator of mortality. Berthou et al. (1987) conducted a study on the European flat oyster, *Ostrea edulis*, and the Pacific oyster, *Crassostrea gigas*, after the "Amoco Cadiz" oil spill in Brittany. The histopathological analyses revealed the highest incidence of lesions in the digestive tract, followed by the interstitial tissue and the gills. These effects were accompanied by an increased mortality in the populations during the first three months after the accident.

Gold-Bouchot et al. (1995) reported in *Crassostrea virginica* tissues from oyster populations in three coastal lagoons in Mexico, that more than half of all specimens showed histopathological lesions, which could be related to salinity but also to the concentrations of cadmium and hydrocarbons in their environment. Gregory et al. (1999) investigated the possibility that changes in the surface morphology of gill filaments of the mussel *Perna perna* may be used to indicate the relative toxicity of pollutants in the marine environment. In Hg exposed specimens, the authors described a gradual increase in a number of histopathological parameters, such as diameters of microvilli, depletion of abfrontal cilia, increase in necrotic cells and of cilia on the lateral gill surfaces. These results were recently confirmed by Bigas et al. (2001).

Vaschenko et al. (1997) analysed histological alterations in the gonads of the scallop *Mizuhopecten yessoensis* sampled from six stations in the Sea of Japan and demonstrated a retardation of gametogenesis, oocyte resorption, autolysis of spermatozoa and their phagocytosis in the gonads of scallops from polluted sites. Additionally, the percentage of gonadial hermaphrodites was about 6% against 0.3–0.4% in the scallop populations from clean areas.

3.3.5. Developmental effects

From an ecological perspective, one of the most important responses of organisms to pollution is the impairment of reproduction due to either direct effects on the formation of germ cells (as described above) or to interference with normal development processes in animal early life stages. Additionally, it is well documented that early life stages of many species are several orders of magnitude more sensitive to pollutants than the adults. Among the molluscan embryonic or larval biotests, the so-called oyster embryo bioassay (OEB) with early life stages of *Crassostrea gigas* is in widespread use (Beiras and His, 1994; His et al., 1997b), especially for the assessment of toxicity in coastal sediments (Chapman and Morgan, 1983). Even in international programmes this bioassay has been validated as a useful biological tool for marine environmental quality assessment (Williams et al., 1986; Butler et al., 1992; Chapman et al., 1992). One of the main problems of this test is the availability of more or less homogeneous biological material during the entire year. Because cryopreserved, cloned, or established laboratory strains of marine test species are currently not available, techniques for long term preservation of environmental samples are required. Beiras et al. (1998) investigated the effects of temperature and duration of storage on the toxicity of estuarine sediments using the OEB. Sediments ranging from unpolluted (controls) to extremely polluted with heavy metals (>100 mg/kg Hg, Cu, Zn, and Pb) and total hydrocarbons (>1000 mg/kg) were collected from sites in southwest France and northern Spain. Development of oyster embryos was significantly reduced in polluted sediments. A prolonged storage of fresh sediments at 4°C resulted in a loss of toxicity, which was more rapid in the less contaminated sediments. Deep frozen sediments (−196°C) were highly toxic regardless of origin and storage time. Because deep freezing caused spurious toxicity in the control samples, the authors recommended not to consider a freezing of sediments at such low temperatures for biomonitoring studies. Should the sediments require prolonged storage, freezing at −20°C appeared to be the best choice.

His et al. (1997a) utilised the OEB for the identification of polluted sediments in mud flats of Arcachon Bay in western France, where oyster farmers discarded old coal-tarred material with a resulting PAH contamination of up to 10.5 mg PAH/kg. Vaschenko et al. (1997) recorded marked effects in the development of offspring of the scallop *Mizuhopecten yessoensis* sampled in Peter the Great Bay, Sea of Japan. At polluted sites, a decrease in fertilisation success, diminution in percentage of normal trochophores, D-veligers, veligers, and a retardation of larval growth were recorded. Those scallop populations inhabiting the most polluted areas of Peter the Great Bay seemed to be incapable of normal reproduction. Dixon and Pollard (1985) assessed embryo abnormalities in the ovoviviparous snail *Littorina saxatilis* as an indictor of pollutant exposure in coastal waters and Koster and van den Biggelaar (1980) described impairment of development in the scaphopod genus *Dentalium* after the "Amoco Cadiz" oil spill in northern France.

Dregolskaya (1993) investigated the effects of environmental pollutant exposure on development of early life stages in two freshwater gastropod species, the pulmonate *Lymnaea stagnalis* and the prosobranch snail *Bithynia tentaculata*. In both molluscs, the most vulnerable development stage to toxicant exposure was the last stage before metamorphosis, when the reduction of the protonephridium occurred and a definitive kidney had not yet been built up. The author described furthermore a species-specific difference in the sensitivity to Cu in the snails, but because pond snail embryos were obtained from an unpolluted region, while *Bithynia* eggs came from a region receiving industrial water discharges, the observed differences might be due to specific adaptations in these populations.

3.3.6. Community effects

Analysis of biological communities with respect to environmental changes was primarily focussed on the effects of introduced exotic species in a certain habitat with the zebra mussel *Dreissena polymorpha* being in the centre of interest in the past (e.g. Dermott and Kerec, 1997; Karatayev et al., 1997; Strayer et al., 1998). Nevertheless, benthic community analyses have been shown to be a sensitive measure to reflect the impact of pollution on marine and freshwater life, changes in community structure being directly related to the ecological "health" of the environment and were thus recognised in the Joint Assessment and Monitoring Programme (JAMP) of the Oslo and Paris Commissions (OSPARCOM). The established role of benthic community analysis in monitoring pollution has already provided a number of species, particularly molluscs, which are recognised as stress indicators.

McCarthy et al. (1997) published a case study conducted at the Slave River in Canada between 1990 and 1994. The catchment of this river receives discharges from industrial and agricultural processes providing a certain background pollution level. The main objective of the five-year programme was to establish a baseline data set for comparison purposes in future monitoring programmes. Special emphasis was laid on the assessment of benthic invertebrate populations, since it was recognised that such a survey could be important for biomonitoring purposes. Abundance of organisms, taxon diversity, and presence or absence of sentinel species were used to assess environmental contamination. The study concluded that the abundance of benthic invertebrates at the numerous sites examined in the Slave River was very low and organisms that had been used in other biomonitoring studies, especially bivalve molluscs or large oligochaetes were rare or totally absent. Over 90% of the invertebrates collected from the Slave River were chironomids or small oligochaetes and comparisons of benthic invertebrate communities in the Slave River Delta indicated that few changes in percentage composition or diversity had occurred over a 10-year period. The authors reported that molluscs were the rarest invertebrate group in the entire river system, indicating their dedicated susceptibility to pollutants in freshwater systems.

Statzner et al. (2001) investigated benthic invertebrate communities in European freshwater systems to illustrate how multiple biological traits could provide a measure for the large scale biomonitoring of the functional composition of communities. Their measure considered the relative abundance of 63 categories of 11 biological traits (such as size, reproductive and dispersal potential, food and feeding habits) that indicate various ecological functions. Comparing this measure for 10 French reference regions with 37 other most natural stream types scattered across Europe demonstrated an extremely high spatial and temporal stability of the functional composition of natural invertebrate communities at the European scale. The authors identified highly significant differences between natural reference and human-impacted communities, especially in regulated stream sites below dams and in rivers receiving sewage inputs.

Gardner (2000) investigated the bivalve community composition in the Cook Strait region, New Zealand and found evidence that environmental parameters, particularly the seston quality, were crucial for the distribution of the mussels *Aulacomya maoriana*, *Mytilus galloprovincialis* and *Perna canaliculus*.

3.4. Tributyltin as an integrated case study

Tributyltin (TBT) compounds are mainly used as biocides in antifouling paints, but also in various other formulations. They produce a variety of malformations in aquatic animals with molluscs as one of the most TBT-sensitive groups of invertebrates (for review Bryan and Gibbs, 1991; Fent, 1996). As the impact of TBT on nontarget organisms became apparent in the early 1980s, France was the first European country to draw up regulations to control TBT emission and banned the use of TBT antifoulings on small boats (length <25 m) in 1982. The French legislation was adopted by other countries since 1987 almost worldwide, but in the following years TBT pollution of coastal waters was found to have remained on a high level or even increased further in many regions (e.g. Oehlmann et al., 1993; Minchin et al., 1995, 1996, 1997; Huet et al., 1996). This was the main reason for the decision of the International Maritime Organization (IMO) in autumn 2001 to ban the application of TBT-based paints on all boats by January 2003 and the presence on ship hulls by January 2008.

The first adverse effects of TBT on wildlife molluscs were observed in the Bay of Arcachon on the west coast of France, one of the main centres of oyster aquaculture in Europe. Alzieu et al. (1980, 1986) described ball-shaped shell deformations in adult oysters, *Crassostrea gigas*, and found that the annual spatfall also declined dramatically. Both effects resulted in a break-down of oyster production in the bay with marked economic consequences. Later, comparable effects were also observed in southern England and Ireland. Detailed laboratory and field investigations revealed that TBT, leached from antifouling paints of yachts and other smaller vessels in marinas and moorings near the oyster fields, was the causative agent for the adverse responses in oysters with trace concentrations as low as 10 to 20 ng TBT/l in ambient water being already effective (Bryan and Gibbs, 1991). The induction of shell deformities in oysters, but also in further bivalve groups, has been successfully applied as a biological marker of TBT effects in the following years (e.g. Alzieu et al., 1986; Dyrynda, 1992; Page et al., 1989, 1996; Phelps and Page, 1997). Another effect of TBT in molluscs was first described in a number of regions worldwide in the early 1970s without identifying the organotin compound as the responsible cause at that time: A virilisation of female prosobranchs, which has been termed as imposex (Smith, 1971) or pseudohermaphroditism (Jenner, 1979). The imposex phenomenon of prosobranchs, i.e. the formation of a penis and/or vas deferens on females of these gonochoristic species is induced at lower concentrations than all other described TBT effects. Furthermore, it is a specific response of organotin compounds, so that the use of imposex offers the unique possibility for a highly sensitive biological effect monitoring for a special group of man-made chemicals in the environment.

Molluscs are effective bioaccumulators of organotin compounds, a favouring aspect for their extreme sensitivity compared to other systematic groups. They exceed the highest reported TBT bioaccumulation factors (BAFs) for algae (3.0×10^4), annelids (3.0×10^3), crustaceans (4.4×10^4) and fish (3.0×10^4) by at least one order of magnitude (for review Oehlmann, 1994). BAFs of 1.5×10^3 to 3.0×10^5 were measured in marine molluscs and values of 8.3×10^3 to 4.5×10^5 for freshwater bivalves and snails (Bryan and Gibbs, 1991; Schulte-Oehlmann et al., 1995; Oehlmann et al. 1996a, b, 1998b). Due to this fact, molluscs and especially freshwater and marine bivalves have widely been used as bioaccumulation indicators of TBT contamination in the past. Examples for such approaches are the investigations of Becker et al. (1992) and Becker-van Slooten and Tarradellas (1995) in Swiss lakes, using the zebra mussel *Dreissena polymorpha*, and the analyses of Short and Sharp (1989), Higashiyama et al. (1991), Page (1995), Phelps and Page (1997), Morcillo et al. (1999), Jacobsen and Asmund (2000) with different marine bivalves.

A variety of biological markers on the sub-organism level in molluscs were applied for the assessment of TBT contamination, especially in the coastal environment. It is virtually impossible, to give a representative overview of all the different techniques and endpoints, which have been used in the past for this purpose, but at least some examples will be provided additionally to those already covered by the reviews of Bryan and Gibbs (1991) and Fent (1996). Brick and Deutsch (1993) and Sundermann et al. (1998) used ultrastructural endpoints for marine prosobranch snails. HSPs and other stress protein levels were investigated by Ludebye et al. (1997), Clayton et al. (2000) and Smith et al. (2000). Jha et al. (2000b) applied genetic markers like the SCE assay in mussels and Cima et al. (1999) used markers of immunotoxicity in the clam *Tapes philippinarum*. The majority of TBT-related biochemical marker studies was dedicated to the assessment of effects on key enzymes of the phase I and phase II metabolism and the use of these endpoints in biomonitoring studies. Special emphasis was laid on the cytochrome P-450-dependent MFO system (e.g. Livingstone et al., 1989; Ronis and Mason, 1996; Morcillo et al., 1998a, b, 1999). In contrast to other organic contaminants, TBT does not induce the MFO system of molluscs, but causes a marked reduction of both MFO activities and cytochrome P-450 content. These findings are in line with the results of Spooner et al. (1991), Schulte-Oehlmann et al. (1995), Bettin et al. (1996), Oehlmann and Bettin (1996), Morcillo and Porte (1997) and Morcillo et al. (1998a, b, 1999), who described an increase of testosterone concentrations in TBT exposed molluscs, most probably due to an inhibition of the cytochrome P-450-dependent aromatase.

The imposex response is known today for more than 150 prosobranch species. The gradual virilisation of imposex affected females can be described by a development scheme with six stages, furthermore divided in up to three different types (a-c) (Gibbs et al., 1987, further developed by Fioroni et al., 1991), which has the advantage to be applicable for all affected species worldwide. Females are sterilised in the imposex stages 5 and 6 by one of the following mechanisms: (a) blockade of the pallial oviduct (Fig. 8), as in *Nucella lapillus* at ambient TBT concentrations above 2.0 ng as Sn/l or (b) by a split bursa copulatrix and capsule gland (stage 5c) as in *Ocenebra erinacea* (threshold concentration 8 ng TBT as Sn/l). The first possibility prevents the deposition of egg capsules, resulting in an accumulation of abortive capsular material in the pallial oviduct (stages 6a, b); the second mechanism prevents copulation and capsule formation. In young and sexual immature specimens of some muricid species a protogyne sex-change can be induced by TBT concentrations, e.g. above 10 ng as Sn/l in *N. lapillus* (Gibbs et al., 1988; Oehlmann et al., 1991) and above 2 ng as Sn/l in *Ocinebrina aciculata* (Oehlmann et al., 1996a)

The classification in six different stages is the basis of the VDS (vas deferens sequence) index, calculated as the mean imposex stage of a population. This parameter allows the assessment of imposex intensities in natural populations and laboratory groups. It has been shown that imposex intensities, measured as the VDS index in a range of affected prosobranch species, show a highly significant correlation with TBT concentrations in ambient sea water, as demonstrated for the dog whelk, *Nucella lapillus* in Figure 9a. Consequently, the degree of coastal TBT pollution can be assessed with high precision by a determination of imposex intensities in prosobranch populations. A further advantage of the VDS index as a measure of imposex is the possibility to perform interspecific comparisons of TBT sensitivities of different species and that the index is also a measure of the reproductive capability of a given population (for details cf. Oehlmann et al., 1996b). The imposex phenomenon has been successfully used in TBT biomonitoring studies in Scotland (e.g. Bailey and Davies, 1988a, b, 1989, 1991), England (e.g. Gibbs et al., 1990, 1991), Ireland (e.g. Minchin et al., 1995, 1996, 1997), France (Oehlmann et al., 1993; Huet et al., 1996), the

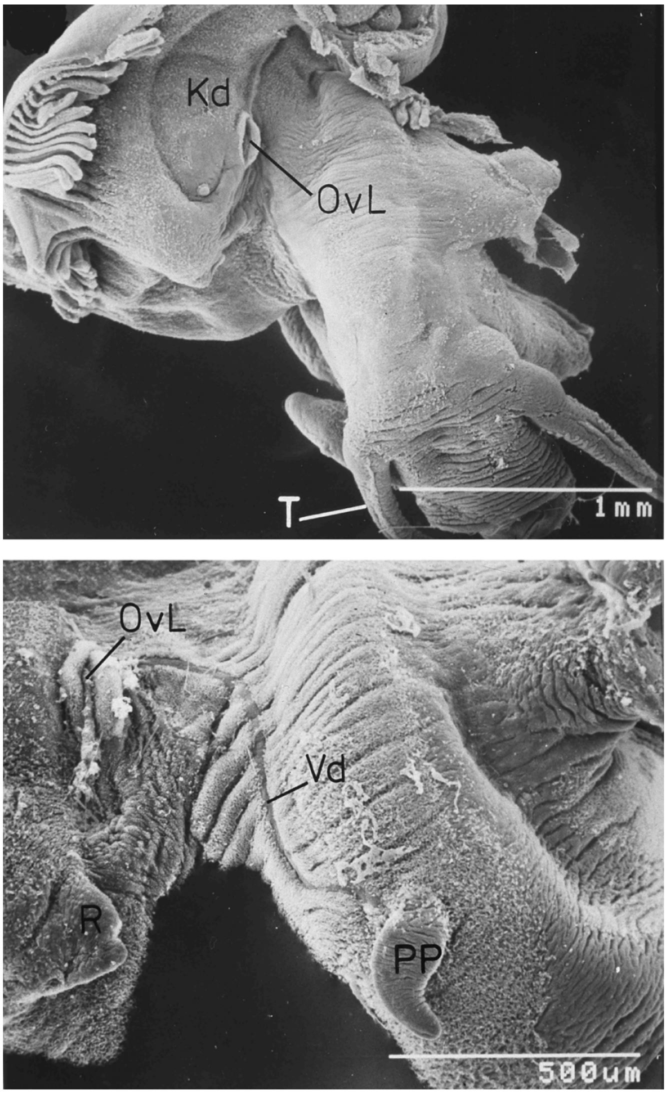

Figure 8. *Hydrobia ulvae*. Scanning electron micrographs of female specimens with their mantle cavity opened. Above: normal female without imposex; below: sterilised female in the final stage of imposex development with blocked oviduct. Abbreviations: Kd, capsule gland; OvL, Ooparous opening of oviduct (oben above; blocked below); PP, Penis; T, tentacle; Vd vas deferens.

Mediterranean (e.g. Terlizzi et al., 1999) and outside Europe, e.g. in the United States (e.g. Short et al., 1989; Saavedra Alvarez and Ellis, 1990), Canada (e.g. Bright and Ellis, 1990), southeast Asia (e.g. Ellis and Pattisina, 1990; Horiguchi et al., 1997, 1998), New Zealand (e.g. Stewart et al., 1992) and Australia (e.g. Kohn and Almasi, 1993; Wilson et al., 1993).

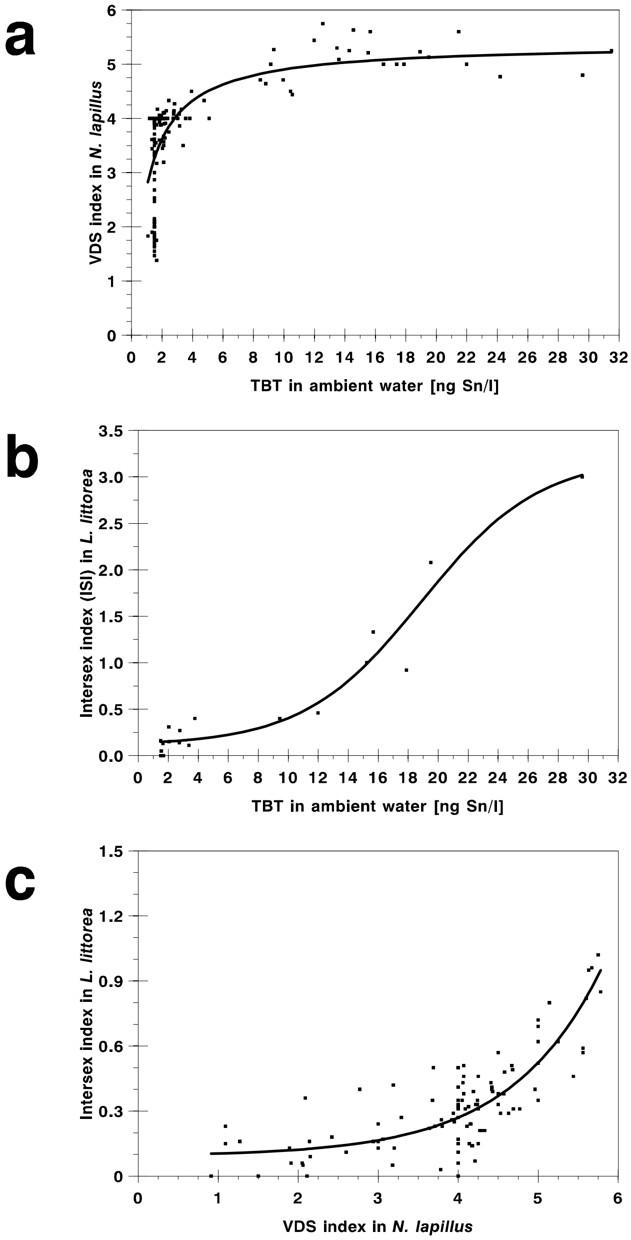

Figure 9. Relationship between ambient TBT concentrations in water and imposex intensities in *Nucella lapillus* populations (a) or intersex intensities in *Littorina littorea* populations (b). In (c), the relationship between the intersex index in *L. littorea* populations and VDS indices in sympatrically living populations of *N. lapillus* is presented. (a) $y = 5.54x/(1.12 + x)$; $n = 151$ population samples from 81 stations; $r = 0.688$; $p < 0.0005$; (b) $y = 3.1/(1 + e^{-0.254(x-18.9)}) + 0.111$, $n = 18$ population samples from 11 stations, $r = 0.827$, $p < 0.0005$. (c) $y = 63.2/(1 + e^{-0.89(x-10.4)}) + 0.092$, $n = 103$ population samples from 12 French and 91 Irish stations.

In German coastal waters, but also in other European regions, the established TBT biomonitoring species are absent. The periwinkle *Littorina littorea* does not develop imposex but especially in direct proximity to harbours and marinas malformations of the female genital tract were found, which were termed as intersex (Bauer et al., 1995, 1997). The female specimens affected by intersex were either characterised by the development of male features on female pallial organs (inhibition of the ontogenetic closure of the pallial oviduct) or female sex organs were supplanted by the corresponding male formations. The intersex phenomenon of *L. littorea* is a gradual transformation of the female pallial tract, which can be described by an evolutive scheme with four stages (Bauer et al., 1995).

Intersex development causes restrictions of the reproductive capability of females. In stage 1, a loss of sperm during copulation is possible and consequently the reproductive success is reduced. Females in stages 2–4 are definitively sterile because the capsular material is spilled into the mantle cavity (stage 2) or the glands responsible for the formation of the egg capsule are missing (stages 3 and 4). Due to female sterility, populations of *Littorina littorea* can be in decline but are not likely to become extinct because of the planktonic veliger larvae of the species. Veligers produced by populations with lower intersex intensities can guarantee a minimum abundance of periwinkles even at sites suffering from high TBT contamination and reproductive failure.

The assessment of intersex intensities in periwinkle populations bases on the same principle as described for the VDS index. The intersex index (ISI) is the average intersex stage in a population. A value of 0.0 indicates that only normal females (stage 0) occur and no restrictions of the reproductive capability have to be expected. ISI values above 0 show that intersex affected females can be found and that reproductive success may be reduced. Intersex intensities are highly significantly correlated to ambient TBT concentrations (Fig. 9b) and can therefore be used together with or as an alternative to imposex assessments for the determination of the degree of coastal TBT pollution especially in regions with a relatively high level of contamination. In these areas periwinkles are very common and can be sampled in sufficient numbers because *L. littorea* (a) is tolerant of high TBT levels, (b) recruits from the plankton and (c) can occur in areas where dog whelks have expired.

Imposex in dog whelks and intersex in periwinkles have been used as combined biological markers for the convention-wide biological TBT effect monitoring of OSPARCOM (Oslo and Paris Commissions, 1996). For such large-scale surveys the interspecific comparison of intersex and imposex and the geographical uniformity of these two responses have to be clarified as prerequisites. Gibbs et al. (1991) demonstrated the geographical uniformity of the imposex response for *Nucella lapillus* and Oehlmann et al. (1998a) for the same species and additionally for intersex in *Littorina littorea*. The interspecific comparison of ISI values in periwinkle populations with VDS indices in sympatrically living dog whelk populations was performed by Oehlmann et al. (1998a) and is demonstrated in Figure 9c. The relationship between the ISI and VDS index showed that the use of intersex and imposex has specific advantages over different contamination ranges. Imposex in *N. lapillus* should be utilised for the assessment of lower TBT exposure levels in only slightly and moderately contaminated areas (ambient TBT concentrations <2.0 ng as Sn/l) whereas ISI values in

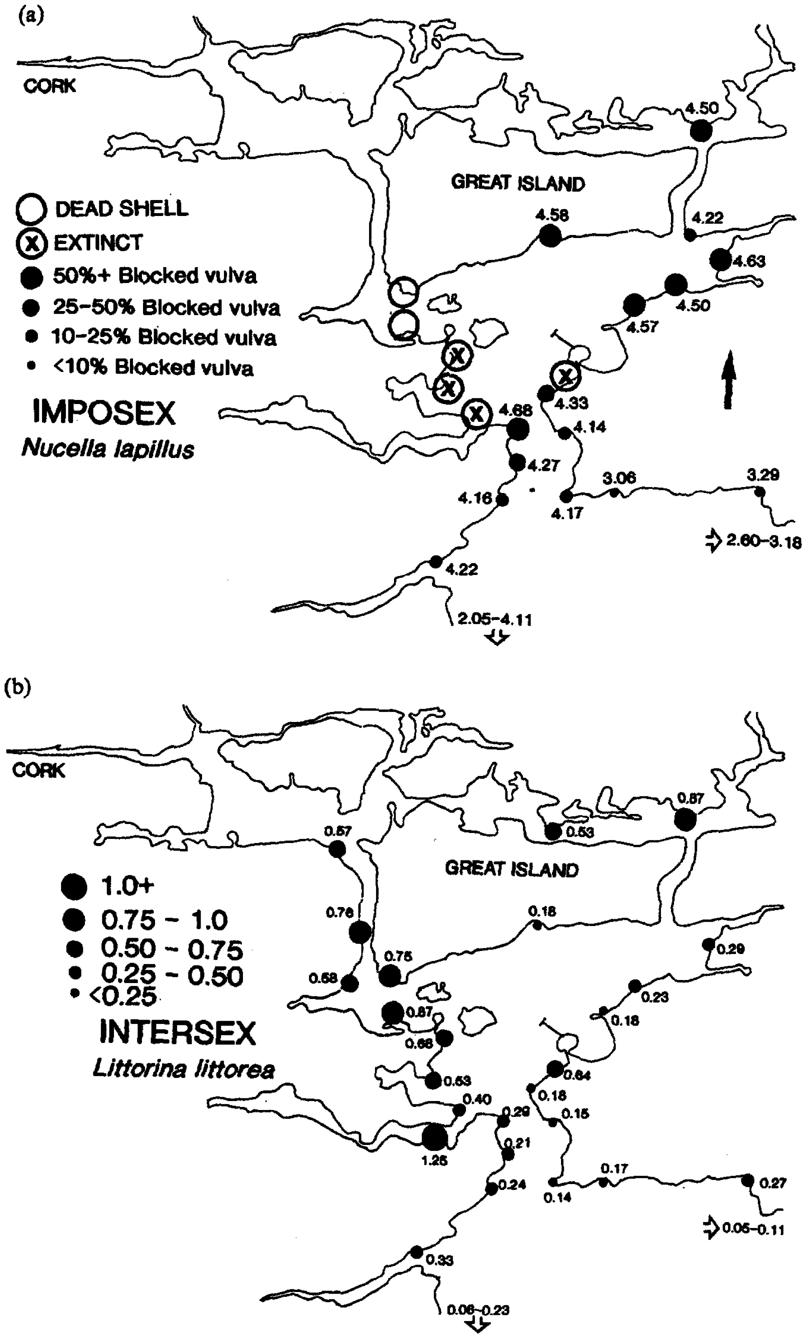

Figure 10. Biological parameters for (a) imposex intensities, measured as the VDS index and percentage of sterilised females in *Nucella lapillus* populations, and (b) intersex intensities in *Littorina littorea* populations, measured as the intersex index, in the Cork harbour area, Ireland (from Minchin et al., 1996).

L. littorea exhibit a kind of background noise with values between 0 and 0.4. At highly contaminated sites with ambient TBT concentrations above 2.0 ng TBT-Sn/l, VDS indices in dog whelks exceed 4.0 and the population becomes endangered due to progressive female sterilisation. It is in this contamination range that ISI values begin to increase. Should periwinkle populations exhibit an ISI of 0.5 or more, all females in a dog whelk population (which lives sympatrically) will have become sterile and the *Nucella* population will expire.

An example of the common use of both TBT bioindicators, *Nucella lapillus* and *Littorina littorea*, and their specific biological TBT responses is the analysis of Minchin et al. (1996) in the Cork harbour area in southern Ireland (Fig. 10). In the inner harbour, dog whelks had become extinct in the two decades before the analysis due to the existing level of contamination, but the second monitoring species, the periwinkle, was still available. Towards the open sea with decreasing TBT concentrations, where *Nucella* could survive, dog whelks were better suited as bioindicators and allowed a more appropriate differentiation of the contamination levels compared to periwinkles.

The biological TBT effect monitoring within the Joint Assessment and Monitoring Programme (JAMP) of OSPARCOM is one of the few examples of established quality assurance and quality control measures in biological environmental monitoring. The QUASIMEME (Quality Assurance Laboratory Performance Studies for Environmental Measurements in Marine Samples) office at the Marine Laboratory in Aberdeen organises regular training workshops and conducts exercises where identical subpopulations of dog whelks and periwinkles are analysed for imposex and intersex, respectively (Davies et al., 1999). Two reference laboratories, the marine laboratory in Aberdeen and our own laboratory in Frankfurt, are responsible for the training of participants and for the fixation of biological samples, which can be used as a standard reference material in all participating laboratories.

A monitoring of TBT effects on the community level and even beyond the scale of populations was performed by Rees et al. (1999) and Waldock et al. (1999). The authors conducted a number of subsequent surveys of the inter- and subtidal fauna of the River Crouch in southeast England between 1987 – the year of implementation of TBT restrictions in the UK – and 1992 and compared their results with older reports before the introduction of TBT-based antifouling paints. Overall, directional trends in community level attributed at a number of analysed stations suggested a moderate improvement in environmental conditions over the sampling period, which was coincident with a marked decline in TBT concentrations at the same stations. However, reference to historical data indicated that certain taxa that were previously frequent or common, especially the snails species *Doto coronata*, *Facelina* spec., *Archidoris pseudoargus*, *Lacuna crassior*, *Nassarius reticulatus* and *Nucella lapillus*, were only rarely recorded or still absent in the 1992 survey.

In contrast to these already institutionalised biological TBT effect monitoring programmes in coastal waters, by far less attention has been paid to comparable attempts in freshwater ecosystems, although even here an increasing pollution can be assessed due to the wide application of organotin compounds. Especially high TBT concentration in raw sewage, sewage sludge and even in effluents from sewage treatment plants are a cause for concern (e.g. Fent et al., 1991; Donard et al., 1993). The

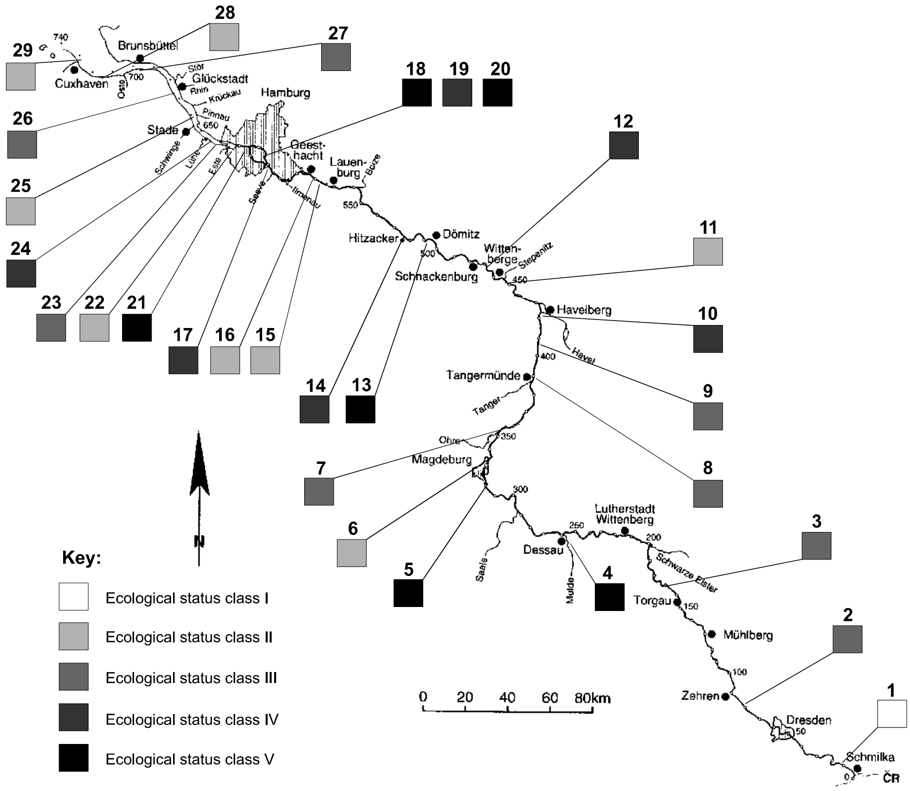

Figure 11. Graphical representation of androgenic activities in sediments of the River Elbe in the year 2000, expressed as ecological status classes according to the European Water Framework Directive. The activities were assessed by using the imposex response in the netted whelk, *Nassarius reticulatus*, in the laboratory (Schulte-Oehlmann et al., 2001).

ramshorn snail *Marisa cornuarietis* was proposed by Schulte-Oehlmann et al. (1995) and the hydrobiid snail *Potamopyrgus antipodarum* by Schulte-Oehlmann (1997) as suited biological effect monitor species for freshwater environments. The latter and the netted whelk *Nassarius reticulatus* were employed by Schulte-Oehlmann et al. (2001) for a monitoring of androgenic activities in sediments of the River Elbe (Fig. 11). The majority of sediments exhibited marked androgenic activities and some of them, assigned to the ecological status classes IV and V according to the European Water Framework Directive, caused a maximum increase of imposex intensities in the netted whelk within four weeks. Although TBT was the main responsible pollutant for imposex development, an even stronger biological effect was observed as could be expected on the basis of analytically measured TBT concentrations for a number of sediments. This indicates that next to TBT further, not yet identified compounds may have contributed to the androgenic activities in these sediments.

Recently, it has been shown that marine and freshwater prosobranchs respond to an exposure of other endocrine active compounds such as estrogens, further androgens and antiandrogens in the laboratory with a characteristic set of toxicological effects (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000; Tillmann et al., 2001) so that it can be assumed that in the near future these endpoints will also be used for biological effect monitoring in the field. Estrogens caused primarily an induction of so-called "superfemales" resulting in an increased female mortality by the enhancement of spawning mass and egg production. The main effects of androgens were – as already demonstrated for tributyltin – a virilisation of females by imposex development and a marked decrease of the fecundity. Antiandrogens induced a growth reduction of the penis and of accessory male sex organs (e.g. penis sheath, prostate).

4. Conclusions

Molluscs have been successfully used as bioindicators in monitoring programmes in the past. Terrestrial ecosystems were much less considered than the aquatic environment but even for the latter it can be stated that bivalves, gastropods and especially the other molluscan classes have not yet received the attention they probably deserve, speaking in terms of their ecological importance. Major emphasis was laid in the past on the assessment of bioaccumulation in aquatic ecosystems, whereas the monitoring of biological effects in marine and freshwater environments has plaid a clearly secondary role. The example of the already institutionalised biological TBT effect monitoring programme within the JAMP of OSPARCOM with its extraordinary sensitivity for a special environmental pollutant and the relevance of effect measures for populations and higher levels of biological integration demonstrates the potential of such attempts with molluscs. It can be assumed that there is no reason to suppose that such far reaching effects of pollutants on mollusc populations are in any sense unique. The limited number of comparable successful monitoring programmes with molluscs is partially due to the fact that this special group of invertebrates was very much neglected in basic ecotoxicological research in the last decades and that therefore deleterious effects in the field, following an exposure to pollutants, may easily be missed or simply be unmeasurable at present.

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