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# 14 Soil Biology and Ecotoxicology

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## Terrestrial Gastropods and Their Impact on the Cycling of Nutrients and Trace Elements

During their evolution, gastropods repeatedly have achieved terrestriality (Barker, Chapter 1, this volume). Indeed, they are among the most successful invertebrates in terrestrial ecosystems, including the most inhospitable arctic (Kerney and Cameron, 1979), high alpine (Baur and Raboud, 1988) and arid desert (Crawford, 1981; Arad *et al.*, 1993) areas. In the central alpine region of Europe, for instance, more than 20 species of gastropods live at altitudes over 2000 m above sea level, and still four species occur at altitudes of 3000 m or above (Kerney *et al.*, 1983). This is remarkable, since terrestrial gastropods depend on sufficient amounts of water in order to maintain their activity (Stöver, 1973; Ward and Slotow, 1992). Further, they have developed efficient mechanisms of coping with freezing (Riddle, 1981; Riddle and Miller, 1988), starvation and desiccation (Machin, 1967; Schmidt-Nielsen *et al.*, 1971; Burla and Gosteli, 1993). Nevertheless, gastropods pay a high price for their occupancy of dry and cold terrestrial environments, which is reflected, for instance, in slow growth rates and high stochastic mortality in species living in bleak habitats (Baur, 1984, 1988; Lazaridou-Dimitriadou and Kattoulas, 1991).

In spite of such biological and physiological limitations, and considering the known negative relationship between individual size of a species and its population density (see, for instance, Perry and Arthur, 1991), gastropods in certain woodland and forest habitats can reach very high population densities (e.g. Mason, 1970; Reichardt *et al.*, 1985). Relatively high population densities have been recorded even for alpine (e.g. Baur, 1993) and desert-dwelling species (e.g. Hermony *et al.*, 1992) living under particularly adverse climatic conditions. From an ecophysiological

point of view, most terrestrial gastropods are detritivorous animals, feeding on decaying litter along with bacteria, fungi and algae thriving on the surface of the dead plant material. On the other hand, some species, particularly those in ruderal systems of the northern hemisphere, are regarded as primary consumers exhibiting high rates of food digestion and relatively high assimilation efficiencies (Wieser, 1978; Lazaridou-Dimitriadou and Kattoulas, 1991).

In addition to such general patterns of feeding, many species of terrestrial gastropods, particularly those of snail form, strongly depend on calcium as a major macronutrient constituent of their body. This element is the main component of virtually all gastropod shells and opercula, and in many cases also of the eggs. Calcium is also present, either as carbonate or as pyrophosphate, within storage vesicles of connective tissue cells and of basophil cells in the digestive gland of these animals (Simkiss and Mason, 1983). Most of the calcium absorbed by terrestrial gastropods may enter the animal's body via the epithelium of the intestine (Dexheimer, 1963; Beeby and Richmond, 1988), although some authors have suggested that a certain fraction of the element may also be taken up across the integument (Simkiss and Wilbur, 1977; Ireland, 1982). The calcium can be mobilized readily from the intracellular storage sites during periods of increased demand, such as reproduction (Tompa and Wilbur, 1977), hibernation (Dexheimer, 1963) and shell repair (Abolins-Krogis, 1968). Thus, these animals are able to fix calcium by a process of intracellular and extracellular biomineralization (Simkiss, 1976), hence probably contributing significantly to the retention of calcium in the upper layers of soil ecosystems.

Against this background, it has to be expected that gastropods have a strong impact on nutrient cycling by diverting fluxes and changing availabilities of macronutrients in terrestrial ecosystems. In spite of this likely importance, only a few quantitative studies are available from which we can make an assessment of how much terrestrial gastropods can contribute to nutrient cycling. Most studies relate to temperate ecosystems, and information is particularly available for arid and tropical terrestrial ecosystems. If only ingestion rates are considered, it seems that the role of gastropods as detritivores or herbivores in most indigenous temperate ecosystems is rather modest (e.g. Mason, 1970). However, the sample of studies is not large, and they do not cover adequately the range of species richness, functional diversity and population densities that are known to occur in temperate woodlands, forests and grasslands. In some instances, consumption rates in indigenous ecosystems can be considerable (Mason, 1970; Jennings and Barkham, 1979). Furthermore, the importance of terrestrial gastropods for nutrient cycling in these temperate ecosystems probably lies more in their ability to alter the resource base for microbial-driven decomposition processes. Their feeding fragments coarse pieces of plant material, providing for more rapid access by microorganisms. The deposition of mucus and faeces provides 'resource hot spots' in the litter and soil, and, starting from these, microorganisms may

spread and accelerate decomposition processes of the surrounding substrate (Herlitzius and Herlitzius, 1977). Thus high microbial biomass and activity in the mucus and faecal material contributes to high carbon and nutrient turnover. Theenhaus and Scheu (1996a), for example, demonstrated by means of experimental microcosms with *Arion ater* (Linnaeus) (Arionidae) that beech (*Fagus Linnaeus*) (Fagaceae) litter decomposition was enhanced by the amendment that resulted by deposition of mucus and faeces by the gastropods. These faecal and mucus 'hot spots' also increase the heterogeneity of the soil system, which might be of considerable importance for maintenance of a diverse soil microflora and fauna (Schaefer, 1991).

The importance of gastropods in macronutrient cycling may be expected to be higher in cold or moisture-stressed environments where the rate of nutrient turnover is lower than in more equable temperate regions. In desert ecosystems, for example, terrestrial gastropods often constitute the predominate standing biomass in the detritivore and herbivore guilds. *Euchondrus albulus* (Mousson) (Enidae) and closely related species in the Negev Highlands feed on endolithic lichens, along with some of the rocky substrate on which lichens thrive, and deposit their faeces on the soil under the rocks. In this way, these gastropods transfer considerable amounts of nitrogen from the lichens to the soil, thus facilitating establishment and production of higher plants in this arid region (Jones and Shachak, 1990, 1994).

At the same time, some species have been reported to be very selective in their preferences for habitat types (e.g. Chang and Emlen, 1993) and their choice of food (e.g. Baur *et al.*, 1994; Hanley *et al.*, 1995; Linhart and Thompson, 1995). As a consequence, herbivorous terrestrial gastropods can exert considerable selective pressure on plant communities and thus profoundly influence their species composition (e.g. Barker, 1991b; Speiser and Rowell-Rahier, 1991; Speiser, Chapter 6, this volume). Thompson *et al.* (1993) have shown that selective herbivory by gastropods led to a reduction in soil ammonia nitrogen, and to an increased availability of phosphate in the soil, thus affecting plant community composition.

Closely connected to the cycling of macronutrients is the cycling of essential and non-essential trace elements (van Hook *et al.*, 1977; Zöttl, 1985; Kratz, 1991). It has been shown repeatedly that terrestrial gastropods possess an exceptional affinity for certain trace elements, concentrating metals such as copper, zinc, cadmium and lead in their soft tissues (Coughtrey and Martin, 1976; Ireland, 1979, 1981) or shells (Beeby and Richmond, 1989; Mulvey *et al.*, 1996), often far above environmental levels (Dallinger, 1993). This is particularly evident in gastropods from metal-polluted environments (Martin and Coughtrey, 1982; Greville and Morgan, 1989a; Berger and Dallinger, 1993; Rabitsch, 1996), but can also be observed, at least with respect to certain metals, in gastropods in apparently uncontaminated habitats (Knutti *et al.*, 1988). It is also significant that the uptake and storage of certain trace elements by these animals often are connected intimately with the uptake and storage of calcium

(Beeby and Richmond, 1988; Beeby, 1991). The physiological basis for this observation lies in the fact that a series of trace elements in gastropod tissues follows the pathway of calcium along a process of biomineralization within cellular vesicles (Simkiss, 1981; Simkiss *et al.*, 1982). Certain trace elements, such as cadmium, can also be immobilized by detoxification and binding to metallothioneins (see below) (Dallinger and Berger, 1993; Dallinger, 1996).

The increasing exploitation of natural resources by human activities during the past few centuries has adversely affected the global balance of trace elements (Nriagu, 1990), causing a gradual increase of concentrations and availabilities of metals in soil ecosystems (van Hook *et al.*, 1977; Beyer *et al.*, 1985; Zöttl, 1985). Processes of acidification additionally may increase the availability of most trace elements to invertebrates in the soil (van Straalen and Bergema, 1995). The role of terrestrial gastropods in this process of continuous accumulation is not yet clear. However, given the significant contribution of these animals to nutrient cycling (see above), and considering their central position in terrestrial food chains (Laskowski and Hopkin, 1996a), it can be suggested that gastropods must also have a strong impact on the turnover of trace elements. It has to be expected, for instance, that an increasing proportion of essential and non-essential trace elements is retained in upper soil layers due to the presence of terrestrial gastropods which may act as biomineralization sinks and 'buffers' for certain metals (Beeby, 1985), hence changing their biological availability (Dallinger, 1993). At the same time, the activity of terrestrial gastropods may also be responsible for an acceleration of metal cycling in the soil ecosystem as a whole.

## Interactions with Other Soil Biota

As ecological key components of terrestrial foodwebs, gastropods may also contribute significantly to the transfer of pollutants from plants to primary consumers (Carter, 1983) and terrestrial predators (Martin and Coughtrey, 1976; Reichardt *et al.*, 1985). Unfortunately, only a few examples exist in which the role of terrestrial gastropods has truly been analysed for the transfer of pollutants in terrestrial habitats. One reason for this lack of information might be that the quantification of food chain transfer of chemicals by terrestrial gastropods is made difficult by the ability of many species to reduce their activity or to aestivate under unfavourable climatic or ecological conditions (Laskowski and Hopkin, 1996a). It has been shown clearly, however, that in transferring nutrients and pollutants through terrestrial food chains, gastropods interact with a variety of other soil organisms, thereby significantly influencing pollutant fluxes and availabilities.

Soil particles, and especially the living and decaying plant and fungal material, exhibit a high capacity for retaining certain trace elements and organic pollutants on their surface or within their organic structure

(Somers, 1978; Bohác *et al.*, 1990; Calderbank, 1994; Alberti *et al.*, 1996). Terrestrial gastropods feeding on this material will take up pollutants in relation to their biological availability in the food source, being able to accumulate metals far above the element levels encountered in their food source (Williamson, 1979; Carter, 1983; Dallinger, 1993; Gomot and Pihan, 1997). The interspecific differences in the capacity of plants to accumulate certain trace elements are well documented (Hunter *et al.*, 1987a). Similarly, dead plant matter and decaying litter, as well as microorganisms thriving on this material, may accumulate trace elements to different degrees. Some species of macrofungi are able to accumulate high concentrations of trace elements in their fruiting bodies (Stijve and Besson, 1977; Kojo and Lodenius, 1989) on which some species of gastropods seem to feed normally (Beyer *et al.*, 1985). It is speculated that under such circumstances, terrestrial gastropods influence metal fluxes through soil ecosystems simply by their preferential selection of certain types of food (see above).

The absorption of pollutants in the gastropod's gut is also influenced by interactions with microorganisms from the substrate or bacteria colonizing the animal's alimentary tract. Valuable examples of this have been provided in connection with the accumulation of certain trace elements. The uptake of zinc by *Cantareus aspersus* (Müller) (Helicidae), for instance, depends on microorganisms in the alimentary tract and the abundance of bacteria in the soil substrate (Simkiss and Watkins, 1990, 1991). Furthermore, sulphate-reducing bacteria in the gastropod oesophageal crop can facilitate the absorption of copper (Simkiss, 1985). Another kind of interaction between gastropods and bacteria is exemplified by the inoculating effect which gastropod faeces exert on the soil substrate, leading to increasing bacterial and fungal biomasses, and hence to rising decomposition rates (Theenhaus and Scheu, 1996b). As a consequence, organic matter becomes more accessible to other soil organisms, and this probably increases the availability of nutrients and pollutant metals. Their mobilization from the substrate may also be enhanced due to interactions of terrestrial gastropods with other soil invertebrates such as earthworms (Thompson *et al.*, 1993).

Terrestrial gastropods are likely to play an important role in directly transferring nutrients and certain pollutants to higher trophic levels of terrestrial food chains, with these animals often serving as prey or hosts for a variety of other animals (e.g. Reichardt *et al.*, 1985). It has been shown, for instance, that a decline in gastropod abundance due to processes of soil acidification can adversely affect populations of forest passerines which need calcium for their eggshells (Graveland and Vanderwal, 1996).

Concerning trace elements, it has been argued that the availability of certain metals for potential predators may depend on the chemical form in which the metal is immobilized in the gastropod's body (Dallinger, 1993; Laskowski and Hopkin, 1996a), even if the gastropod itself may be considered rather tolerant towards the metal (Greville and Morgan, 1991). It can be assumed, for instance, that zinc and lead, which are accumulated

in cellular granules along with calcium, may be less available to predatory animals than cadmium, which is mainly bound to chelating proteins such as metallothioneins (Dallinger and Wieser, 1984b; Dallinger *et al.*, 1993a; Berger *et al.*, 1994). In this context, the question arises of whether terrestrial gastropods can contribute to biomagnification of metals along terrestrial food chains. However, it has been stated (Laskowski, 1991) that biomagnification of trace elements normally does not occur in terrestrial ecosystems, at least for metals. On the other hand, it should be considered that even low transfer rates for trace elements between trophic levels may suffice to exert adverse effects on predators due to metal toxicity (Dallinger, 1993).

## Environmental Contamination and Sequestration

### *Trace elements*

Contamination of terrestrial environments by trace elements leads to an increasing uptake of metals by soil invertebrates (Strojan, 1978a; Beyer *et al.*, 1985; Hunter *et al.*, 1987b), and hence to adverse effects of toxic elements on the terrestrial invertebrate fauna (van Straalen and Bergema, 1995), or on the functioning of the soil ecosystem as a whole (Strojan, 1978b). The first studies on terrestrial gastropods as metal-accumulating animals in polluted terrestrial habitats were carried out more than 20 years ago (Meincke and Schaller, 1974; Coughtrey and Martin, 1976). Since that time, many studies on the role of terrestrial gastropods in metal accumulation have been performed (for an overview, see, for instance, Dallinger, 1993), and it has become evident that gastropods belong to those invertebrate species which exhibit the highest capacities for metal accumulation throughout the animal kingdom.

The process of metal accumulation by terrestrial gastropods is determined by some important prerequisites. First, the animals are confronted – as are many other organisms – with trace elements exerting essential and non-essential biological functions (see, for instance, Underwood, 1977). Secondly, the metabolism of many trace elements in terrestrial gastropods is pre-determined by the metabolic pathway of calcium, which is a major constituent of the animal's body (see above). Thirdly, metal accumulation in terrestrial gastropods is conditioned by their obligate need to avoid excessive water loss. Probably as a consequence of such constraints, terrestrial gastropods have developed strategies of metal sequestration at both the cellular and molecular levels (Dallinger, 1993).

The uptake of most trace elements by terrestrial gastropods follows the route of food uptake via the alimentary tract (Hopkin, 1989; Dallinger, 1993; Triebkorn and Köhler, 1996a); moreover, it has to be assumed that terrestrial gastropods do not possess efficient mechanisms allowing them selectively to exclude certain trace metals from being absorbed through the wall of their gut. Available concentrations of trace elements in soil

organic matter on which the animals feed are, therefore, the primary extrinsic factors which determine the rates of metal uptake (Dallinger, 1993). For some elements, an additional pathway of entry into the animal's body seems to be the skin (Ryder and Bowen, 1977; Ireland, 1982).

For most trace elements, the main site of accumulation in the gastropod's body is the digestive gland (or midgut gland) (Dallinger, 1993). This organ can be regarded as central to the animal's metabolism, serving as a site of enzyme synthesis, nutrient absorption and decomposition, as well as metal storage and detoxification (Janssen, 1985). In individuals of *C. aspersus* from different contaminated field sites, for example, significant concentrations of cadmium, zinc and lead were detected in the digestive gland (Coughtrey and Martin, 1976; Cooke *et al.*, 1979), whereas only minor amounts of these metals were found in other tissues. Similar results were reported for *Helix pomatia* Linnaeus (Helicidae), *Arianta arbustorum* (Linnaeus) (Helicidae) and *A. ater* after exposure to metals in the laboratory (Schötti and Seiler, 1970; Ireland, 1981; Dallinger and Wieser, 1984a; Marigómez *et al.*, 1986a; Berger and Dallinger, 1989a). A few studies involving the tropical species *Achatina fulica* (Bowdich) (Achatinidae) have confirmed the importance of the digestive gland as a major site of metal accumulation (Ireland, 1988; Ireland and Marigómez, 1992). Other important tissues for accumulation of certain trace elements include, to various degrees, the foot sole, the mantle and the intestine (Coughtrey and Martin, 1976; Dallinger and Wieser, 1984a; Berger and Dallinger, 1989a).

The metabolic pathway of a trace element within the animal's tissues depends mainly on the physicochemical properties of the metal involved. Following a proposal by Nieboer and Richardson (1980), metals can be classified according to their tendency to form ionic or covalent compounds, and hence their preferences in ligand binding. According to this scheme, metals of class A include the alkali and earth alkali elements together with aluminium. In forming chemical compounds, these metals exhibit a clear preference for oxygen-carrying ligands. Metals of class B comprise, for the most part, non-essential heavy metals such as silver, gold, lead (IV) and bismuth. They prefer sulphur-bearing ligands in their chemical compounds. The third category (class C) contains the so-called borderline metals, most of them being essential trace elements such as iron, manganese, zinc and copper(II), or non-essential metals such as cadmium and lead(II). These elements can behave, depending on their chemical environment, like metals either of class A or class B (for more detailed information, see Nieboer and Richardson, 1980).

Class A also includes calcium, potassium and magnesium, which can be regarded as essential macronutrients. The important role of calcium in nutrient metabolism of terrestrial gastropods has already been stressed (Ireland, 1991). As already mentioned above, many trace elements in gastropod tissues follow the pathway of calcium.

Once assimilated, calcium serves a variety of tasks, being an essential constituent of many of the animal's structures and molecules. Sufficient

amounts of calcium should, therefore, always be available to meet the gastropod's varying requirements. On the other hand, the intracellular activity of free calcium ions must be kept as low as possible, since this element exerts toxic effects towards cellular structures at elevated concentrations (Taylor *et al.*, 1988). Probably as a consequence of this metabolic dilemma, calcium in gastropod tissues (but not only there; for a general view of this topic, see Brown, 1982) becomes precipitated by a process of biomineralization within intracellular, membrane-surrounded granules, thus forming an energetically convenient and inactivated kind of storage and/or detoxification product (Beeby, 1991). There exist several cell types in gastropod tissues which contain calcium granules. Simkiss and Mason (1983) have distinguished between calcium-containing storage cells and detoxification cells. According to this classification, the most important storage cells are the connective tissue calcium cells which are found in connective tissues of the mantle, foot, visceral complex, and other organs. They contain granules of amorphous calcium carbonate which seems to be readily available upon metabolic demand (Simkiss and Mason, 1983).

More interesting with regard to detoxification processes are the basophilic calcium cells, as well as the digestive and the excretory cells within the gastropod's digestive gland. The basophilic calcium cells contain vesicles with granular, concentrically structured deposits of calcium salts (Abolins-Krogis, 1970; Simkiss, 1976). As shown in *C. aspersus*, the calcium in these granules is present in an amorphous form of pyrophosphate (Simkiss and Mason, 1983). Detailed analyses of such granules have revealed that they contain about 18% water, 5% organic matter and 76% inorganic material, with predominant amounts of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{P}_2\text{O}_7^{4-}$ , and minor proportions of the trace elements zinc and manganese (Howard *et al.*, 1981). Through a series of studies, it became clear that these calcium granules can also be regarded as intracellular detoxification sites for trace elements (Simkiss, 1981; Mason and Simkiss, 1982; Simkiss *et al.*, 1982; Almedros and Porcel, 1992a). Cytological studies have shown that the calcium granules of the basophilic cells are formed in close connection to the Golgi system (Simkiss and Mason, 1983), showing elevated activities of acidic phosphatase (Almedros and Porcel, 1992b). It seems that at least in some gastropod species, these granules can be released into the tubular lumen of the digestive gland (Mason and Simkiss, 1982), hence providing a route of trace element liberation.

Trace elements following the calcium pathway into detoxifying granules of basophilic cells can belong to all of the three classes (A, B and borderline) defined by Nieboer and Richardson (1980), although metals of class A and borderline metals are, due to their binding preferences, expected to show a more pronounced affinity for such granules than elements of class B. Traces of aluminium, a metal of class A, were observed in calcium granules of *C. aspersus* (Almedros and Porcel, 1992a) but, in contrast to these findings, Brooks and White (1995) have shown that most of the aluminium assimilated by this species can be detected in excretory

granules of the excretory cells (see below). Other metals of class A which become incorporated into calcium granules of the basophilic cells are barium, strontium and beryllium (Simkiss, 1981). Moreover, a variety of borderline metals are also precipitated in this manner, and include manganese (Howard *et al.*, 1981; Mason and Simkiss, 1982; Greaves *et al.*, 1984; Taylor *et al.*, 1988), zinc (Howard *et al.*, 1981; Dallinger, 1993; Triebkorn and Köhler, 1996a), copper (Ireland, 1979; Marigómez *et al.*, 1986a) and possibly lead (Triebkorn and Köhler, 1996a). In contrast to this, few metals of class B have been reported to occur in granules of the basophilic calcium cells (e.g. silver) (Simkiss, 1981; Almedros and Porcel, 1992a).

The digestive gland of terrestrial gastropods contains, besides the basophilic cells, additional cell types involved in metal detoxification: the digestive cells and the excretory cells. The excretory cells of *C. aspersus*, for instance, characteristically contain large, yellowish excretory granules. The predominant elemental constituents of these granules are sulphur, phosphorus and calcium. Only recently has the important role of these granules in the detoxification of aluminium been discovered (Brooks and White, 1995). The digestive cells have a function in the sequestration of lead (Dallinger, 1993).

Apart from cellular compartmentalization, certain trace elements in gastropod tissues can be sequestered by complexation to metallothioneins (Dallinger, 1995, 1996). These are low-molecular weight, metal-binding proteins with a high affinity for certain borderline metals and some trace elements of class B such as cadmium, zinc and copper (I) (Kägi and Schäffer, 1988). Other outstanding features of these proteins are their high cysteine content and their lack of aromatic amino acids. The cysteine residues of the polypeptide chain are highly conserved in their sequential position, being arranged in several Cys-X-Cys motifs, with 'X' designating any other amino acid except cysteine. The metals are bound to the protein backbone by means of several bridging sulphur atoms of the cysteine residues, forming tetrahedrally coordinated metal-thiolate clusters located in two separate protein domains (Kägi and Schäffer, 1988). So far, metallothioneins have been discovered in species from nearly all phyla of the animal kingdom (Kägi, 1993). The metallothioneins from molluscs, and especially from terrestrial gastropods, differ in some of their structural and biochemical features from those of most species in other phyla (Dallinger *et al.*, 1993b; Berger *et al.*, 1995a; Dallinger, 1996). In spite of these differences, gastropod metallothioneins probably perform functions similar to those of most other metallothioneins, being involved in both metal detoxification and trace element regulation (Dallinger, 1996).

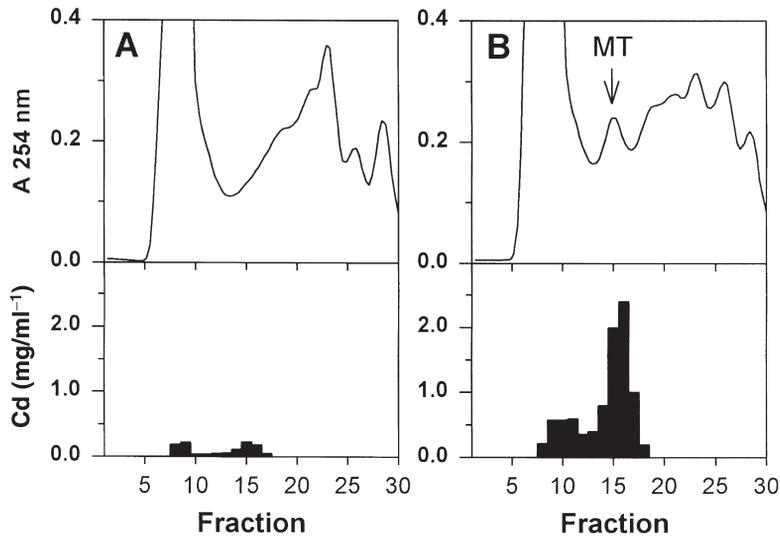
The first indication of the presence of a water-soluble, cadmium-binding protein in gastropods was provided by Cooke and colleagues for *C. aspersus* (Cooke *et al.*, 1979); due to its high molecular weight and because of a lack of information about its amino acid composition, it is not clear whether this protein can be regarded as a metallothionein. True

metallothioneins have been fractionated by gel permeation chromatography (Dallinger and Wieser, 1984b) and characterized further by amino acid composition and sequencing from *H. pomatia* (Dallinger *et al.*, 1989a, 1993b), *A. arbustorum* (Dallinger *et al.*, 1989a; Berger *et al.*, 1995a), *A. ater* (Ireland, 1981) and *Arion lusitanicus* Mabilille (Dallinger *et al.*, 1989b; Janssen and Dallinger, 1991). As shown by amino acid sequencing, terrestrial gastropod metallothioneins consist of 63–66 amino acids, 18 of which are cysteine residues (Dallinger, 1996; Berger *et al.*, 1997). The molecular weight of the proteins is about 6.4–6.6 kDa.

A fascinating aspect of the biological activity of metallothioneins in gastropod tissues is their involvement in metal detoxification on the one hand, and trace element regulation on the other. It was argued that the function of metallothionein in terrestrial gastropods may have shifted from trace element regulation towards a more efficient detoxification of cadmium (Dallinger and Berger, 1993). This applies mainly to the animals's digestive gland. This organ is by far the most efficient of all gastropod tissues in accumulating exceptionally high amounts of cadmium (Dallinger and Wieser, 1984b). As shown by quantitative chromatographic fractionation of the digestive gland in *H. pomatia*, 85–95% of the cadmium is tightly bound to an inducible, cadmium-specific metallothionein isoform (Dallinger *et al.*, 1993b; Dallinger, 1996). The inducibility of metallothionein synthesis by cadmium exposure (Janssen and Dallinger, 1991; Berger *et al.*, 1995a), along with the subsequent sequestration of this metal by the induced isoform (Dallinger, 1996) and the long-term persistence of cadmium in the gastropod's digestive gland (Dallinger and Wieser, 1984a), can therefore be regarded as a true detoxification process. The exceptional efficiency of this kind of detoxification also lies in the fact that the trace elements zinc and copper are additionally present in the animal's digestive gland, but are bound by different molecules that do not seem to interfere with the cadmium-specific metallothionein isoform (Berger *et al.*, 1993, 1994; Dallinger *et al.*, 1993a). Typical gel chromatographic elution patterns showing metallothionein induction and cadmium sequestration in the digestive gland of uncontaminated and cadmium-exposed individuals of *H. pomatia* are exemplified in Fig. 14.1.

Cadmium sequestration by metallothionein binding in the digestive gland may confer to the animals a high degree of tolerance against this toxic metal, at least in terms of individual survival, as demonstrated for *C. aspersus* (Russell *et al.*, 1981) and *H. pomatia* (Berger *et al.*, 1993). However, exposed animals exhibit reduced growth rates at even moderate concentrations of cadmium (Russell *et al.*, 1981).

Recently, a copper-specific metallothionein isoform was isolated and characterized from the mantle tissue of *H. pomatia* (Berger *et al.*, 1997). In contrast to the cadmium-specific isoform of the digestive gland, the copper-binding protein in the mantle cannot be induced at all by cadmium. Moreover, this isoform does not bind the metal, neither after cadmium feeding nor by direct administration of cadmium by injection



**Fig. 14.1.** Typical elution patterns after gel permeation chromatography (Sephacryl S-200, Pharmacia Biochemical) of pooled ( $n = 3-4$ ) digestive gland supernatants from *Helix pomatia* (Linnaeus) (Helicidae) for uncontaminated individuals (A) and individuals exposed for 40 days to a dietary cadmium concentration of  $133.5 \pm 41.4 \mu\text{g g}^{-1}$  dry weight (B). Note the very low concentrations of cadmium in animals on an uncontaminated diet, and the high cadmium concentrations and the presence of induced metallothionein (MT) in animals exposed to contaminated food.

into the mantle tissue. The function of this strictly copper-specific metallothionein isoform must, therefore, be related to the metabolism of the essential trace element copper, probably in connection with the synthesis of haemocyanin (Dallinger *et al.*, 1997).

It is concluded that the role of metallothioneins in tissues of terrestrial gastropods certainly is not restricted to metal detoxification. However, at least some species synthesize organ- and metal-specific metallothionein isoforms by virtue of which they become able to detoxify the non-essential cadmium efficiently, and at the same time maintain the homeostatic regulation of the essential trace element copper, possibly for the benefit of haemocyanin synthesis. So far, this clear sharing of tasks among metallothionein isoforms in handling of essential and non-essential trace elements is unique to gastropods among the animal kingdom (Dallinger *et al.*, 1998). This evident diversification of metallothionein isoforms is probably also one of the main keys to a better understanding of the exceptional tolerance of terrestrial gastropods towards exposure to cadmium and copper in the environment.

### **Organic pollutants**

In contrast to the bulk of detailed research on metal relationships, relatively few studies have been published dealing with the uptake

and metabolism of organic pollutants in terrestrial gastropods. The only exception in this respect are studies focusing on molluscicides (see, for example, Triebkorn and Künast, 1990; Triebkorn and Schweizer, 1990; Triebkorn, 1991).

Some studies dealing with the accidental uptake and accumulation of DDT and related compounds by terrestrial gastropods were published 20–30 years ago. As an example of this kind of research, a thorough examination on the uptake of DDT by *Cepaea hortensis* (Müller) (Helicidae) has demonstrated that after a single meal, DDT residues were accumulated and retained in the digestive gland (Dindal and Wurzinger, 1971). Considerable concentrations of DDT were also detected in the ovotestis of this species, while most of the other organs accumulated the pesticide to only moderate degrees. Moreover, it was reported that concentration factors (referred to the soil substrate on a dry weight basis) for DDT residues are generally lower in terrestrial snails (with values varying from 0.1 to 1.0) than in slugs (with values in the range 2.2–17.9). It was suggested that terrestrial gastropods, as non-target organisms for DDT, may serve as a source for the transfer of this pesticide to vertebrate predators (Dindal and Wurzinger, 1971), thus contributing to the biomagnification of DDT in terrestrial food chains.

Many other studies on the uptake of organic chemicals by terrestrial gastropods were interested primarily in the efficiency of dietary absorption of chemicals and their efficacy as molluscicides, with pestiferous species being target organisms (see, for instance, Briggs and Henderson, 1987). However, a chapter dealing with the accumulation and action of molluscicides is included in a related volume (see Henderson and Triebkorn, 2001), and is, therefore, not a subject of this article.

## Terrestrial Molluscs as Biological Indicators of Environmental Pollution

According to a very general definition, biological indicators can be regarded as species which indicate the condition or state of the environment in which they live (Spellerberg, 1991). This broad definition includes the so-called 'bioindicators of accumulation'. These are animal or plant species which indicate, by virtue of their capacity to accumulate certain environmental pollutants, the presence of these toxicants at concentrations possibly hazardous to the biota of the respective ecosystem. The use of terrestrial gastropods as biological indicators so far has been restricted mainly to metal pollution, by employing these animals as accumulation indicators of contaminant trace elements in terrestrial ecosystems (see, for example, Martin and Coughtrey, 1982).

As discussed above, many species of terrestrial gastropods have been shown to concentrate trace elements in their tissues owing to the efficient mechanisms of sequestration which they possess. Accordingly, the final concentration of a metal reached in the gastropod's body is the

result of a process of accumulation involving metal uptake, storage and elimination (Dallinger, 1993). The idea behind the concept of biological indication in this respect is based on an expected quantitative or semi-quantitative relationship between the metal concentration in the animal's tissues and the metal levels detected in the environment (Williamson, 1979). This relationship ideally can be expressed by a simple equation (Dallinger, 1994):

$$y = a.x + b$$

where  $y$  is the metal concentration in the gastropod's body or tissue,  $x$  is the metal concentration in the environment (or substrate),  $a$  is the biological concentration factor and  $b$  is the background burden of the metal in the animal's body or tissue.

The biological concentration factor ( $a$ ) in this linear equation predicts by how much a terrestrial gastropod will concentrate a metal in relation to environmental levels. Considering the animal as a whole, a terrestrial gastropod may therefore be classified according to its biological concentration factor with respect to a certain trace element. Species which accumulate a metal far above environmental concentrations (with  $a > 2$ ) have been defined as 'macroconcentrators', whereas species accumulating a trace metal in equal proportion to environmental levels ( $a = 1-2$ ) or below ( $a < 1$ ) have been classified as 'microconcentrators' and 'deconcentrators', respectively (Bohác and Pospíšil, 1989; Dallinger, 1993). It appears, for instance, that some helioid species such as *H. pomatia*, *C. aspersus*, *Cepaea nemoralis* (Linnaeus), *A. arbustorum* and arionid species can be regarded as 'macroconcentrators' for cadmium and copper, while they mostly behave as 'microconcentrators' with respect to zinc and lead (Dallinger, 1993; Laskowski and Hopkin, 1996a).

Whereas the concept of quantitative bioindication has been used quite successfully for soil invertebrates such as isopods (Wieser *et al.*, 1976; Hopkin *et al.*, 1986; Dallinger *et al.*, 1992), the linear relationship explained above might be applied to terrestrial gastropods only with some reservations. The reason for this lies in the fact that in contrast to terrestrial isopods which normally feed on a rather homogeneous substrate, the foods utilized by terrestrial gastropods are remarkably varied (Martin and Coughrey, 1976; Reichardt *et al.*, 1985; Hopkin, 1989; Speiser and Rowell-Rahier, 1991). Moreover, metal concentrations in the litter substrate on which terrestrial gastropods feed can display a high degree of variability (Carter, 1983), probably due to different states of decomposition. Additional factors which adversely interfere with the concept of quantitative bioindication in terrestrial gastropods are variabilities of metal concentrations in the animal tissues depending on their body size (Coughtrey and Martin, 1977; Williamson, 1980; Greville and Morgan, 1990; Berger and Dallinger, 1989b; Dallinger and Berger, 1992), species-specific peculiarities in metal accumulation (Greville and Morgan, 1990; Gomot and Pihan, 1997), seasonal factors (Williamson, 1979; Ireland, 1981, 1984; Berger and Dallinger, 1989b, 1993; Greville and

Morgan, 1989b) and prevailing temperature (Meincke and Schaller, 1974). Some studies also suggest that the bioindication approach probably does not work at all for certain metals such as copper (Berger and Dallinger, 1993).

In spite of such adversities, terrestrial gastropods have been employed as biological indicators of environmental metal contamination with a fair degree of success (Martin and Coughtrey, 1982; Berger and Dallinger, 1993). In this respect, it appears that snails are probably more appropriate for the purpose of biomonitoring than slugs. This is explained by the fact that even closely related species of slugs can display large intra- and interspecific variabilities in their metal body burdens (Greville and Morgan, 1989a; Berger and Dallinger, 1993), thus rendering a correct interpretation of the data more difficult. In spite of these circumstances, however, some slugs such as *A. ater* have been shown to be suitable animals for biomonitoring in metal-contaminated soil ecosystems (see, for instance, Popham and D'Auria, 1980).

One major difficulty often encountered in using terrestrial gastropods as biological indicators of environmental pollution is the apparent lack of a significant correlation between metal concentrations in the gastropod tissues and concentrations of the respective metals in plants or leaf litter on which the animals probably feed. This absence of correlation may be explained by the technical problem of identifying the food item the animals had consumed before they were collected, and the selectivity in choice of food displayed by many species of terrestrial gastropods. Accurate identification of the food source is important considering the variability of metal concentrations in different plant species or in litter substrate of mixed composition (Martin and Coughtrey, 1982; Hunter *et al.*, 1987a). Jones and Hopkin (1991) have shown that there may exist better correlations in cadmium concentrations between the gastropods *C. aspersus*, *C. hortensis* and *Monacha cantiana* (Montagu) (Hygromiidae) and the isopods *Porcellio scaber* (Latreille) and *Oniscus asellus* (Linnaeus) (Oniscidae) from the same sites in the field, than between snails and their nettle (*Urtica dioica* Linnaeus) (Urticaceae) diet. Likewise, body tissue burdens of cadmium and lead have been demonstrated to be remarkably similar between the gastropod species *A. arbustorum* and the isopod *P. scaber* collected at the same sampling points in the urban area of Innsbruck, Austria (Dallinger *et al.*, 1989c; Berger, 1990; Dallinger and Berger, 1992).

A semi-quantitative approach to the use of terrestrial gastropods as bioindicators of metal contamination has been presented by Berger and Dallinger (1993). Concentrations of cadmium, lead, copper and zinc were first measured in individuals of *A. arbustorum* collected at different urban and rural sites of known metal contamination levels near Innsbruck (Austria). The results from this reference data set were then compared with all available data on metal concentrations in terrestrial gastropod species from many European sites. In this way, three distinct levels (classes) of environmental contamination could be established for

cadmium, zinc and lead, corresponding to unpolluted reference sites (class 1), areas moderately contaminated by traffic exhausts or urban emissions (class 2) and heavily polluted sites due to mining activity or metal-working industry (class 3), respectively (Berger and Dallinger, 1993) (Fig. 14.2). This system has been applied successfully to moderately contaminated areas in urban environments (Dallinger and Berger, 1992). The sensitivity of the biomonitoring approach with terrestrial gastropods might be improved further by using the digestive gland tissues only, instead of whole animal preparations, for the metal analyses, as recently proposed by Rabitsch (1996).

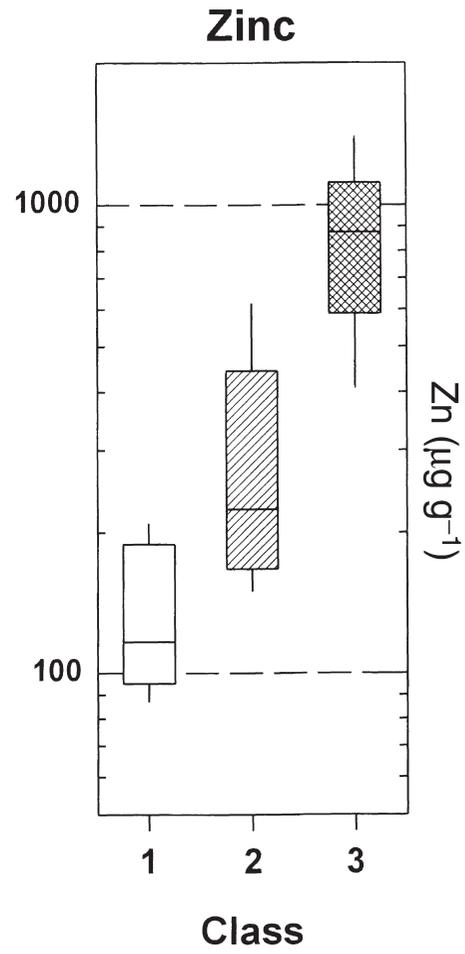
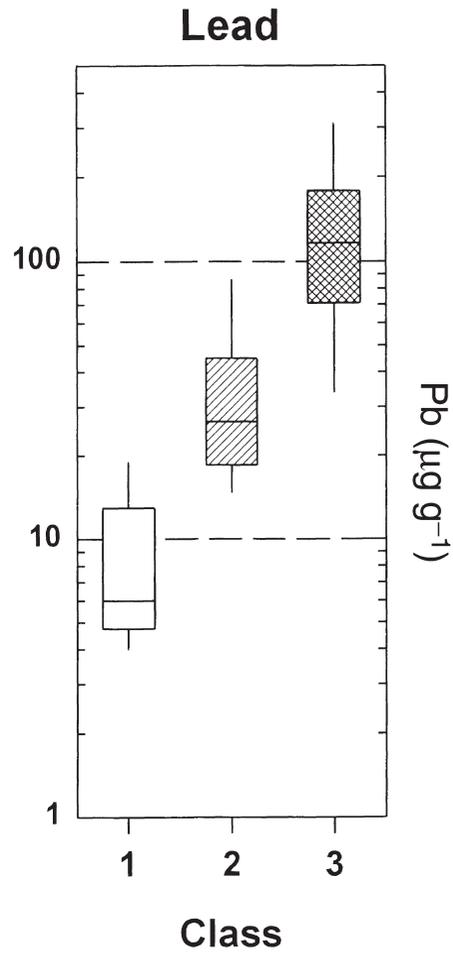
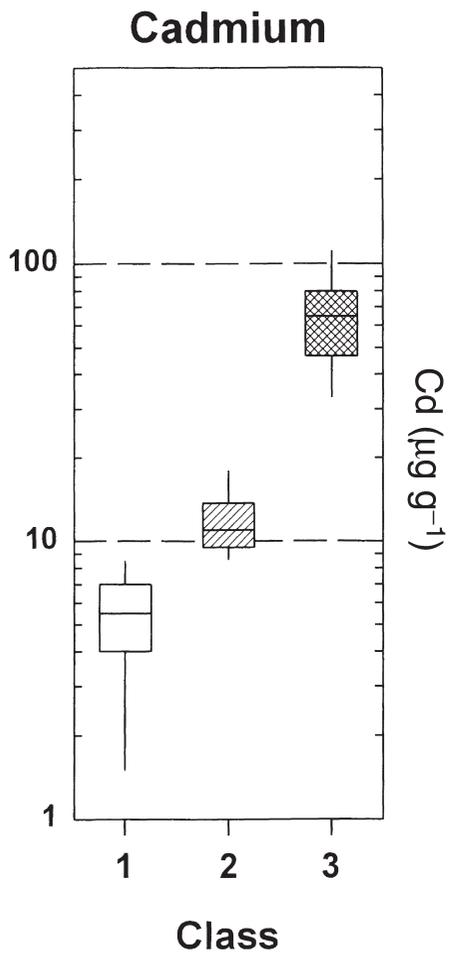
## Using Biomarkers in Terrestrial Gastropods

'Biomarker' is a relatively recent concept and currently under much discussion (see, for instance, van Gestel and van Brummelen, 1996). Sanders (1990) suggested a biomarker concept based on the measurement of molecular, biochemical, cellular and physiological parameters in animals at the subindividual or individual levels in response to a pollutant chemical as screening tools in environmental toxicology. Biomarkers are – in contrast to bioindicators – not species, but subindividual parameters. Their potential use in terrestrial environmental toxicology presently is being researched by many investigators (see, for instance, Kammenga *et al.*, 1996). A commonly used biomarker approach, for example, is that based on the cytochrome P450 monooxygenase system. Surprisingly, this concept so far has not been applied successfully to terrestrial gastropods. Instead, three other biomarker approaches have been followed in these animals: cellular biomarkers, and biochemical biomarkers with metallo-thioneins on the one hand, and stress proteins on the other.

### **Cellular biomarkers in terrestrial gastropods**

As summarized by Braunbeck and Storch (1989), histological and ultra-structural alterations in certain organs of animals can be used as biomarkers to detect individual responses to toxicant exposure, with the ultimate purpose of evaluating the toxic potential of a given chemical in the environment (see also Storch, 1988; Triebkorn *et al.*, 1991). In a study with mercury-exposed individuals of *A. ater*, for example, Marigómez and colleagues proposed the use of this species as a sentinel organism assessing cellular biomarkers of exposure to metallic pollutants (Marigómez *et al.*, 1996).

As in the field of human medicine, light and electron microscopy are used to detect cellular and subcellular symptoms of injury resulting from poisoning by organic xenobiotics or toxic elements (Cajaraville *et al.*, 1989; Marigómez *et al.*, 1990; Triebkorn *et al.*, 1991; Triebkorn, 1995). Both techniques can be applied to determine symptoms preceding cell



death (Sparks, 1972; Bowen, 1981), or to disclose sublethal reaction (or compensation) in animals tolerating elevated concentrations of toxicants induced through processes of biotransformation or detoxification (Köhler and Triebkorn, 1996; Triebkorn and Köhler, 1996b; Triebkorn *et al.*, 1996).

It has to be noted, however, that interpretation of cytotoxicity as a tool for environmental diagnosis is influenced by a variety of endogenous and exogenous factors, such as the nutritional and developmental state and gender of the animal, and prevailing environmental temperature and humidity, as shown for many species of terrestrial invertebrates (Carstens and Storch, 1980; Alberti and Storch, 1983; Storch, 1984; Neumann, 1985; Hryniewiecka-Szyfter and Storch, 1986; Triebkorn and Schweizer, 1990; Fischer and Molnár, 1992). Therefore, cytotoxicity refers to a cellular status quo that results from several factors which include the possible toxic impact of a xenobiotic substance. Moreover, cellular reactions in animals exposed to pollutants often differ not qualitatively, but quantitatively from those occurring in the 'normal' metabolic state. These subcellular effects observed after toxic impacts often reflect modifications of established metabolic pathways intensified by pollutants (Moore, 1985; Köhler and Triebkorn, 1998), rather than being the development of completely new metabolic pathways and related structures. A further consideration in interpreting cellular diagnosis is the fact that cells in a particular organ often do not react simultaneously, with the consequence that 'hot spots' of cellular injury are distributed unevenly throughout the organ.

In order to overcome such disadvantages in histological and ultrastructural diagnosis, the 'control status', that is the diversity and plasticity of cellular reactions and structures occurring in an organ under normal conditions, has to be well known before evaluating the cytotoxicological state of an organ after chemical exposure. This already indicates the comparative character of biomarkers. Furthermore, a series of tissue samples from each of several individuals per treatment is needed, in order to cope with individual and organ-specific variations in controls and contaminated animals. Additionally, a combined approach involving both light and electron microscopy is indispensable. Light microscopic histological studies permit a gross examination of the target organ and allow the researcher to select areas suspected to have developed ultrastructural damage. These tissue areas can, in turn, be analysed in more detail by electron microscopy.

**Fig. 14.2.** (Opposite) Diagrams (modified after Berger and Dallinger, 1993) showing three classes (x-axes) of environmental contamination with cadmium (left), lead (middle) and zinc (right) as reflected by metal concentrations ( $\mu\text{g g}^{-1}$  dry weight) in whole animals of several species of terrestrial gastropods sampled at a range of sites, expressed on a logarithmic scale (y-axes). Boxes represent ranges (25 and 75% percentiles) of metal concentrations, with medians (horizontal lines within the boxes) and 10 and 90% percentiles (vertical lines) for each class, corresponding to uncontaminated environments (class 1), moderately contaminated environments (class 2), and heavily polluted environments (class 3), respectively.

In terrestrial gastropods, the digestive gland has been found to be the major site of metal accumulation (see above), and is the organ in which cellular alterations are most evident after exposure to organic and inorganic pollutants (Marigómez *et al.*, 1986b; Récio *et al.*, 1988; Triebkorn, 1989; Triebkorn and Künast, 1990; Felder, 1992; Bradley and Runham, 1996; Marigómez and Dussart, 1996; Triebkorn *et al.*, 1996). Consequently, ultrastructural changes in basophilic and digestive cells, the two major cell types in the digestive gland of terrestrial gastropods, can be used successfully as biomarkers in toxicant-exposed animals (Triebkorn and Köhler, 1996a) (Table 14.1).

In the basophilic cells, alterations of the granular endoplasmic reticulum are the most prominent reactions to toxicant exposure. Symptoms such as dilatation, vesiculation and degranulation of the endoplasmic reticulum, as well as formation of circular arrays can be related to induced processes of biotransformation or to an activation of the animal's metabolism (Triebkorn and Köhler, 1992) (Table 14.1). Furthermore, an increase in the number of electron-dense calcium granules (spherites) can be related directly to metal pollution (Hopkin, 1989; Triebkorn and Köhler, 1996a). Bradley and Runham (1996) have described alterations in calcium granules of basophilic cells in the digestive gland of *C. aspersus* after exposure to manganese, and a necrosis of basophilic cells after exposure to zinc. Marigómez and Dussart (1996) have suggested a shift in 'responsibility' for metal detoxification from digestive to basophilic cells in the digestive gland of arionid species exposed to copper, zinc, mercury and lead.

In the digestive cells, the 'status of reaction' to exposure to metals or pesticides is reflected mainly by an increase in cellular vacuolization and lysosomal instability (Triebkorn, 1989; Triebkorn and Künast, 1990) (Table 14.1). Moreover, mitochondria are swollen and stocks of storage products become reduced. Above a certain threshold of pollution (representing the 'status of destruction'), cell membranes are ruptured, the lysosomal system breaks down and cell death occurs, each readily apparent in electron microscopy. Such a type of necrosis has been described, for instance, in the digestive gland of *C. aspersus* (Bradley and Runham, 1996) and *Deroceras reticulatum* (Müller) (Agriolimacidae) (Triebkorn and Köhler, 1996a) after exposure to zinc.

Apart from the digestive gland, mucocytes of the gastropod's skin and intestine have also been found to exhibit prominent alterations after exposure to environmental pollutants (Triebkorn and Ebert, 1989; Triebkorn and Schweizer, 1990). Characteristic ultrastructural symptoms reflecting toxicant-induced reactions of these cells (which are related to an increased mucus production and extrusion) include a dilatation of the endoplasmic reticulum and an increase in the number and dilatation of Golgi cisternae and vesicles. Again, the membrane system of the cells breaks down above a certain threshold of pollutant exposure, and the whole cells are extruded from the epithelium. Such symptoms obviously represent the 'status of destruction' (see above). Bradley and Runham

**Table 14.1.** Ultrastructural changes in the basophilic and digestive cells of the digestive gland of *Deroceras reticulatum* (Müller) (Agriolimacidae) following exposure to cadmium over a period of 21 days in a contaminated lettuce (*Lactuca sativa* Linnaeus: Asteraceae) leaf diet.

		Cadmium concentration in the food (dry weight)			
		Control 0.7 µg g <sup>-1</sup>	59 µg g <sup>-1</sup>	358 µg g <sup>-1</sup>	751 µg g <sup>-1</sup>
<b>Basophilic cells:</b>					
Cytoplasm	Homogeneous	100% of cytoplasm inhomogeneous, partly highly aggregated, partly very electron-lucent	90% of cytoplasm inhomogeneous, 10% of cytoplasm reduced in extent	50% of cytoplasm electron-lucent, 50% reduced	
Nuclei	OK	80% of nuclei very electron-lucent, 5% karyolysis, in 5% of nuclei increased mitosis	80% of nuclei electron-lucent, in 10% of nuclei karyolysis	90% of nuclei electron-lucent, in 10% of nuclei karyolysis	
Mitochondria	OK	OK	60% of mitochondria dilated, with reduced cristae, 20% of mitochondria destroyed	60% of mitochondria dilated, with reduced cristae, 20% of mitochondria destroyed	
Rough endoplasmic reticulum	OK	80% of RER degranulated, vesiculated or dilated	80% of RER with short cisternae, vesiculated or dilated, 5% of RER with myelin bodies	80% of membranes destroyed, forming myelin bodies	
Golgi apparatus	OK	Activated secretion	Dilated or compressed cisternae, increased in number	50% of the cisternae destroyed	
Calcium granules	Concentrically structured	Strongly mineralized, with dark appearance	Large vacuoles with light contents	Concentrically structured, vacuoles with dark contents	
Lipid content	High	Absent	Absent	Absent, lipofuscin appearing	
Glycogen content	Medium	Medium	Medium	High	
<b>Digestive cells:</b>					
Cell apices	Mostly OK	Elongated pinocytotic channels, 5% of cell apices destroyed	50% of cell apices with elongated pinocytotic channels, 50% destroyed	95% of cell apices destroyed	
Microvilli	Few damaged	80–90% of microvilli reduced in number, 10% shortened	80–90% of microvilli reduced in number, 10% shortened	100% of microvilli reduced in number	
Cytoplasm	Homogeneous	100% of cytoplasm inhomogeneous, electron-lucent	90% of cytoplasm inhomogeneous, electron-lucent, 10% of cytoplasm reduced	50% of cytoplasm electron-lucent, 50% reduced	
Nuclei	OK	OK	10% of nuclei dark	10% of nuclei dark, with structural changes in the nucleolus	
Mitochondria	OK	OK	50% of mitochondria dilated	80–90% of mitochondria dilated, with membrane whorls	
Vacuoles	OK	Increased fusion and very light contents	Increased fusion, enlarged in appearance, with partly destroyed membranes	Increased fusion, enlarged in appearance, with partly destroyed membranes	
Lipid content	High	Medium	Very low	Absent	
Glycogen content	Medium	Absent	High	Absent	

For details on tissue preparation and transmission electron microscopy, see Triebkorn and Köhler (1996a).

(1996) have described, moreover, a kind of general necrosis in the foot sole of *C. aspersus* after exposure to copper.

Finally, the epithelial cells of the oesophageal crop have also been shown to represent sensible tools for monitoring environmental contamination. Several authors have demonstrated ultrastructural alterations to occur in these cells after pesticide application (Bourne *et al.*, 1991; Triebkorn, 1989; Triebkorn and Künast, 1990; Triebkorn *et al.*, 1990). The most prominent reaction of epithelial crop cells to pollutant exposure is a reduction in intracellular energy stores such as lipids and glycogen, probably due to the energy-consuming synthesis of biotransformation enzymes, metallothioneins or stress proteins (Triebkorn and Köhler, 1996a).

### **Gastropod metallothioneins as biomarkers**

The strong inducibility of metallothionein synthesis by certain trace metals and their subsequent binding to the induced protein (see above) make this molecule a potential biomarker for environmental metal pollution (Dallinger, 1994, 1996; Berger *et al.*, 1995b). The fact that metallothionein synthesis in many animal species additionally can be promoted by non-metallic inducers such as organic chemicals and other stress factors (see, for instance, Kägi and Schäffer, 1988) further increases the importance of this protein for biomonitoring purposes (Dallinger *et al.*, 2000).

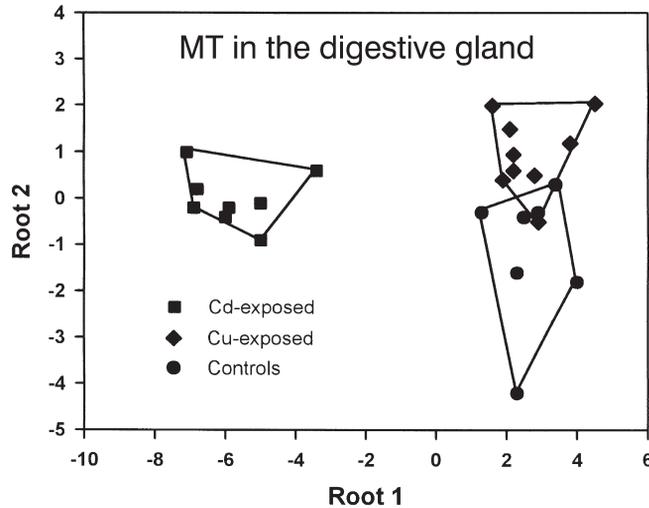
So far, the most important parameter reflecting the metallothionein status in an animal is, of course, its concentration in different tissues (see, for example, Roesijadi, 1993; Berger *et al.*, 1995b). Methods allowing metallothionein quantification at the level of the expressed protein can be regarded as direct approaches. They are based mainly on saturation assays in which metals such as cadmium (Bartsch *et al.*, 1990), silver (Scheuhammer and Cherian, 1986) and mercury (Dutton *et al.*, 1993) are added to the metallothionein to saturate its binding sites completely by replacing other metal ions having a lower affinity for the protein molecule. An indispensable prerequisite for this kind of quantification is the knowledge of the metallothionein's metal-binding stoichiometry (e.g. Dallinger *et al.*, 2001). Apart from direct approaches, there exist some indirect methods for assessing metallothionein synthesis which are based mainly on molecular techniques such as quantification of metallothionein mRNA (Unger and Roesijadi, 1993).

A cadmium saturation method (the so-called Cd-Chelex assay) has been used to measure metallothionein concentrations in terrestrial gastropods (Berger *et al.*, 1995b). This approach allows not only the quantification of the cadmium-binding metallothionein pool, but also the assessment of the native protein's saturation with this toxic metal. In this way, the metallothionein status in the digestive gland of metal-exposed individuals of *H. pomatia* was assessed in terms of the protein's concentration and its saturation with cadmium, and related to critical threshold

values observed in animals that were moribund after long-term exposure. It appeared that the mortality of metal-exposed *H. pomatia* significantly increased above concentrations of (Cd)-metallothionein in the digestive gland of about 1000  $\mu\text{g g}^{-1}$  (fresh weight), and at a cadmium saturation of the (Cd)-metallothionein pool of more than 60% (Berger *et al.*, 1995b).

The Cd-Chelex assay recently has been modified in order also to detect concentrations of copper-binding metallothionein and of total metallothionein (Dallinger *et al.*, 2000) in the digestive gland and the mantle of some species of helioid species. Figure 14.3 depicts an application of this method, showing significant differences in the status of different metallothionein pools in the digestive gland of uncontaminated and metal-exposed *H. pomatia* individuals.

The extent to which the metallothionein approach in terrestrial gastropods can be applied to field conditions as a biomarker for environmental pollution remains to be established. One possible approach in the future will be to correlate metallothionein concentrations in terrestrial



P-levels

	Controls	Cd-exp.	Cu-exp.
Controls	--	0.01	0.01
Cd-exp.	0.01	--	0.01
Cu-exp.	0.01	0.01	--

Factor structure matrix

	Root 1	Root 2
Total-MT	-0.47	-0.81
Cd,Zn-MT	-0.79	-0.18
Cu-MT	-0.11	-0.92

**Fig. 14.3.** Top: Graphic representation of a discriminant function of the total metallothionein concentrations, (Cd, Zn)-metallothionein and (Cu)-metallothionein in the digestive gland of *Helix pomatia* (Linnaeus) (Helicidae) after different exposure treatments (controls, cadmium-exposed and copper-exposed). Bottom: tables showing the significance levels (*P*-levels) of differences between treatments (left) and the factor structure matrix (right) for concentrations of total metallothionein (Total-MT), (Cd, Zn)-metallothionein (Cd, Zn-MT) and (Cu)-metallothionein (Cu-MT), respectively.

gastropods with sublethal parameters of toxicity. Such parameters include, for example, growth and fertility, as recently studied in metal-contaminated individuals of the species *C. aspersus* (Laskowski and Hopkin, 1996b; Gomot, 1997).

### ***Stress proteins of terrestrial gastropods as biomarkers***

First discovered in the species group of the fruit fly, *Drosophila melanogaster* Meigen (Drosophilidae) in response to increased temperature (Tissières *et al.*, 1974), a set of inducible proteins, originally called heat shock proteins, since has been found to occur ubiquitously in animal tissues after exposure to a variety of chemicals, but also in response to viral infections and both minor and massive damage to tissues (e.g. Schlesinger *et al.*, 1982, 1990; Nover, 1984). The term 'heat shock proteins' has been replaced by the expression 'stress proteins' for this class of molecules (Gething and Sambrook, 1992). Despite their apparent non-specificity in induction, the suitability of stress proteins as molecular biomarkers for chemical exposure and cellular stress has become more and more evident during the past few years (Peakall and Walker, 1994; Sanders and Dyer, 1994).

According to their molecular weight, eukaryotic stress proteins are classified into different protein families. Invertebrate animals possess: (i) a heterogeneous group of low molecular weight stress proteins with molecular masses of about 15–40 kDa; (ii) a group of so-called stress-60 proteins (hsp60, chaperonin, cpn60 and tcp1; mol. mass: 58–60 kDa); (iii) proteins of the prominent stress-70 family (hsp70 and BiP; mol. mass: 66–78 kDa); (iv) groups of stress-90 (hsp90; mol. mass: 83–90 kDa); and (v) high molecular weight stress proteins (mol. mass: 100–110 kDa) (Sanders, 1993). Additionally, a small protein of 7 kDa, called ubiquitin, which is involved in non-lysosomal protein degradation, as well as a variety of associated proteins assisting the aforementioned protein groups in their physiological function, are usually assigned to the stress proteins.

The physiological rationale for using stress proteins as biomarkers is based on the mode of induction of hsp70 which so far represents the best investigated family of stress proteins. Hsp70 is involved in intracellular protein folding and membrane translocation (Pelham, 1986; Morimoto *et al.*, 1990). Thus, whenever a stressor leads to an increase in denatured or malfolded protein molecules in the cell, the elevated presence of uncoiled polypeptide chains protruding from those proteins promotes a cascade of induction (see Gething and Sambrook, 1992), resulting in an enhanced synthesis of hsp70 ('abnormal protein hypothesis'; see Edington *et al.*, 1989; Sorger and Nelson, 1989; Craig and Gross, 1991). Based on this background, it is now well accepted that the main advantage of the hsp70 proteins as biomarkers is their ability to integrate effectively a variety of adverse effects on protein integrity collectively termed proteotoxicity (Sanders, 1993). The increased level of stress proteins indicates

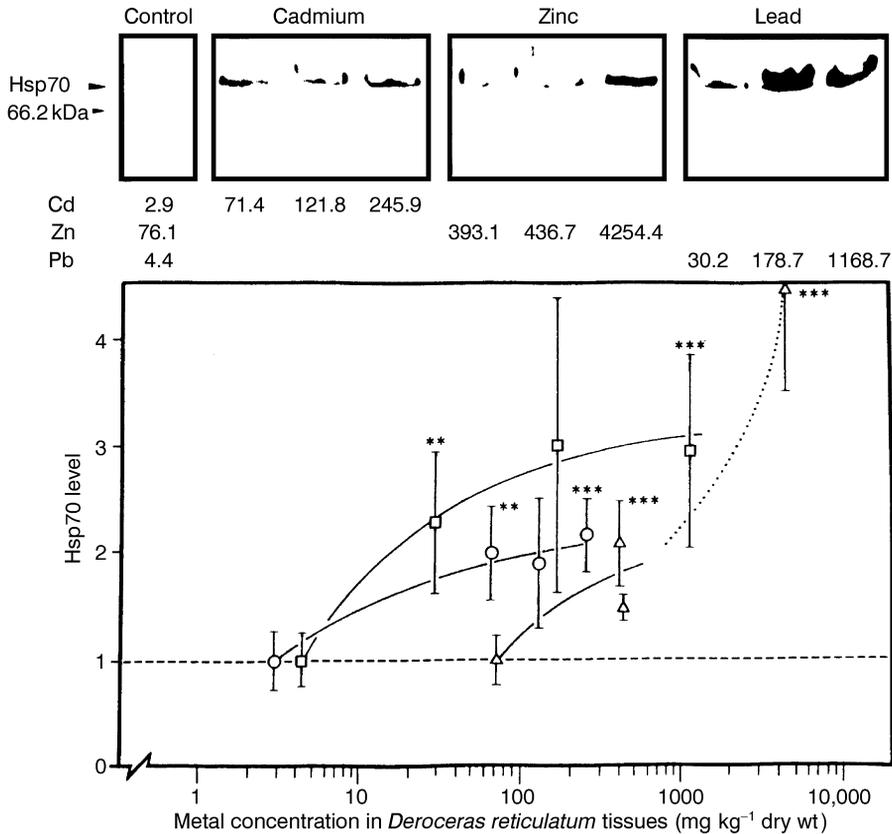
the presence of a stressor, but usually does not give any information about its physical or chemical nature (Peakall and Walker, 1994). Some of the numerous members of the aforementioned stress protein families, however, are thought to be stressor-specific (Sanders and Dyer, 1994).

The use of stress proteins as biomarkers for environmental hazards has gained increased prominence during the last decade. Most of the research in this field, however, has been directed towards animals from aquatic environments (e.g. Sanders, 1993), with only a limited number of studies focusing on soil invertebrates (Köhler *et al.*, 1992, 1996a). The supposed ubiquity and structural conservatism of at least the three major families of stress proteins (hsp90, hsp70 and hsp60) imply their presence also in terrestrial gastropods. However, evidence for this so far has only been provided for hsp70 and hsp60 in *A. ater* and *D. reticulatum*.

In individuals of *D. reticulatum* exposed to a set of environmentally pollutant chemicals, hsp70 seems to be a 'better' biomarker than hsp60 due to its higher sensitivity in response to stressors (Rahman, 1994). Accordingly, it has been shown that poisoning in *D. reticulatum* and *A. ater* by carbamate molluscicides leads to an induction of hsp70 proteins (Köhler *et al.*, 1992). Moreover, subchronic exposure of *D. reticulatum* to elevated concentrations of pentachlorophenol over a period of 2 weeks resulted in a dose-dependent induction of these proteins (Rahman, 1994).

Most of the work on stress proteins as biomarkers in terrestrial gastropods has been carried out with trace elements as inducing agents. Subchronic exposure of *D. reticulatum* to metal ions ( $Zn^{2+}$ ,  $Cd^{2+}$  and  $Pb^{2+}$ ) over a period of 3 weeks, for instance, resulted in the induction of hsp70 in a concentration-dependent manner (Köhler *et al.*, 1994, 1996a) (Fig. 14.4). This has been demonstrated at the level of protein expression by means of accumulation of the stress protein itself, as well as at the mRNA level (Köhler, 1996). In the exposure experiments with cadmium and zinc, the levels of hsp70 accumulated after 3 weeks in animal tissues were found to be good predictors of mortality in life cycle exposure experiments. Accordingly, RT-PCR (reverse transcription-polymerase chain reaction) studies have shown an elevated transcription rate of the hsp70 gene, and/or an increased longevity of the hsp70 mRNA after 2 weeks of exposure, to reflect (and possibly to result in) a decline in fecundity and viability of eggs in *D. reticulatum* under conditions of lifetime exposure (Köhler, 1996; Köhler *et al.*, 1996b).

The molecular stress response in terrestrial invertebrates is especially useful as a biomarker for sublethal effects of chemicals. Like other biochemical biomarkers, the stress protein response is maximal with initial increases in intensity of the stressor, but then decreases above a certain threshold level of exposure. This commonly is explained by a pathological inhibition of gene transcription and protein synthesis at too high intensities of exposure. Such a pattern of response has been demonstrated for the hsp70 levels in the isopod *O. asellus* (Eckwert *et al.*, 1997) and the nematode *Caenorhabditis elegans* (Maupas) (Rhabditidae) (Güven *et al.*, 1994). It might also be expected to occur in the tissues of terrestrial



**Fig. 14.4.** Accumulation of the stress protein hsp70 induced by exposure of *Deroceas reticulatum* (Müller) (Agriolimacidae) to cadmium-, zinc- and lead-enriched food and substrate over a period of 21 days under constant laboratory conditions (temperature: 10°C; photoperiod: 16/8 h light/dark) (data taken from Köhler *et al.*, 1996a). Top: representative western blots of equal amounts of total protein, extracted from whole body homogenates of unaffected (Control) and metal-exposed animals (Cadmium, Zinc, Lead), showing increasingly dense bands of hsp70 reflecting increasing concentrations (in mg kg<sup>-1</sup> dry weight) of metals in the animals's soft tissues, as specified below the blots. Visualization of hsp70 bands was achieved by monoclonal antibodies raised against hsp70. Bottom: densitometric quantification of hsp70 bands (hsp70 level) generated by western blot analysis with increasing metal concentrations (means and standard deviations) in animal tissues (mg kg<sup>-1</sup> dry weight). The mean value of the hsp70 band in controls was set, as a reference, to 1.00 (dashed horizontal line). Circles, cadmium; squares, lead; triangles, zinc. For zinc exposure only, the experiment resulting in the highest metal accumulation (up to 1168.7 mg Zn kg<sup>-1</sup> tissue dry weight; dotted line) was terminated after 9 days instead of 21 because of the high mortality rate in this group. Significance levels (Student's *t*-test, difference from controls) were as follows: \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .

gastropods. It is concluded, therefore, that a proper interpretation of the effects of pollutant exposure might only be achieved by combining the stress protein response with cellular biomarkers, and, depending on the intensity of exposure, taking into account pathological alterations.

It is not clear yet to what extent the biomarker concept in terrestrial gastropods might contribute to a more efficient impact assessment in

polluted terrestrial ecosystems (see Treweek, 1996). Much work in this novel field of ecotoxicology is still in progress.

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