WATER QUALITY AND STRESS INDICATORS IN MARINE AND FRESHWATER ECOSYSTEMS: LINKING LEVELS OF ORGANISATION (INDIVIDUALS, POPULATIONS, COMMUNITIES)

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Metallothionein as an indicator of water quality – assessment of the bioavailability of cadmium, copper, mercury and zinc in aquatic animals at the cellular level

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The study of metallothioneins (MTs) has greatly improved our understanding of body burdens, metal storage and detoxification in aquatic organisms subjected to contamination by the toxic heavy metals, Cd, Cu, Hg and Zn. These studies have shown that in certain organisms MT status can be used to assess impact of these metals at the cellular level and, whilst validation is currently limited to a few examples, this stress response may be linked to higher levels of organisation, thus indicating its potential for environmental quality assessment. Molluscs, such as *Mytilus* spp., and several commonly occurring teleost species, are the most promising of the indicator species tested.

Natural variability of MT levels caused by the organism's size, condition, age, position in the sexual cycle, temperature and various stressors, can lead to difficulties in interpretation of field data as a definitive response-indicator of metal contamination unless a critical appraisal of these variables is available. From laboratory and field studies these data are almost complete for teleost fish. Whilst for molluscs much of this information is lacking, when suitable controls are utilised and MT measurements are combined with observations of metal partitioning, current studies indicate that they are nevertheless a powerful tool in the interpretation of impact, and may prove useful in water quality assessment.

Introduction

The primary impact of a pollutant is at the cellular level, through disturbance of cell membrane integrity/permeability properties, or by interaction with cellular macromolecules, leading to inhibition of essential cellular metabolic or physiological processes. Thus measurement of sub-lethal responses at the cellular level could provide a very sensitive measure for assessment of water quality and environmental impact of pollutants. Many physiological responses are far too unspecific to be of practical use for diagnosis of the effects of specific compounds; however, several biochemical effects are specific to a particular pollutant or class of pollutants. Two such responses, at the level of induction of gene transcription, are that of the metal-binding protein, metallothionein (MT), to heavy metal exposure – which is discussed in this article – and induction of the mixed function oxygenase enzyme, CYP1A, to polyaromatic hydrocarbon exposure – which is discussed in the following contribution in this volume, by Livingstone *et al.*

As for linking levels of organisation, there is much evidence to indicate that MT induction, coupled with disturbances in metal distribution among cytosolic ligands, are a direct reponse to metals. The appearance of excess metal in "abnormal" metal pools, resulting from saturation of MT – the so-called "spillover" effect – has been associated with the onset of deleterious events and has led to the "early-warning" indicator concept. In cases where causal links are established it is not difficult to see the value of cellular indices in assessing the health of the individual or population. Thus the saturation of hepatic MT synthesis and binding to MT

results in inhibition of essential membrane-bound enzymes in fish liver (George 1989), more direct toxic effects, including mortalities, are associated with MT saturation and spillover in oysters (Engel 1983), whilst a sudden increase in cytosolic (low molecular weight) cadmium in the polychaete *Neanthes arenaceodentata*, for example, coincides with a decline in reproductive potential, which has obvious ecological significance (Jenkins & Mason 1988). In more general terms it may be postulated that MT induction in response to metal stress could result in the diversion of energy reserves away from normal requirements. There are as yet, however, no satisfactory indications regarding the share of the energy budget diverted into detoxification, or the related costs of tolerance (MT-gene multiplication, loss of diversity), though it is clear that these processes could influence the general health of the organism or population. Thus, being able to determine whether or not a population is attempting to adapt under stress, through observation of biochemical indices such as MT, does in some respects represent a method for detecting the first stages of ecosystem change. For the most part, however, MT studies seem unlikely to be of value in predicting specific functional changes at the highest levels of organisation (community and ecosystem) and this was never the primary goal of MT-related research. Equally, an ecological study, *per se*, is unlikely to be an acceptable indicator of stress unless accompanied by some chemical/biochemical information on causative agents and mechanisms of effect. Thus different forms of stress, whether due to physical disturbance or contaminants, often manifest themselves in common fashion in communities (e.g. reduced abundance and species diversity). Without some indication of specific cause and effect (whether it be for example, metal accumulation, MT induction or, in the case of organic pollutants, enzyme activity) it would be difficult for the environmental manager to make an appropriate assessment of damage and take the correct remedial action.

In this paper we will briefly discuss the use of a cellular parameter, the metal-binding protein, metallothionein, as a sublethal bioindicator of the environmental bioavailability of the toxic metals cadmium, copper, mercury and zinc. The occurrence of heavy metals and MT in aquatic organisms have been recently reviewed (George 1990; Langston 1990; Roesijadi 1992) and a critical appraisal of the application of MT measurements, especially in fish, to biomonitoring of coastal waters and estuaries, and the various assay procedures, have been reviewed in greater detail by George & Olsson (1994). Biological and physicochemical parameters affecting metal uptake and partitioning in aquatic organisms have also been reviewed (George 1980; Bryan & Langston 1992; Langston & Spence 1994); thus the reader is referred to these papers for fuller literature citations.

Role of metallothionein in metal homeostasis

Chemically reactive metals which bind to essential prosthetic groups of enzymes inhibit their action and are therefore toxic. However, when they are also essential to life processes and are present at sub-optimal concentrations they may limit functionality and thus efficiency of bodily functions. Thus when availability is limiting, by increasing their bioavailability a "beneficial" dose-response curve is observed (Fig. 1, curve A). Copper and zinc are examples of two such metals. The cell regulates or "buffers" the intracellular concentrations of these two essential metals by maintaining a small "store" in the form of a protein complex, metallothionein (MT). Thus over a limited range of intracellular concentrations where Cu and Zn are present in excess of their metabolic requirements, they are sequestered by MT and the dose/effects plateau (Fig. 1, curve B), i.e. homeostasis is maintained. When the capacity of MT for metal-binding is exceeded, essential life-processes are inhibited and toxicity becomes apparent (Fig. 1, curve C).

When effects of increasing concentrations of non-essential metals such as cadmium and mercury are considered, little or no stimulation of life-processes occurs. Due to their chemical

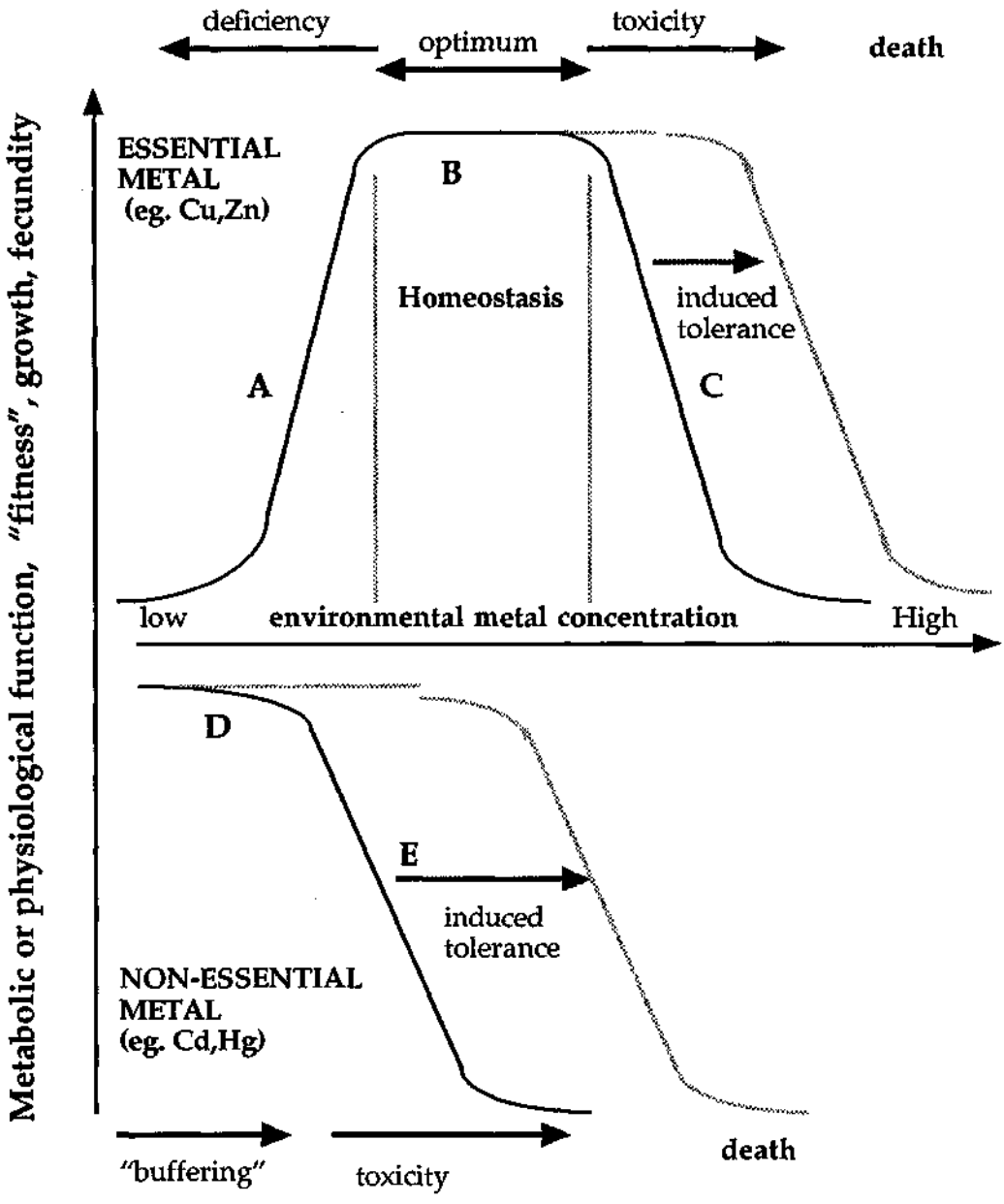

Figure 1. Influence of metal bioavailability on functioning of an organism. (Modified from George 1990).

similarity to Cu and Zn (ionic radii and affinity for -SH co-ordination), at low concentrations they displace Zn from MT, i.e. they are detoxified, and no adverse effects are observed (Fig. 1, curve D). As their concentrations are increased and MT is saturated, inhibition of life-processes and toxicity become apparent (Fig. 1, curve E). Thus important functions of MT are maintenance of the homeostasis of the essential metals Cu and Zn and detoxification of the non-essential (or pollutant) metals Cd and Hg.

What is metallothionein?

Metallothionein, a metallo-derivate of the sulphur-rich protein, thionein, was first isolated by Vallee's group as a Cd-binding protein from equine renal cortex. The structure and metal binding properties of MT have been extensively studied. MTs generally have a molecular weight of about 6,000–7,000 Daltons and consist of some 60–62 amino acids. The protein is unique in containing some 20 cysteine residues (i.e. 33%) which do not form disulphide bridges. In most species different charge isoforms are found; however, in all MT isoforms the sequence position and number of cysteine residues are highly conserved within the protein.

Vertebrate MT binds 6–7 gram atoms of Cd or Zn by tetrahedral co-ordination with the cysteine residues. MT is usually saturated with Zn and this can be displaced by varying amounts of Cd from a $CdZn_6MT$ to a Cd_7MT. The metals are organised in two domains: the α-domain, extending from amino acid 31 to 61, holds four metal ions, whilst the ß-domain, extending from amino acid 1 to 30, holds three metal ions. These features for MT in plaice are shown in Figure 2, which is a computer model derived from the rat structure (obtained by X-ray crystallography) and the deduced amino acid sequence of the plaice protein which we obtained by gene cloning. Both domains are globular, with diameters of 1.5–2.0 nm and are linked by residues 30 and 31 to form a prolate ellipsoid; thus on size-exclusion chromatography the protein behaves as though it has an apparent molecular weight of *c.* 12–15,000 Daltons. Copper and mercury form tetragonal complexes and thus 11–12 gram atoms of these metals are bound by MT; the folding of CuMT and HgMT are not yet known.

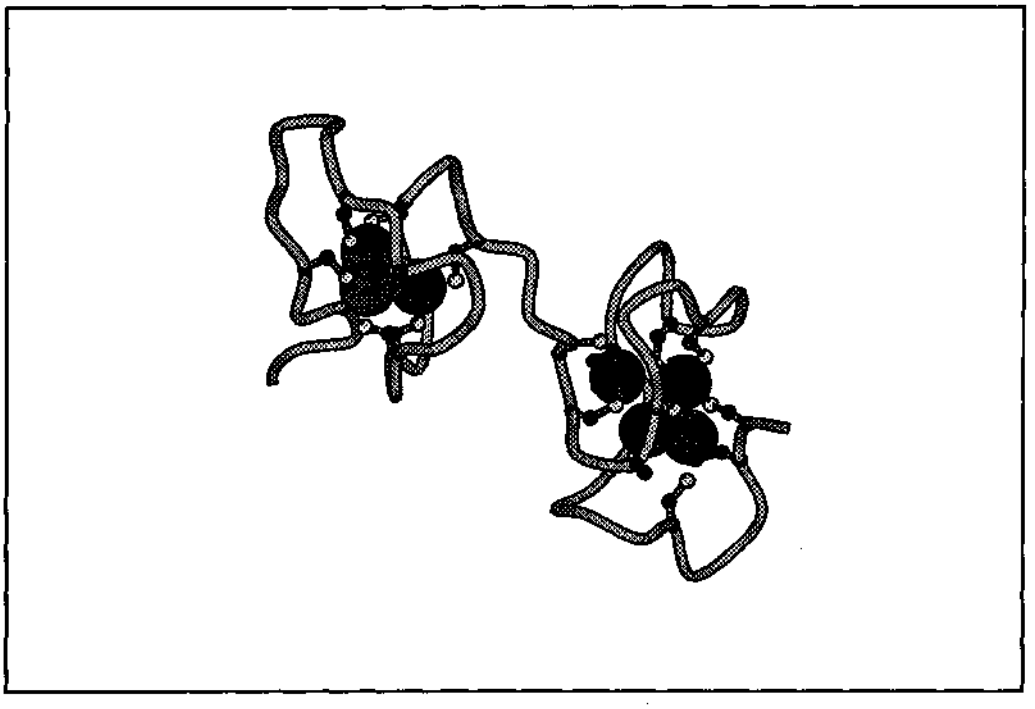

Figure 2. Structure of plaice (*Pleuronectes platessa*) metallothionein (MT), computer-modelled from X-ray structure of rat (*Rattus norvegicus*) MT, showing the backbone of the amino acid chain and the side chains of the cysteine residues with their sulphur atoms co-ordinated to cadmium and zinc atoms.

Occurence of metallothionein in aquatic organisms and choice of animals for biomonitoring

The presence of MTs and MT-like proteins has been reported in practically all animal phyla. In aquatic organisms amino acid sequence data have only been obtained for a few species (Table 1), although from purification studies positive identification of characteristic MTs has been obtained in numerous other species. As noted earlier there is a very strong degree of homology amongst vertebrate Class I MTs (see Roesijadi 1992 for review), although the presence of only four residues before the first cysteine at the amino terminus of piscine MTs, compared with five residues in mammals, results in a lack of immunogenic cross-reactivity. Structural homology is not as well conserved in invertebrate MTs, although they do contain many conserved cysteine-containing structural motifs and evidence supports the classification of many of these proteins as Class I MT, e.g. crab *Scylla serräta*, mussel *Mytilus edulis*, and oyster *Crassostrea virginica*, whilst positions of cysteine residues in species such as the sea urchin *Strongylocentrotus purpuratus* and the nematode *Caenorhabditis elegans* are only distantly related to equine renal MT, and their metal-binding proteins are regarded as Class II MT which do not appear to be inducible by metal-exposure.

Table 1. *Metallothioneins of aquatic organisms characterised by amino acid or nucleotide sequence data.*

*The vertebrate MTs show a very high degree of structural conservation. Whilst the invertebrate MTs are also cysteine-rich and contain many conserved structural motifs they differ in both sequence and molecular sizes.

Phylum	Species	Common name
Pisces*	*Oncorhynchus mykiss*	rainbow trout
	Pleuronectes platessa	plaice
	Psuedopleuronectes americanus	winter flounder
	Esox lucius	pike
	Barbatula barbatulus	stone loach
Crustacea	*Scyla serrata*	crab
Mollusca	*Mytilus edulis*	mussel
	Crassostrea virginica	American oyster
Nematoda	*Caenorhabditis elegans*	worm
Echinodermata	*Stongylocentrotus purpuratus*	sea urchin

Care must be taken in selection of organisms for monitoring purposes. A few fish species appear to be metal-tolerant, e.g. the freshwater stone loach *Barbatula barbatulus*, although the mechanism of this tolerance is not yet known. A few other fish species, e.g. the white perch *Morone americana* and the squirrelfish *Holocentrus brufis*, appear to have a metabolic defect resulting in hepatic accumulation of copper which is akin to certain disease states of man, and their use in monitoring programmes should be avoided. To date, sufficient validation has been performed for Perciformes, Pleuronectidae and Salmonidae to enable their use in environmental monitoring.

Compared with vertebrates, the body burden of metals in invertebrates is commonly very much higher and these are found in a diversity of pools (see George 1982; Viarengo & Nott 1993; Langston & Spence 1994) and thus interactions are complex. This is particularly notable in crustaceans where the major pools of Cu and Zn include CuMT and ZnMT, the Cu-containing respiratory pigment haemocyanin, insoluble metal-containing lipofuschin granules and also Ca/Zn phosphate granules. There is also the added complication of the arthropod moult cycle which affects metal dynamics considerably, and MT plays a major role in this

normal physiological process. Whilst Cd bound to MT accumulates in crabs and lobsters exposed to elevated metal levels, fluctuations due to metabolic needs just noted, as well as possible artifacts due to the susceptibilities of CdMT to protease degradation, displacement of Cd by Cu, and oxidation during homogenisation, would in our opinion make the adoption of MT assays in these species somewhat hazardous for routine use as sentinel organisms. In other invertebrates, notably clams, whelks, scallops and some worms, proteins other than MT capable of binding large amounts of heavy metals such as Cd have been identified (reviewed by Stone & Overnell 1985). The unqualified adoption of MT assays using invertebrates, is further inhibited by a number of reports suggesting the absence of MT, or at least the apparent lack of induction in response to metal contamination, among certain groups, especially commonly occurring worms and clams which would otherwise prove to be convenient test organisms. However, it should be pointed out that until more organisms are investigated premature generalisations are best avoided.

With the above caveats in mind it will be essential to limit the disscussion of "MT as a stress indicator" to those invertebrates for which (a) validatory studies have been carried out and (b) their use as indicator organisms of water quality is a practical proposition. With present knowledge the choice essentially becomes restricted to several species of molluscs (and possibly some crustaceans), some of which are described below.

Control of metallothionein levels

Metallothionein sequesters metals so strongly that thionein, i.e. the apoprotein, cannot be detected in cells. Synthesis of MT is transcriptionally controlled by activation of mRNA synthesis through cis- and trans- acting factors acting on regulatory regions of the gene. The newly synthesised mRNA then acts as a template for *de novo* protein synthesis. Present evidence indicates that MT mRNA has a relatively short half-life. Factors influencing MT gene expression in mammals and fish are shown in Table 2. No equivalent data are so far available for invertebrate MTs.

Table 2. *Factors inducing MT gene expression in fish and mammals.*

Agent		Mechanism of action	Magnitude of induction
Divalent metal ions	Cd, Cu, Hg, Zn (Ni, Ag)	MREs in gene	strong, up to 100-fold
Hormones	Corticosteroids	GRE in gene	up to 2-fold
	Progesterone	GRE in gene	up to 2-fold
demonstrated in mammals but not in fish:			
Tumour promoters	phorbol esters		up to 2-fold
Protein kinase C affectors	interleukin 1		up to 2-fold
	inflammatory agents		
	interferon		

Use of metallothionein measurements in biomonitoring

Inducibility of teleost MT, exogenous and endogenous influences

In most fish species basal levels of hepatic Zn are sequestered by MT, the Zn and MT concentrations in the liver displaying a linear relationship in the maintenance of cellular metal homeostasis (Fig. 3). However, the exact proportionalities between hepatic concentrations of Cu + Zn and MT appear to vary with age, sex, species and populations. In the pleuronectid

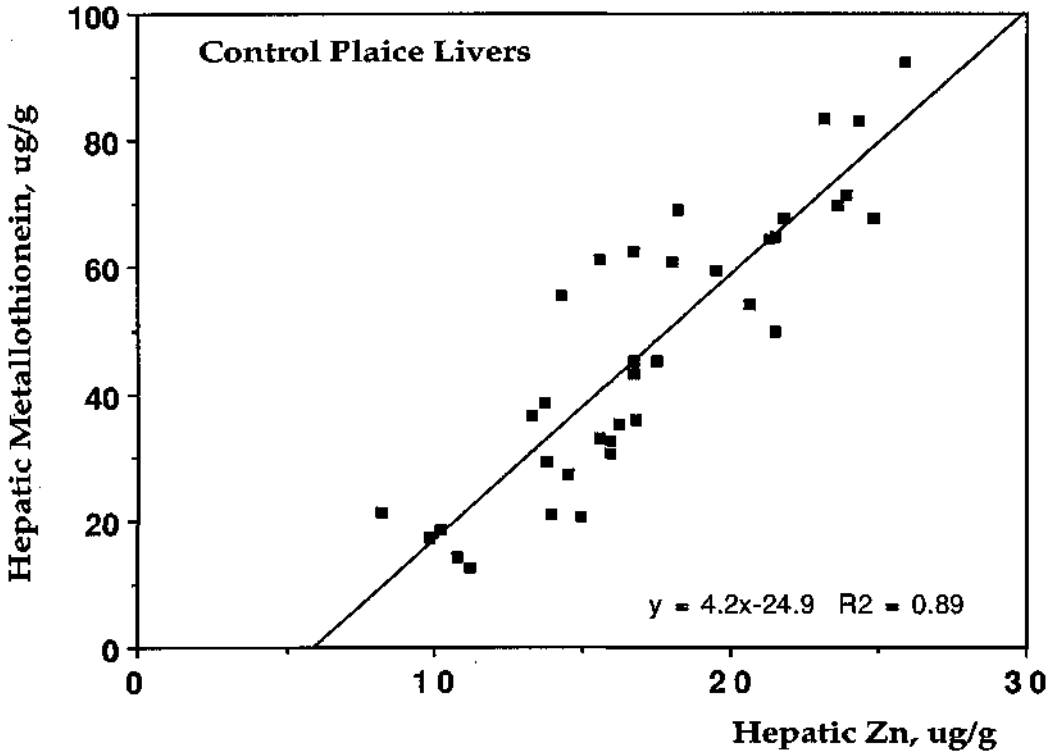

Figure 3. Relationship between hepatic zinc and metallothionein concentrations (μg g^{-1}) in plaice (*Pleuronectes platessa*) from a non-polluted environment.

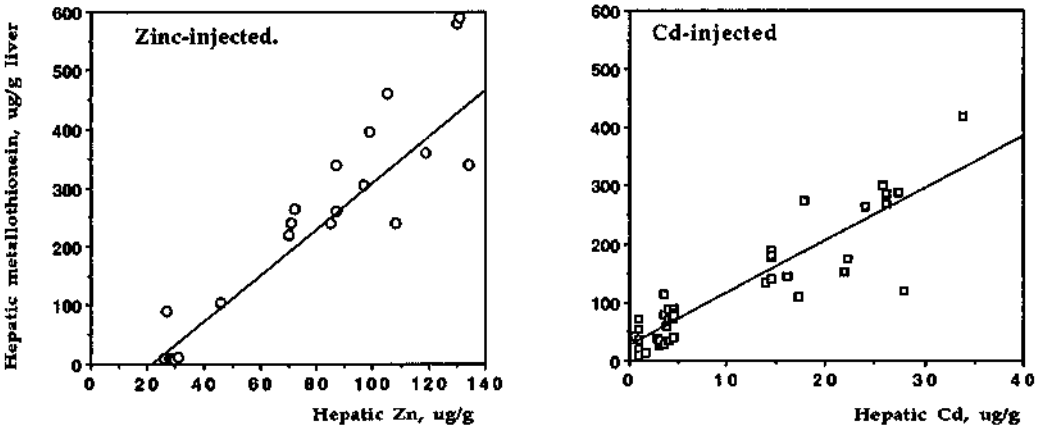

Figure 4. Relationships between hepatic concentrations of metallothionein and zinc or cadmium (μg g^{-1}) after administration of metals to plaice (*Pleuronectes platessa*) by intraperitoneal injection.

flatfishes, *Pleuronectes platessa, Psuedopleuronectes americanus* and *Platichthys flesus* from non-contaminated environments, basal levels of 15–80 μg MT g^{-1} (wet wt) are present. In salmonid livers the basal levels of Cu are very high (150–350 μg MT g^{-1}), reflected by high levels of MT. Thus in *Onchorhynchus mykiss* liver, values of 140–240 μg MT g^{-1} are normal whilst in Atlantic salmon *Salmo salar*, returning to non-polluted rivers, values of *c.*100 μg MT g^{-1} are found.

Hepatic MT levels have also been shown to vary with time of year, reproductive state, water temperature and developmental state in both *O. mykiss* and *P. platessa*. In juvenile fish, seasonal variations in MT of 2- to 3-fold are most probably related to altered metabolism during acclimation to lowered water temperature, although effects of altered feeding or photoperiod may also be influential. Hepatic MT levels are also elevated during sexual maturation and spawning. In male fish, MT levels rise about 2-fold, whilst in female fish much larger increases in MT protein levels of up to 7-fold are observed at the cessation of the period of exogenous vitellogenesis prior to spawning. Studies with isolated hepatocytes and cell lines have indicated that the rise in male fish may be due to progesterone, whilst in females the elevation appears to be due to oestradiol-induced mobilisation of Zn and Cu for metabolic purposes rather than a direct hormonal effect. Other *in vitro* studies have shown that the MT levels may also be induced up to about 2-fold by "stress" hormones such as glucocorticoids and noradrenaline, and in an *in vivo* study it has been shown that capture-stress when taking fish from the field could cause a small elevation in MT levels in striped mullet *Mugil cephalus*.

Thus there are a variety of non-metal related conditions that will ultimately result in a relatively small induction of MT levels which must be taken into account when sampling fish and in the interpretation of data. Generally these can be avoided by selection of males or immature fish and use of fish from reference sites, at the time of sampling, as controls. There are also data to suggest that measurements of MT levels in gills of fish may be a better indicator of environmental quality than hepatic measurements which may be affected by local dietary effects (see George & Olsson 1994).

Inducibility of teleost MT as an indicator of metal exposure

Experimental determinations of dose-response relationships between metal exposure and MT levels are important for calibration purposes when considering MT measurements as a tool in environmental monitoring and as an early warning system.

Laboratory experiments performed on several fish species and fish cell lines have shown that teleost MTs are inducible by Cd, Cu, Zn and Hg, that they are bound to MT and both MT protein and MT mRNA levels increase in correlation with the dose of administered heavy metals. In *P. platessa* injected intraperitoneally with Zn or Cd, good correlations between metal and MT levels are obtained (Fig. 4). A central theme to the debate on the role of MT as a defence system/stress response concerns its rate of synthesis, turnover and affinity for metals: if the protein is to be effective as a detoxifying agent, synthesis must be sufficiently rapid and binding sufficiently strong to prevent excessive binding to other more sensitive structures. Thus for higher doses of Cd, when metal overload and hepatotoxicity becomes apparent, this relationship between metal burden and MT levels deviates from linearity (George 1989). In *O. mykiss*, exposed to Cd in the water (200 μg l^{-1}), a linear relationship was again attained. Similar linear relationships have also been observed in feral *P. fluviatilis* and *O. mykiss* from contaminated areas.

The response-time of MT induction has been determined in laboratory experiments where Cd has been administered by intraperitoneal injection in plaice and turbot *Scophthalmus maximus*. Whilst MT mRNA levels rise in both liver and kidney within 24 hours of

administration (peaking at 2–4 days) and remain elevated for at least 21 days, there appears to be a lag-period of several (3–5) days before protein levels rise significantly. Experimental determination of MT turnover-time in plaice has shown that the half-life of the protein is around 1 month. Thus the effect of metal exposure is sufficiently long lasting to be of practical use in monitoring.

Inducibility of invertebrate MTs as an indicator of metal exposure

Whilst MTs have been characterised from several molluscs and crustaceans and the response to metal exposure studied, the interactions between metal pools and effects of moult cycle, diet etc. in crustaceans are so complex, as noted earlier, that constraints of space excludes their inclusion in the present review. Summaries of the potential value of MT as an indicator of metal exposure, for some of the most well studied molluscs commonly found in European waters, are presented below.

(a) Mussels: Mytilus spp. Some 4–5 isoforms of MT are present in Cd-exposed *M. edulis*; the isoforms have a cysteine content of around 25% and can exist individually or as dimers (apparent sizes 10 and 20 kDa). Basal levels of MT in mussels, determined polarographically, are of the order of 2–3 mg MT g^{-1} dry wt (*c.* 0.4–1.0 mg MT per gram wet weight), which is some 4- to 50-fold higher than in teleost fish and is presumably reflective of their naturally high tissue trace metal contents. Following laboratory exposures, increased levels of MT have been found in animals exposed to Ag, Cu, Cd and Hg but not Zn (referenced in George 1990). It is interesting to note that there is apparently no significant difference in MT induction rates between mussel species; 400 μg Cd l^{-1} gives rise to a 30-day net increase from about 2.5 to 9 mg MT g^{-1} dry wt, i.e. a 3- to 4-fold increase on basal levels, following a lag-time of 7 days for both *M. edulis* and *M. galloprovincialis* (Fig. 5 A).

Although utilisation of a single species is preferable in surveys, it would seem that the responses of *M. galloprovincialis* and *M. edulis* (which interbreed where their ranges overlap) are sufficiently similar to allow some intercomparison of data, at least in initial stages of impact assessment. Curves for MT induction in mussels, shown in Figure 5, are for whole soft tissues. Though for a variety of reasons the use of single tissues might be considered preferable, in the case of *Mytilus* similar responses have been observed for gills and whole animal and the latter may be more practical when sample size is limited. As a general rule MTs appear to be concentrated in gills, digestive gland (hepatopancreas) and kidney in most invertebrates, commensurate with their respective roles in uptake, storage and excretion of metals.

The proposal that mussel MT functions as a detoxification system/stress response is well founded. Field data show that Cd, Cu and Hg are effectively bound by mussel MT at contaminated sites and laboratory experiments confirm that the rate of MT production appears to be sufficient to keep pace with the influx of Cd, even at extremely high exposure levels (giving rise to the linear relationship shown in Fig. 5 B). Inevitably, however, some association of Cd with other intracellular ligands takes place and partitioning thus reflects exposure conditions. At high levels of exposure, net MT production and Cd accumulation eventually slow down (and peak at around 12 and 1 mg g^{-1} dry wt respectively) indicating the approach of equilibrium and saturation of MT with Cd (inset, Fig. 5 C). The proportion of metal bound to MT is therefore a function of exposure concentration and time and such measurements add significantly to the interpretation of body-burden data. Turnover rates for MT and different metals in *M. edulis* vary. The protein is thought to be degraded in the lysosomes and the released metal becomes re-sequestered by newly-synthesised MT. Copper, zinc and cadmium have different tissue distributions (the highest concentrations usually appearing in the digestive

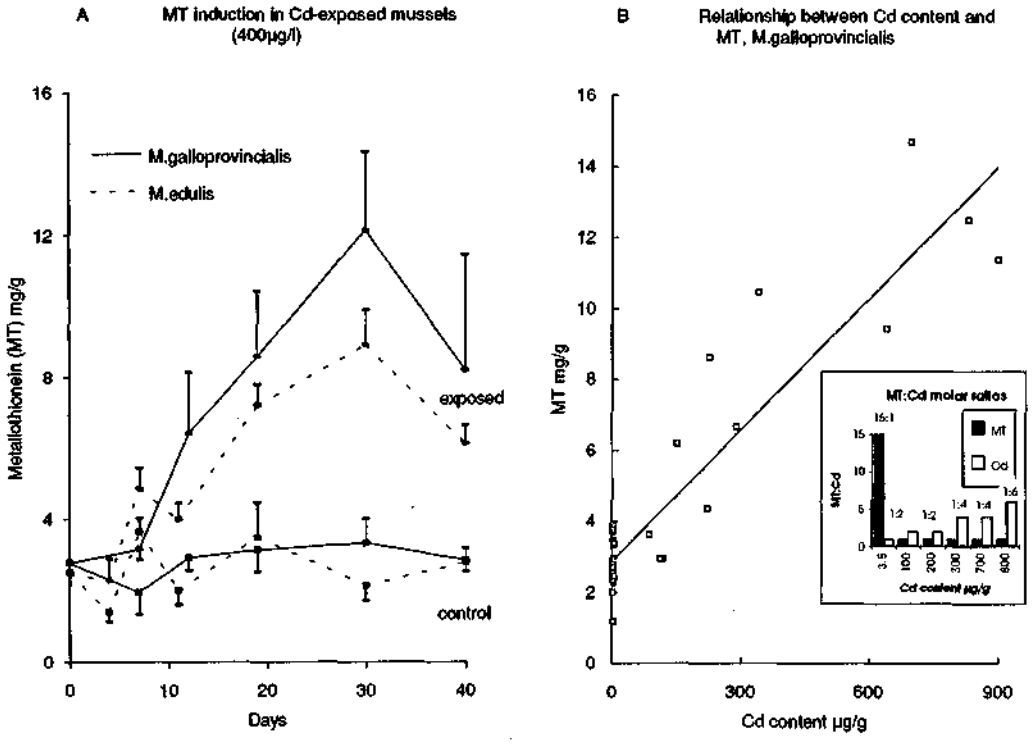

Figure 5. A, Induction of metallothionein (mg g^{-1}dry wt) with time in two species of mussels (*Mytilus edulis* and *M. galloprovincalis*) exposed to cadmium at a concentration of 400 µg l^{-1}, compared with controls; B, relationship between MT and Cd concentrations (µg g^{-1}) in tissues of *M. galloprovincialis*. (Redrawn from Langston *et al.* 1989; Bebianno & Langston 1992).

gland and kidney) and are eliminated at different rates (with half-lives of 10, 60 and 300 days, respectively). It was proposed that these were explicable by differential susceptibility to lysosomal degradation and varying rates of turnover of their respective MTs (George & Viarengo 1985), a hypothesis since confirmed for mammalian MTs. Recently it has been shown that CdMT is degraded with a half-life of about 25 days, whilst the half-life of Cd is about 300 days (Bebianno & Langston 1993). MT synthesis does not appear to be induced by Zn-exposure of mussels; this may be reflective of the rapid transport of Zn to the kidney where very high levels accumulate, and become immobolised, in tertiary lysosomes.

Since mussels are easily collected and widely used in pollution assessment exercises, they are obvious practical candidates for metal-binding studies. The response-time for MT induction in mussels is similar to that in fish, and laboratory experiments have shown a linearity of response of mussel MT to Cu, Cd and Hg exposure. It would appear that measurement of mussel MT levels, preferably backed up by metal-partitioning studies, will provide a valid monitoring tool for recent metal impact, though further field validation and examination of natural variability is desirable.

(b) Winkles: Littorina littorea. Gastropod molluscs are often found to be excellent bioindicators of contamination, at least for non-essential metals (see for example Bryan *et al.* 1985). Proteins with MT-like characteristics have been described in several species including chitons, whelks, limpets and winkles although none have been characterised by amino acid

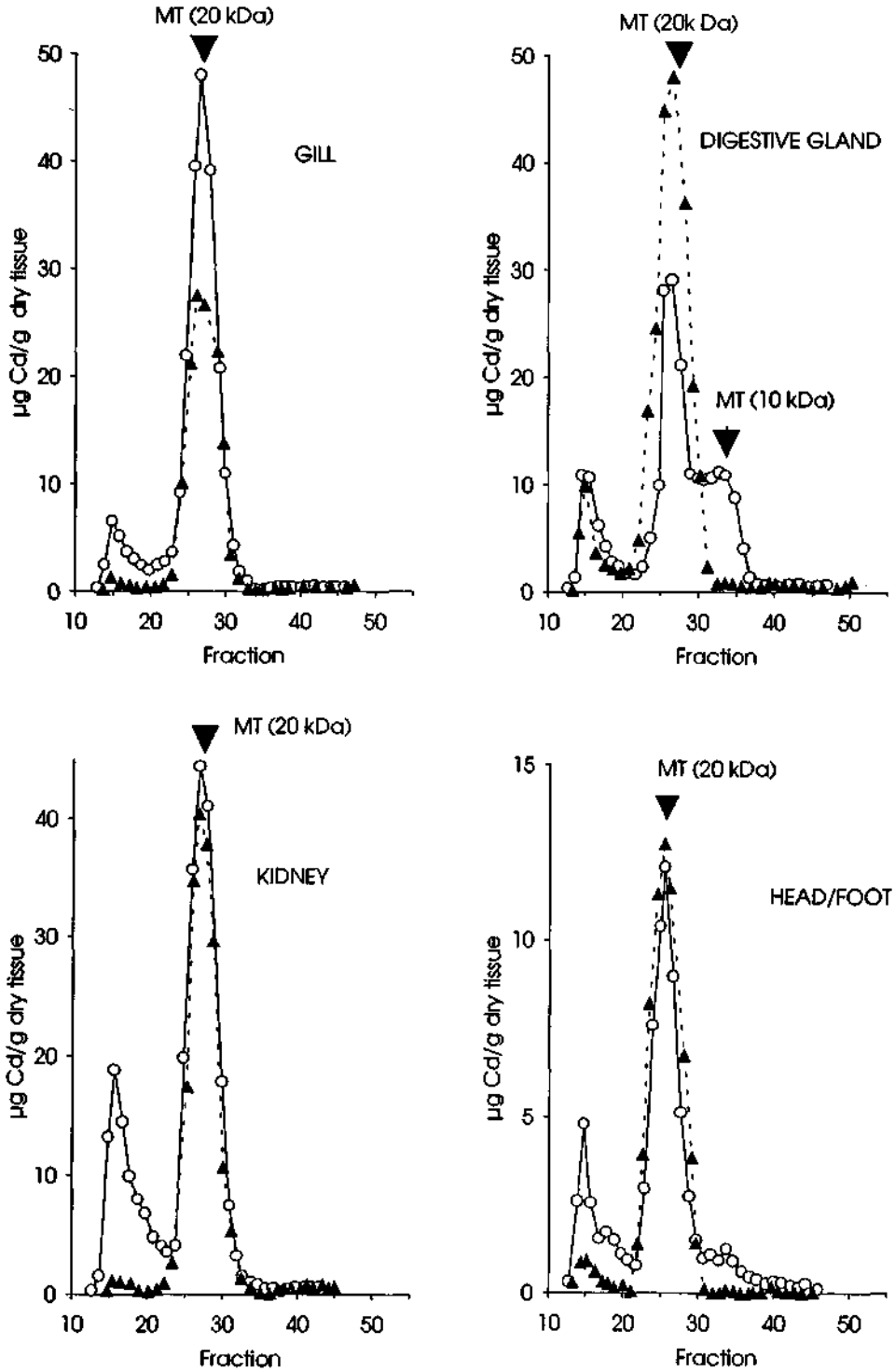

Figure 6. Distribution of cadmium in cytosol of individual tissues (µg Cd g^{-1} dry tissue) following exposure of winkles (*Littorina littorea*) to 0.4 mg l^{-1} Cd for 39 days (solid lines) and subsequent depuration for 197 days (broken lines). (Redrawn from Langston & Zhou 1987).

sequencing. Metals bound by MT in the winkle *L. littorea*, for example, include Cd, Cu, Hg, Ag but not Zn.

Unfortunately from the standpoint of water quality assessment, there are a number of complexities concerning the use of MT analysis in this group of molluscs (as with various other invertebrates) and brief discussion of these problems here might prove relevant for those contemplating MT determination on previously untested organisms. Thus, kinetic studies with *Littorina* have indicated that the Cu-containing respiratory pigment haemocyanin (HCY) may be involved in complexation and inter-organ transport of several metals, and the partitioning of Cd between HCY, MT and other ligands is a dynamic process reflecting exposure history. Chromatographic analysis of metal-partitioning is therefore an essential component of data interpretation. In *Littorina* from clean sites, trace amounts of Cd can be detected as a CdMT dimer (apparent molecular weight of 20 kDa) whilst profiles from tissues of metal-exposed winkles demonstrate an increasing build up of Cd associated both with high molecular weight proteins (including HCY) as well as MT (Fig. 6). In digestive gland there are also indications of *de novo* synthesis of a MT-like protein (*c.* 10 kDa) when Cd burdens are high. During depuration of Cd-exposed winkles in clean sea water, high molecular weight proteins continue to offload Cd to MT, notably in the digestive gland, and the protein once more becomes the major site for storage (Fig. 6). Despite subcellular and tissue redistribution of Cd during depuration, however, there is virtually no reduction in the total body burden of Cd in winkles even after one year in Cd-free water (Langston & Zhou 1987).

Careful evaluation of metal-binding behaviour therefore gives important clues about the contamination history of the organism. However, the presence of haemocyanin and other interfering ligands, together with exceptionally high basal levels of MT in the digestive gland (presumably because it is involved in regulation of Cu during HCY turnover in this tissue), highlights further problems when making direct measurements of MT (Langston *et al.* 1989). Despite a large influx of Cd into the digestive gland of exposed winkles and association of the metal with MT, polarographic determinations indicate there is little net increase in MT relative to controls in this tissue, which indicates that it is displacing Zn from ZnMT. Thus *Littorina* digestive gland is clearly unreliable for use in environmental impact assessment. In contrast, induction of MT after Cd-exposure can be determined in kidney and to a lesser extent in gill tissues, where the response is proportional to accumulated Cd. Generally the magnitude and timing of this response is of a similar order to that decribed above for mussels. Despite a 3- to 4-fold increase in MT concentrations from basal levels of about 3 mg MT g^{-1} wet wt, the small size of these tissues somewhat reduces their practical value as indicators of water quality.

Metallothionein measurements as an indicator of water quality: field validations

Two examples of the value of MT measurements in fish are provided by analyses of flatfish from the Forth estuary in Scotland, and the North Sea. Metal contamination of the Forth estuary is primarily due to a point-source discharge from the petrochemical, foundry and paint manufacturing industries based at Grangemouth and Falkirk. Although discharges have decreased dramatically over the past decade, the hydrography of the estuary is such that there is a very slow flushing time (*c.* 1 yr) and contaminants tend to be "pumped" upstream from Grangemouth during tidal cycling. Thus metals have accumulated in the sediments at the turbidity maximum of the estuary around Kincardine (Fig. 7). Measurement of hepatic MT mRNA and protein levels in flounder *Platichthys flesus* (a species which appears to display little migration from month to month) at different stations along the estuary, and in fish from the outer Forth, Clyde and Ythan estuaries as reference areas, show that the highest MT levels are in fact found in livers of fish obtained from the area of the turbidity maximum in the Forth

estuary (Sulaiman *et al.* 1991; Sawyer & George, unpublished results). These data demonstrate that the metals are not immobilised in the sediments but are bioavailable and the fish have responded by an increased concentration of hepatic MT.

In another study carried out during the ICES/IOC Bremerhaven workshop in March 1990, dab *Limanda limanda* were sampled at various stations along a cruise transect in the German Bight, from the Elbe/Weser estuaries towards the Dogger Bank, and a number of chemical and biological parameters were measured, including MT, (the MT analyses were carried out by Hylland and Harvey, see collected workshop papers in Stebbing *et al.* 1992). Sediments at coastal stations closest to the estuaries contained generally elevated metal concentrations, a gradient of Zn contamination being evident. Hepatic MT and Zn concentrations in female fish appeared to be overridingly influenced by sexual maturation (tissue Zn mobilisation). Whilst MT levels in male fish livers were more closely correlated with total Cd and Cu levels, they did not reflect the sedimentary metal concentrations but reflected benthic invertebrate metal concentrations. However, branchial MT and concentrations of Cd, Cu and Zn displayed a clear relationship with sedimentary metal concentrations which were elevated at the coastal stations. These data clearly illustrate the necessity for avoiding mature female fish in sampling programmes and also indicate that local dietary influences can confound hepatic data. In this case branchial MT levels appeared to provide a more satisfactory integration of environmental metal bioavailability.

Because of the problems described in earlier sections, validation of MT assays in invertebrates from the field is more complex than for teleosts. Nevertheless the data shown in Table 3 for *Littorina* and *Mytilus* provide evidence that such assays will be of value in

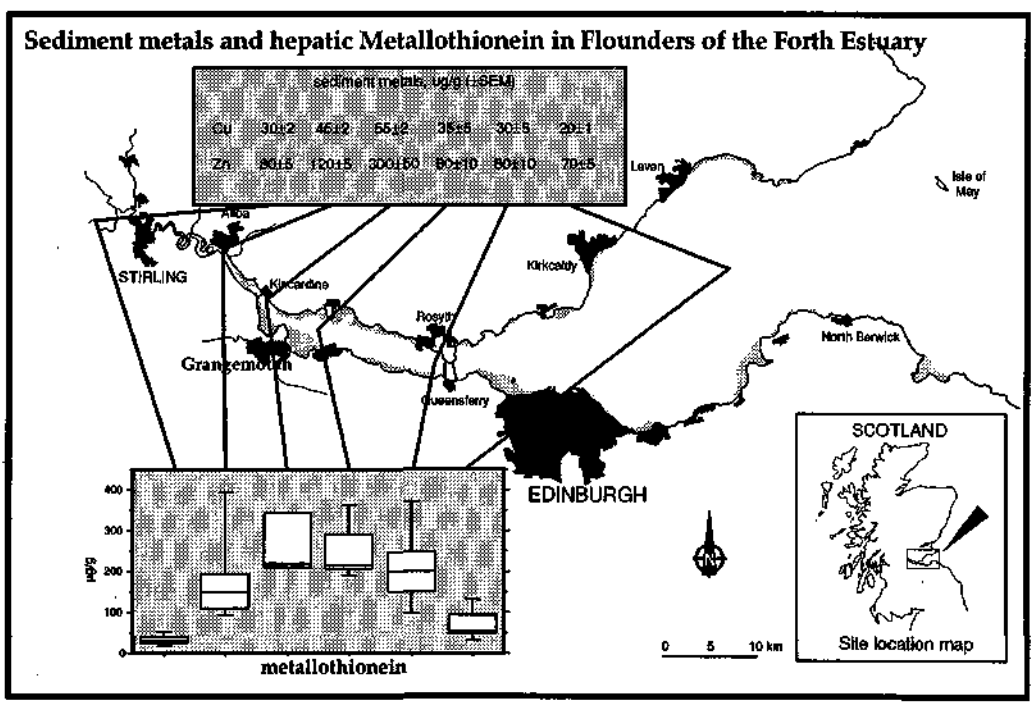

Figure 7. Hepatic metallothionein concentrations (µg g^{-1}; histograms, medians and percentiles) and sedimentary metal concentrations (µg g^{-1} dry wt; mean and S.E.) in flounders (*Platicthys flesus*) caught at various stations along the Forth estuary. (Data from Sulaiman *et al.* 1991 and unpublished results).

demonstrating biological response to metal contamination (Langston & Zhou 1976, and Langston unpublished results; see also Roesijadi 1992 for review of other invertebrates). Furthermore, the availability and sedentary habit of many invertebrates may compensate for their apparent lack of sensitivity compared with fish.

Table 3. *Effect of environmental cadmium concentrations on tissue burdens of cadmium and metallothionein (MT) in indicator organisms from English estuaries.*

Concentrations are expressed as micrograms per gram dry weight (*Fucus* and molluscs) or per litre of sea water, and given as means and standard errors.
*MT determined by polarography.

Sites	Water Cd	*Fucus* Cd	*Mytilus* (whole) Cd	MT*	*Littorina* (gills) Cd	MT*
Contaminated (W. Cumbria)	0.4 - 7.5	16 ± 10	39 ± 30	6.8	36 ± 33	5.7 ± 0.7
Reference (SW England)	< 0.01 - 0.04	1 - 1.8	6 ± 2	2.8 ± 1	2.1 ± 0.8	2.4 ± 1

Conclusions

The advantages of using cellular indices of water quality such as MT stem from their sensitivity, specificity, and potential for indicating and predicting damage to the individual/population.

With minor exceptions (noted earlier) competing systems for metal sequestration are not present in fish, the protein and gene structures of MTs from several species have been elucidated, we have quite a thorough knowledge of endogenous and exogenous controls and variations in MT expression, and reasonably thorough validation has been performed to accept their use in monitoring programmes. In juvenile fish the humoral and temporal influences noted above produce only small (2- to 3-fold) variations in MT levels and these may be reduced or avoided if measurements are not carried out during periods of rapidly changing seasonal variations in water temperature. Relatively large variations are found in sexually maturing fish, especially females, where hormonally-mediated mobilisation of metals occurs and thus it is recommended that determinations should not be carried out during the period of sexual maturation, i.e. only juveniles should be utilised. The response to metal exposure is sufficiently long-lasting to provide reasonable integration of exposure. It is recommended that fish from reference sites collected at the same time should be used as controls in all sampling programmes.

For invertebrates, disadvantages arise from proportionately lower comparative increases in MT levels compared with fish, a lack of knowledge of the basal function of MTs in these phyla, and from the fact that MTs exhibit complex taxon-specific responses to metal contamination, particularly with regard to metal–metal interactions. In addition there is often competition for metals with other binding ligands and sequestration systems. Valuable insights into the role of MT can be gained from chromatographic profiles of cytosolic metals, provided that potentially competing elements, such as Cd, Cu, Hg and Zn, are analysed together to examine the possibility of displacement and the occurrence of other metal-binding pools. For some invertebrates, however, the presence of interferences and competing sequestration systems detracts from their application as monitoring tools. Thus, the digestive gland of *Littorina* contains inherently high levels of MT which reflects the involvement of this tissue in turnover of the Cu-containing pigment haemocyanin, and although MT becomes involved with Cd detoxification there is little or no demonstrable *de novo* synthesis in response to

environmental Cd exposure. Similarly, MT is a metal-regulating constituent of crustaceans such as lobsters and crabs, and unless a specific tissue can be identified for the purpose, variability in protein levels associated with naturally-occurring events such as moulting and growth cycles are likely to mask pollutant-induced responses. In contrast, metal-induced synthesis of MT has been demonstrated in gill and kidney of *Littorina* and in several tissues of *Mytilus*, including the digestive gland. Several isoforms of *Mytilus* MT have been characterised at the level of protein sequencing, and there is also adequate knowledge of competing sequestration systems to enable MT measurements in mussels to be utilised as an indicator of Cd, Hg and possibly Cu contamination, but not of Zn exposure.

A number of observations outlined here therefore support the hypothesis that *direct* analysis of MT induction may be useful in identifying the responses of metal-exposed aquatic organisms, though field validation is still in its infancy and routine application may be some way off. There is also much work to be done in equating MT induction with ecological response. Without doubt, however, studies on the intracellular mechanisms of metal binding, involving MT and other ligands, are an essential component in assessing the toxicological status of metal-exposed organisms. This can only improve our ability to interpret impact at higher levels of organisation, not least by establishing much needed cause–effect relationships.

Dedication

This paper is dedicated to the memory of our longtime colleague, the late Geoff Bryan, whose personality and wit we will always remember along with his tremendous contributions to knowledge in the field.

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