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Chapter 1

ESTABLISHING METAL EXPOSURE – DOSE – RESPONSE RELATIONSHIPS IN MARINE ORGANISMS: ILLUSTRATED WITH A CASE STUDY OF CADMIUM TOXICITY IN TELLINA DELTOIDALIS

A. M. Taylor and W. A. Maher
Ecochemistry, Institute for Applied Ecology, University of Canberra, ACT, Australia

ABSTRACT

The effects of metal contaminants in natural ecosystems are diverse, complex and often unpredictable. Establishing relationships between organism metal exposure, internal dose and associated biological effects is necessary to understand the fate and effects of metals in the environment. The accumulation and sequestration of biologically available metals by aquatic organisms, particularly bivalve molluscs, has led to their use as biomonitors of contamination, as it this portion which is of interest in pollution effects assessments. Biomarker measurements can provide evidence that organisms have been exposed to contaminants at levels that exceed their detoxification and repair capacity, thus establishing the link between contaminant exposure and ecologically relevant effects. This chapter will explore and evaluate approaches to establish relationships between organism exposure, dose and biological response to metals for sediment dwelling bivalve molluscs. Organism dose can be measured by total metal burden and subcellular fractionation of whole tissue used to determine the fraction of the total tissue metal which is in a metabolically available form. Measurements of oxidative stress such as total antioxidant scavenging capacity of cells, lipid peroxidation and lysosomal membrane stability, are good general effects biomarkers for metal exposure. The micronuclei assay, an index of chromosomal damage, can be used to identify genotoxic effects. By identifying relationships between exposure, dose and effects at various levels of biological organisation a better understanding of the mechanisms of organism stress responses to metals in ecological systems should be gained and the predictive capability of ecological risk assessment improved.

INTRODUCTION

Coastal waters are exposed to anthropogenically derived metal contaminants, which are persistent and can be bioaccumulated (Phillips and Rainbow, 1994). Since the early 1970's there has been concern over the deleterious effects of contaminant loads to aquatic ecosystems (Luoma, 1996). Contaminants have also been associated with the decline in marine mammals, fish and bivalves (Luoma, 1996). Hence, there is a need to establish and monitor the links between contaminants in aquatic environments, their bioaccumulation in aquatic organisms and any consequent effects that occur, in order to protect valuable living natural resources and human health.

Monitoring gross effects of environmental pollution has traditionally been carried out by chemical analysis of water, sediments and biota. Biological assessment has included observation and quantification of ecological assemblages and routine assessment of contaminant toxicity, typically using LC_{50} lethality tests (Chapman, 1995; Taylor, 1996). Aquatic organisms have long been known to accumulate significant quantities of metals in their tissues. The degree to which organisms take up and retain metals varies markedly between phyla, and may also differ significantly between individual species within the different phyla. These variations are thought to be a reflection of different evolutionary strategies for detoxifying metals (Phillips and Rainbow, 1994). The accumulation and sequestration of biologically available metals by aquatic organisms has led to their use as biomonitors of contamination, as it this portion which is of interest in pollution effects assessments. They are also considered to provide a time-integrated measurement of contamination, reflecting the average of short term temporal fluctuations in contaminant abundance in the environment (Phillips, 1990). Molluscs, particularly bivalves have been extensively used and studied (Phillips, 1990). Molluscs are effective models for environmental toxicological studies because they are ubiquitous, have highly conserved control and regulatory pathways that are often homologous to vertebrate systems, and are extremely sensitive to anthropogenic inputs (Rittschof and McClelland-Green, 2005). Metals and organic contaminants released into aquatic systems bind to particles and accumulate in estuarine sediments, which become the main repositories and therefore potential sources of contaminants (Byrne and O'Halloran, 2001). Burrowing and feeding by benthic organisms resuspends contaminants, increasing their biological availability both to the benthic fauna and flora, and to the higher order organisms which feed on them.

Sediment toxicity tests, using sediment dwelling bivalves, aid in determining the potential for sediment toxicants to cause adverse effects to the sediment infauna and the potential for these effects to be transferred up food chains.

The history of pollution control and monitoring has been one of slowly evolving standards and techniques, with assessment of the potential effects of metal contaminants on the health of aquatic organisms being given progressively higher priority by many nations (Taylor, 1996). Estimating the extent of biological exposure to metal contaminants in aquatic environments is subject to uncertainties, as is attributing, let alone predicting, the adverse health or ecological effects that result from the exposure. The presence of a contaminant in a segment of an aquatic environment does not; by itself indicate injurious effects, connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects (Widdows and Donkin, 1992).

Exposure to metals is difficult to assess because of the range of exposure routes, (water, sediments and food), differences in the biological availability of metals associated with the different environmental media, and individual and species differences in the metabolic pathways used to sequester or eliminate metals depending on their value or toxic potential. All these processes affect the amount of metal which enters organisms and reaches critical molecular targets. Seasonal changes, feeding habits, reproductive status, or metabolic activity can modify the nature and extent of exposure. Effects of exposure to metals cannot be readily quantified by measuring body burdens because the relationship between body burden and toxic response is complex and not fully understood (McCarthy, 1990). The presence of complex mixtures of metals and other contaminants creates further uncertainty. There is relatively little known about the toxicity of the majority of the tens of thousands of chemicals released to the environment and almost no information on the action of well characterised chemicals when they are in complex mixtures (Phillips and Rainbow, 1994).

It is now recognised that chronic exposure to sub lethal levels of toxicants, with the risk of their accumulation over time and magnification through the food chain, can have severe affects on the survival and reproductive capacity of individual organisms and on species and community diversity. The assessment of metal contamination has therefore broadened to include chronic exposure to sub lethal levels of metals and their effects at physiological, cellular and molecular levels (Huggett *et al.*, 1992).

This approach uses biological markers of exposure and effect that can be defined as measurements of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response (McCarthy and Shugart, 1990). The physiological, cellular and molecular emphasis in aquatic toxicology is consistent with a biomarker approach, with such studies being likely to lead to detection of effects prior to changes at the organism and higher levels of organisation. Since these changes are early and sensitive, they may serve as markers of both exposure and effects (Hinton, 1994).

Exposure – Dose – Response Framework

An understanding of the fate and effects of metals in aquatic environments requires that the causal relationships between chemical exposure, internal dose and associated biological effects be established (Widdows and Donkin, 1992) (Figure 1).

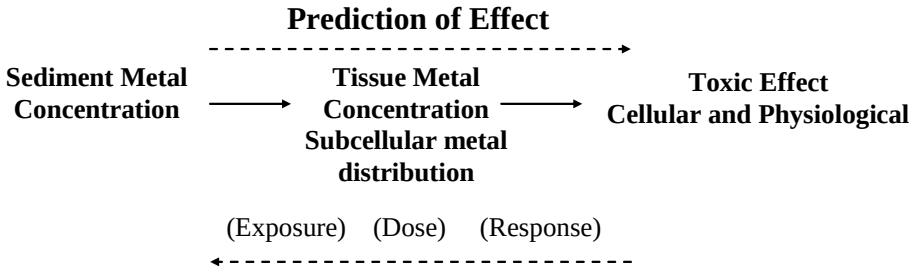

Figure 1. Ecotoxicological framework enabling the prediction and diagnosis of effects resulting from exposure to metals (modified from Widdows & Donkin (1992)).

Traditionally monitoring programs have measured metal contaminant concentrations in the physical environment, water, sediments and suspended matter. These measurements provide only momentary information giving no information on concentrations in and effects on aquatic organisms (Bervoets and Blust, 2003). The diversity and complexity of metal effects in natural ecosystems means they are and often unpredictable (Depledge *et al.*, 1995). Internal dose can differ while the external dose does not as a result of varying exposure routes. If uptake is through food, for example, different feeding habits greatly influence uptake (Chapman, 1995). An ecotoxicological approach enables the differentiation between contamination defined in terms of chemicals, as an artificial increase above back ground levels; from contamination, which implies harm to living resources or risks to human health (Preston, 1989).

Biomarkers help bridge the gap between exposure and effects, providing necessary information to evaluate subtle long term effects. Biomarkers comprise biochemical, physiological and histological endpoints and may be categorised as either biomarkers of exposure, which reflect an organism's attempt to compensate for or tolerate, stressors in their environment; or biomarkers of effect, which reflect deleterious sub lethal organism effects which may be causally linked to one of the four levels of biological organisation; biochemical and cellular; whole organism; population; and community (Chapman, 1995). If an organism that is a bioaccumulator (reaches equilibrium with its environment by detoxification and storage) shows adverse effects and a contaminant in its tissues shows a dose-response relationship then this is a strong indication of cause and effect (Chapman, 1995). The term "biomarker" has been defined by Koeman *et al.* (1993) as a change in a biological response that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals. The term 'biological response' can range from molecular, biochemical, and physiological responses to behavioural responses through to species abundance and composition

External Exposure

External exposure is traditionally characterised through the chemical analysis of discrete water and sediment samples (Salazar and Salazar, 2003). This can provide information on the environmental compartments into which the contaminants are distributed, at what concentrations and the chemical form. Metals are distributed among solution, suspended particles, sediments, pore waters and living and non-living food sources within all of these. Each species' exposure to metals is determined by how the species samples this complex mix and by the accessibility of metals within each compartment of the mix. In biological terms, consideration of a single environmental compartment is artificial and inhibits understanding of bioavailability processes (Luoma, 1996). Metal bioavailability from water and sediments is influenced by physical and chemical factors acting outside the organism, which affect most biota in the same way. These include metal speciation, i.e. complexation of metal ions by inorganic and organic chelating agents, adsorption to particulate matter, precipitation and binding within insoluble matrices, sulphides, and sediment grain size and mineralogy (Borgmann, 2000; Campbell, 1995; Depledge and Rainbow, 1990). Environmental conditions such as pH, temperature, salinity and redox potential, that can cause a shift in the metal

species from one form to another can affect both the bioavailability of the metal and the uptake pathways (Roesijadi and Robinson, 1994; Widdows and Donkin, 1992).

Biological factors acting within or on the surface of organisms, such as exchange surfaces, growth, biochemical composition, reproductive condition, metabolism and elimination can also affect metal bioavailability and can be very species specific (Campbell, 1995; Widdows and Donkin, 1992).

Food and ingested organic particulate matter have been shown to be important metal exposure routes for both molluscs and fish (Handy, 1996; Luoma, 1996; Phillips and Rainbow, 1994). Metal uptake via the gut depends on similar chemical and physical factors to those operating outside the organism, as well as feeding rate, gut transit times, and digestion efficiency (Depledge and Rainbow, 1990). The total metal concentration of the food ingested is not necessarily itself a measure of metal availability, its bioavailability will be subject to the physiochemical conditions in the gut (Depledge and Rainbow, 1990). Although there is limited evidence of biomagnification of metal concentrations along food chains, amplification of a metal concentration can occur if food intake is high, the metal is assimilated efficiently and is not significantly excreted (Rainbow, 1990). Selenium is a notable exception to this. The high efficiency of selenium transfer from one trophic level to the next means biomagnification of selenium between trophic levels occurs more often than not (Luoma and Rainbow, 2008). Assimilation of ingested trace elements differs with food type, and availability from solution differs with geochemical conditions. The two pathways, are additive, therefore neither can be excluded from consideration in bioaccumulation studies (Luoma, 1996).

Sediment dwelling organisms differ in a number of ways from pelagic ones in terms of their exposure to contaminants. Sediment dwelling species are exposed continuously when in contaminated sediments. Burrowing invertebrates may be bathed in the interstitial water of the sediment, or by their own irrigation currents, interacting to varying degrees with the interstitial water. The interstitial water contains dissolved metals in equilibria with sediment-associated metals. Interstitial waters often have redox and other physiochemical conditions very different from those of overlying oxygenated waters (Rainbow, 1990). Pelagic organisms may only be exposed intermittently when foraging in sediments.

Ultimately, it is where and how an organism lives, that dictates the way in which it is most likely to be affected by contamination (Chapman, 1995). Identifying chemical exposure pathways is becoming increasingly important as it is clear that dietary exposure may be controlling toxicity under many exposure conditions, this is particularly so for bivalves where particulate food in sediment can make a substantial contribution to toxicity (Salazar and Salazar, 2003).

Internal Dose

Dose is the concentration of a metal that appears in an organism's tissues and is the starting point for adverse effects (Luoma, 1996). Bioavailability determines dose; it is the relationship between metal concentrations in the environment and uptake into tissues from all sources. Bioaccumulation may not be related to total metal exposure concentrations; nor is it possible to measure one chemical fraction that is universally and exclusively the bioavailable fraction of any chemical (Luoma, 1996).

Once metals have been taken up by biota, systems for transport, storage and detoxification will determine their ultimate fate. The significance of different organisms' tissue concentrations is related to metal accumulation strategies. These accumulation strategies fall along a gradient from the accumulation of all metal taken up, to homeostasis of the body metal concentration at an approximately constant level by balancing metal uptake with excretion; intermediate strategies include degrees of net accumulation when uptake exceeds excretion, and partial homeostasis (Rainbow, 1993). Homeostasis as an accumulation strategy seems to be restricted to the essential metals, zinc and copper, however, if the internal metal dose exceeds the detoxification and excretion capacity of the organism the extra accumulated metal remains biologically available to play a toxic role (Rainbow, 1993). Chapman (1995) suggests that depending on the organism and the contaminant there are two basic accumulation patterns, which are related to contaminant toxicity. The first, are non-regulated metals such as cadmium, lead and mercury which are not essential for growth and may be more predictable from body burden data than from concentrations in external media, (water and sediment). The second, regulated metals such as the essential elements zinc, copper and selenium may be less predictable from internal dose measurements. Internal dose can be characterised by chemically analysing the different tissues of organisms from different environmental compartments from the areas of concern e.g. sediment dwellers and water column. The tissue distribution of metals in fish indicates that they are preferentially accumulated in liver, kidney, spleen and gills (Phillips and Rainbow, 1994). Molluscs have been found to accumulate metals in the gills, liver, gut and in mussels also the byssal gland/threads (Widdows and Donkin, 1992).

Metal Metabolism

The gills of fish and molluscs are the most important tissue for the uptake of the water soluble form of metals and this is mainly a passive-transport process (Livingstone and Pipe, 1992). The digestive gland is the major site for particulate-bound metal uptake via endocytosis, an active-transport mechanism requiring adenosine triphosphate; the endocytotic vesicles subsequently fuse with primary lysosomes (Livingstone and Pipe, 1992).

Accumulation may occur as a result of physiological handling mechanisms in the case of essential metals, where the metal is delivered to tissues requiring the metal for some metabolic function. Metals may be transported to tissues in the haemolymph either dissolved or bound to proteins. Protein binding may be non-specific involving the dominant blood protein haemocyanin, or a specific metal transport protein such as transferrin-like proteins involved in the transport of iron. These proteins will also in addition to iron bind copper, zinc, manganese and plutonium (Depledge and Rainbow, 1990). How metals are transferred from the haemolymph to specific tissues remains obscure. Passive transfer along a cascade of ligands in cell membranes and cytoplasm exhibiting increasing metal affinity is a possibility, but active processes cannot be excluded (Depledge and Rainbow, 1990; Rainbow and Dallinger, 1993). On entering cells, metals are primarily complexed by thiol-containing molecules such as amino acids, glutathione and in particular the metal binding detoxification proteins, metallothioneins. In addition, part of the metal may be compartmentalised in the lysosomal vacuolar system, or trapped in different types of specialised inorganic granules (Livingstone and Pipe, 1992).

Subcellular Tissue Metal Distribution

Physiological effects and toxicity of metals strongly depend on their intracellular localisation and binding to organelles and ligands (Sokolova *et al.*, 2005a). Cadmium, for example, affects the bioenergetics of oyster mitochondria in vitro and in vivo at low concentrations, leading to reduced coupling and impaired ability to produce ATP (Sokolova, 2004). Cadmium accumulation in mitochondria may therefore result in serious disturbances of tissue energy balance and eventually cell death (Sokolova, 2004; Sokolova *et al.*, 2004; Sokolova *et al.*, 2005a).

Mitochondria

Mitochondria are small intracellular organelles which are responsible for energy production and cellular respiration. The provision of energy through the coupling of oxidation to energy transfer via the phosphorylation of adenosine diphosphate (ADP) occurs in the mitochondrial electron transport chain (Winston and Di Giulio, 1991). Adenosine diphosphate respiration in the mitochondria uses pyruvate, formed by glycolysis from glucose, the carrier molecules nicotinamide adenine dinucleotide (NAD+), flavin adenine dinucleotide (FAD+) and oxygen to produce citric acid which is then broken down releasing hydrogen ions and carbon molecules. The carbon molecules are used to make carbon dioxide and the hydrogen ions are picked up by NAD and FAD and recycled. The hydrogen ions produced by the cycle are used to drive pumps that produce adenosine triphosphate (ATP). The release of energy from ATP is achieved via oxygen reduction metabolism, where ATP loses one of its phosphate groups and is converted back to adenosine diphosphate (ADP). The cycling of these two molecules releases energy which is used for cellular functions such as movement, transport, entry and exit of products and cell division (Winston and Di Giulio, 1991). Exposure of oysters *Crassostrea virginica* to cadmium has been shown to result in considerable cadmium accumulation in the mitochondria and a significant impairment of the ATP production capacity and a strong inhibition of the ADP-stimulated respiration (Sokolova, 2004; Sokolova *et al.*, 2005b).

Microsomes

Microsomes are small vesicles found in the endoplasmic reticulum which contain the cell's cytochrome P450 enzymes involved in oxidative metabolism. Microsomal electron transport of aquatic organisms have been studied in detail with respect to oxyradical production. Possible loci of electron transfer to oxygen to produce O_2^- in microsomes are the autoxidation of reduced oxycytochrome P450 and/or autoxidation of flavoprotein reductases (Winston and Di Giulio, 1991). Metals associated with microsomes may be indicative of toxicity as they can induce increased oxidative activity. The cytochrome P4501A hemoprotein located in the microsomes is responsible for oxidation reactions related to xenobiotic biotransformations (Tom *et al.*, 2002). Increased microsomal cytochrome P450 xenobiotic biotransformation activity can also initiate the peroxidative chain in the lipid membranes producing free radicals which can damage microsomal membranes and impair the protein synthesis and transport processes of the endoplasmic reticulum where they are located (Bonneris *et al.*, 2005; Jorgensen *et al.*, 1998; Ribera *et al.*, 1989).

Lysosomes

Metal accumulation in lysosomes of the digestive gland and kidney of mussels has been described (Viarengo, 1989). Tertiary lysosomes accumulate undegradable end-products of lipid peroxidation, oxidised lipid and protein polymers known as lipofuschin. In kidneys, lipofuschin granules have been shown to bind metals in two ways; weakly bound by acidic groups in the outer region of the granules, and so able to dissociate and be in equilibrium with cations in the cytoplasm; and sterically 'trapped' in a non-toxic form in the centre of the developing granules (George, 1983a). Active excretion of these residual bodies by exocytosis enables metal elimination. A second method of elimination has been indicated for copper in the digestive gland, involving the accumulation of copper rich thionein-like proteins in lysosomes, followed by elimination of residual bodies (Carpene, 1993; Viarengo, 1989). Cadmium is not removed via either of these biochemical pathways in mussels and is consequently present in tissues for considerably longer once it is taken up (Viarengo, 1989).

Heat stable proteins – metallothioneins

Metallothioneins are heat stable, low molecular weight, soluble (generally cytosolic), thiol-rich (high cysteine content) proteins with a high metal content (Roesijadi, 1992; Viarengo, 1989). Induction of metallothioneins by metals is specific and metal dependant (Roesijadi, 1996). While their primary role in marine organisms is the homeostasis of the essential metals zinc and copper (Cosson *et al.*, 1991), they can also bind non-essential metals such as cadmium and mercury (Livingstone and Pipe, 1992). There is increasing evidence that metallothioneins are turned over rapidly in cells. Turnover involves lysosomal breakdown and associated production of residual bodies such as metal rich granules which may be stored or excreted. Whether metals handled in this way are available for metabolic utilisation remains unknown (Depledge and Rainbow, 1990).

Inorganic granules and vesicles

A variety of marine molluscs, both bivalves and gastropods, have been shown to sequester metals in inorganic granules as a means of detoxification (Carpene, 1993; Taylor, 1998; Viarengo, 1989). There are two major types identified, copper-sulphur-containing granules and calcium containing granules (Viarengo, 1989). Cells lining the digestive tract of invertebrates (e.g. midgut diverticula, hepatopancreas or caeca) may release metals detoxified in granules into the gut lumen when the epithelial cells complete their cell cycle. The kidney cells of bivalve molluscs also have the ability to excrete metal rich granules (Rainbow, 1990). Metals in granules are inert and not available biologically therefore total metal burden measurements while they may give high concentrations do not give any information about adverse biological effects.

Whatever the handling mechanisms it is clear that metal ions in excess of metabolic requirements are potentially toxic and must be removed from the vicinity of important biological molecules by excretion from the specific tissue. The metal may then be eliminated from the organism or biotransformed prior to storage in specific tissues in inert non-toxic forms.

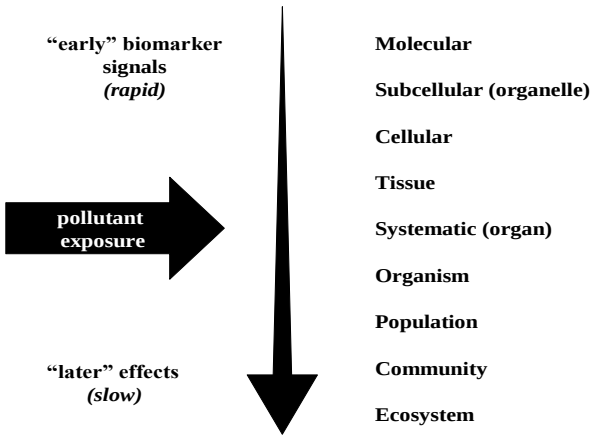

Figure 2. Sequential order of biological responses to toxic stress (modified from (Bayne et al., 1985)).

Biological Response – Biomarkers

Biological response to external metal exposure and consequent internal dose can be assessed at an organism or sub-organism level using a range of biochemical, physiological and histological biomarkers to estimate either internal exposure to toxicants or resultant effects. Metals exert their influence by interaction with a range of biochemical receptors and therefore measurement of these interactions can provide early indication of internal exposure and effects. Toxic effects of metals are influenced by bioavailability, routes of exposure and the level and time of exposure (Koeman *et al.*, 1993). The initial reactions of organisms to toxic compounds is at the molecular and cellular levels of target organs and tissues. Sub lethal compensatory and repair responses may prevent damage during prolonged exposure until cell regulatory systems fail. The effectiveness of specific biomarkers relies on the ability to link them to higher level effects (Figure 2) and thus show that they provide early warning of adverse effects, not just internal exposure (Chapman, 1995).

Biomarker measurements can provide information which cannot be obtained through measurements of contaminants in the environmental media or tissue concentrations. They have the potential to provide evidence that organisms have been exposed to contaminants at levels that exceed their detoxification and repair capacity. This can provide evidence for establishing the link between toxicant exposure and ecologically relevant effects (Koeman *et al.*, 1993). The bioaccumulation of certain persistent environmental chemicals in animal tissues may be seen as a biomarker of exposure to these chemicals, however, these body burdens are not considered to be biomarkers or bioindicators since they do not provide information on deviations related to 'health' (van der Oost *et al.*, 2003). Biomarkers discussed in this chapter will include biological, biochemical, physiological and histological parameters measured inside an organism or its products.

Biomarkers of exposure

Biomarkers of exposure may represent either general or specific responses and have the advantage of quantifying only biologically available toxicants (Mayer *et al.*, 1992). General markers include those that are non-specific for a compound or chemical class but indicate that

exposure to some exogenous chemical has occurred. Changes in some general biomarkers may be caused by environmental variables not related to toxic exposure, such as temperature increases stimulating the production of stress proteins (Stegeman *et al.*, 1993). Specific biomarkers of exposure may be used to demonstrate exposure response to a particular class of compound (Table 1). Biomarkers of exposure include the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between the xenobiotic agent and a target molecule or cell that is measured in a compartment within an organism (van der Oost *et al.*, 2003).

Biomarkers of Toxic Effect

As with biomarkers of exposure biomarkers of effect can be categorised as general or specific responses. While these biomarkers may also demonstrate exposure they can be used to further reveal a toxic effect resulting from that exposure. General biomarkers of toxic effect include measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognised as associated with an established or possible health impairment or disease (van der Oost *et al.*, 2003). These include indicators of cellular and genetic damage such as increase of antioxidant enzyme activity, chromosomal aberrations, and histopathological lesions (Table 1). Chemical or class specific indicators of toxic effect can be used when the mode of action of the chemical is known, such as inhibition of brain acetylcholinesterase by organophosphates and carbamates (Mayer *et al.*, 1992).

The division of biomarkers into categories of exposure or effect is to some extent arbitrary since they are divided according to how they are used rather than by an inherent dichotomy (Suter, 1993). The responses of biomarkers can be seen as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects (van der Oost *et al.*, 2003). Biomarkers of exposure can be used to confirm and assess the exposure of individuals or populations to a particular substance group (metals, hydrocarbons, pesticides etc.), providing a link between external exposure and internal dose. Biomarkers of effect can be used to document either preclinical alterations or adverse health effects due to external exposure and internal adsorption of a toxicant. Biomarkers of susceptibility have also been defined as a separate category by the (WHO, 1993). These help to elucidate variations in the degree of responses to toxicant exposure observed between different individuals and include genetic factors and changes in the receptors which alter the susceptibility of an organism to a specific toxicant exposure.

Biomarker Selection

As with other aspects of study design, biomarker selection depends on the question to be answered. Biological responses and therefore biomarker choice also depends on the mode of action of the chemical of interest and the level of biological organisation being examined.

Table 1. Common biomarkers of exposure and effect, and the compounds they respond to.

Category	Biomarker	Toxicant	Response	Examples of Use
Enzymatic / Biochemical				
Phase 1	Cytochrome P450	PAH, PCB, pesticides	+ / -	Arun *et al.*, 2006; Kim *et al.*, 2004a; Kim *et al.*, 2004b; Peters *et al.*, 1998; Shaw *et al.*, 2004; Stegeman and Hahn, 1994; Watson, 2004
	Ethoxyresorufin O-deethylase	PAH, PCB, pesticides	+	Fossi *et al.*, 2004; Fouchecourt *et al.*, 1999; Kirby *et al.*, 2004; Miller *et al.*, 2004; Whyte *et al.*, 2000
	Aryl hydrocarbon hydroxylase	PAH, PCB, pesticides	+	Bogovski *et al.*, 1998
Phase 2	Glutathione S-transferase	metals, PAH, PCB, pesticides	+ / -	Hoarau *et al.*, 2004; Leaver and George, 1998; Lee, 1988
Antioxidant				
Enzymes	Glutathione peroxidase & reductase	metals, PAH, PCB	+ / -	Cossu *et al.*, 2000; de Almeida *et al.*, 2004; Maity *et al.*, 2008
	Catalase / Superoxide dismutase	metals, PAH, PCB	+ / -	Company *et al.*, 2004; Pedrajas *et al.*, 1995; van der Oost *et al.*, 2003
Cofactors	Total Glutathione GSH+2GSSG	metals, PAH, PCB	+ / -	Canesi *et al.*, 1999; Frenzilli *et al.*, 2004; Regoli *et al.*, 2004
	Reduced :oxidised glutathione GSH:GSSG	metals, PAH, PCB	-	Cossu *et al.*, 2000; Hoffman, 2002; Maity *et al.*, 2008; Tandon *et al.*, 2003
Activity	Total Antioxidant Capacity	metals, PAH, PCB	-	Gorinstein *et al.*, 2005; Moncheva *et al.*, 2004
	Total Oxygen Scavenging Capacity	metals, PAH, PCB	-	Camus *et al.*, 2004; Regoli, 2000; Regoli *et al.*, 2002; Regoli and Winston, 1999
Damage	Lipid Peroxidation	metals, PAH, PCB	+	Charissou *et al.*, 2004; Domouhtsidou and Dimitriadis, 2001
Haematological	Aspartate & alanine aminotransferases	metals, Cd, Cu Hg	+	Benson *et al.*, 1988; Beyer *et al.*, 1996; Blasco and Puppo, 1999; de Aguiar *et al.*, 2004
	δ-aminolevulinic acid dehdratase	metals Pb, Zn	+	Burden *et al.*, 1998; Campana *et al.*, 2003; Perottoni *et al.*, 2005; Rodriguez *et al.*, 1989
Proteins	Heat Shock Proteins	heat, metals	+	Cruz-Rodriguez and Chu, 2002; Feng *et al.*, 2003; Urani *et al.*, 2003
	Metallothioneins	metals	+	Amiard *et al.*, 2006; Lecoeur *et al.*, 2004; Marie *et al.*, 2006
Neurotoxic	Acetylcholinesterase	organophosphate & carbamate pesticides	-	Corsi *et al.*, 2004; Dellali *et al.*, 2001; Lionetto *et al.*, 2003; Pfeifer *et al.*, 2005; Rickwood and Galloway, 2004
Genotoxic	Micronuclei frequency	metals, PAH, PCB	+	Bolognesi *et al.*, 2004; Burgeot *et al.*, 1996; Koukouzika and Dimitriadis, 2008; Scarpato *et al.*, 1990; Williams and Metcalfe, 1992
	DNA strand breaks	metals, PAH, PCB	+	Akcha *et al.*, 2004
	DNA adducts	metals, PAH, PCB	+	Fouchecourt *et al.*, 1999; Kurelec *et al.*, 1990; Pisoni *et al.*, 2004
Reproductive	Vitellogenin	dioxin, endosulphan, pesticides, metals	+	Depledge and Billinghurst, 1999; Funkenstein *et al.*, 2004; Riffeser and Hock, 2002
Cellular	Lysosomal stability	metals	-	Castro *et al.*, 2004; Domouhtsidou and Dimitriadis, 2001; Moore *et al.*, 2006
Physiological	Histopathology	All xenobiotics	-	Au, 2004; Farley, 1988; Sunila, 1988; Wedderburn *et al.*, 2000; Zorita *et al.*, 2006
	Cellular Energy Allocation	All xenobiotics,	-	Cherkasov *et al.*, 2006; Smolders *et al.*, 2004
	Scope for Growth	All xenobiotics	-	Burt *et al.*, 2007; Goldberg and Bertine, 2000; Smolders *et al.*, 2004; Wo *et al.*, 1999
	Condition Index	All xenobiotics	-	Leung and Furness, 2001a; Lundebye *et al.*, 1997

It is necessary to determine whether the study requires biomarkers of exposure to a chemical or group of chemicals, a biomarker of toxic effect, or whether a combination of these is preferred. In most cases, the objectives of studies require or benefit from analysis of multiple biomarkers at several levels of organisation. A combination of sensitive early changes (e.g. molecular) and later changes (e.g. histological) may be particularly useful (Stegeman *et al.*, 1993). The selection of biomarker also depends on the sentinel species used and techniques selected may require laboratory verification before application to field studies (Stegeman *et al.*, 1993).

The following seven criteria for the selection and development of useful biomarkers are suggested based on ideas formulated by (Mayer *et al.*, 1992; Stegeman *et al.*, 1992; van der Oost *et al.*, 2003).

1. The assay to quantify the biomarker should be reliable (with quality assurance), relatively cheap and easy to perform, allowing quantification of multiple individuals.
2. The biomarker response should be sensitive to pollutant exposure and/or effects in order to serve as an early warning parameter.
3. Baseline data of the biomarker should be well defined in order to distinguish between natural variability (noise) and contaminant induced stress (signal).
4. The underlying mechanism of the relationships between biomarker response and pollution exposure in a dose or time-dependent manner should be established so the magnitude of the exposure or effect can be determined.
5. The impacts of confounding factors (i.e., season, gender, weight, and handling) to the biomarker response should be understood and within acceptable limits.
6. The measure must have biological significance. Only biomarkers that can be linked to important biological processes and for which changes can be interpreted should be used.
7. Ideally a suite of interrelated biomarkers based on a cascade of effects should be selected to ensure robustness.

Molecular / Biochemical Biomarkers

Changes at the biochemical level offer specific advantages as biomarkers for two major reasons:

1. Biochemical or molecular alterations are usually the first detectable, quantifiable responses to environmental change, including changes in the chemical environment. Further, biochemical alterations can serve as markers of both exposure and effect. A chemically induced change in biochemical systems, by definition, represents an effect of the chemical (Stegeman *et al.*, 1993).
2. Biochemical system alterations are often more sensitive indicators than effects at higher levels of biological organisation as they usually precede higher order effects and may therefore indicate whether additional effects are likely to occur. Additionally these alterations are a more rapid measurable response to toxicity than the higher order effects which may follow, therefore, allowing remedial intervention to be implemented earlier in the process.

Biochemical systems which are involved in specific responses to toxic chemicals include a number of enzymes and proteins. Many responses are adaptive, but the same systems may be involved in reactions leading to toxic effects (Stegeman *et al.*, 1992). The main systems are:

Biotransformation enzymes

Alterations in levels or activity of biotransformation enzymes are generally the most sensitive effect biomarkers (van der Oost *et al.*, 2003). Their activity may be enhanced or inhibited in response to contaminant exposure.

Phase I enzymes

The initial phase of metabolism of organic compounds involves the addition of polar groups to the molecule through, oxidative, reductive or hydrolytic reactions (Buhler and Williams, 1988). Oxidative reactions are the most important category of phase I reactions (Buhler and Williams, 1988). They are catalysed primarily by cytochrome P450 dependant mixed function oxidase enzymes (MFOs; also referred to as monooxygenases) (van der Oost *et al.*, 2003). These enzymes comprise a large and expanding family of heme proteins which are membrane-bound and predominantly are located in the endoplasmic reticulum of the liver (Stegeman *et al.*, 1992). They metabolise a wide variety of substrates including endogenous molecules (e.g. fatty acids, prostaglandins, steroids) and xenobiotics (e.g. hydrocarbons, pesticides, drugs) (Snyder, 2000).

The toxicity of organic chemicals can be significantly altered by structural transformation. By affecting chemical structures cytochrome P450 enzymes may render a compound non-toxic or may drastically increase its toxicity (Stegeman *et al.*, 1992). The levels of some forms of cytochrome P450 can be increased in response to an organism's exposure to many types of chemicals and as a result the rate of chemical transformation catalysed by these enzymes is altered. Cytochrome P450 can also serve as a highly sensitive indicator of an organism's toxic burden, or the extent to which it has been exposed to chemical inducers in the environment (Stegeman *et al.*, 1992). Ethoxyresorufin O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) are two catalytic probes commonly used for determining the inductive response of the cytochrome P450 system to chemical exposure (van der Oost *et al.*, 2003). Increases in both AHH and EROD catalytic enzyme activity have been measured in many species of fish liver after exposure to organic pollutants and are considered to be sensitive biomarkers of organic chemical exposure which may also precede effects at various levels of biological organisation (Whyte *et al.*, 2000). The phase I biotransformation enzymes, particularly cytochrome P450 are the most sensitive fish biomarkers known at present for indicating exposure to organic compounds (van der Oost *et al.*, 2003).

Phase II enzymes

Phase II (conjugating) enzymes aid in the detoxification and excretion of foreign compounds, including reactive metabolites formed by the phase I cytochrome P450 monooxygenase system, by linking them to various water soluble endogenous compounds present in the cell in high concentrations. These reactions generally result in further increases

in solubility and elimination rates, and reduced toxicity of the compound (Buhler and Williams, 1988). The most widely studied and probably the most important of the phase II enzymes are glutathione S-transferases (GST), UDP-glucuronosyltransferases (UDPGT), and sulphotransferases (ST), which link metabolites to glutathione, glucuronic acid, and sulphate, respectively (Buhler and Williams, 1988; Stegeman *et al.*, 1992). Some xenobiotic compounds possess the required functional groups (e.g. COOH, -OH or $-NH_2$) for direct metabolism by conjugative phase II enzymes, while others are metabolised by an integrated process involving prior action of phase I enzymes (George, 1994). The major pathway for electrophilic compounds and metabolites is conjugation with GST while the major route for nucleophilic compounds is glucuronic acid (GA) conjugation (George, 1994).

Phase I and phase II biotransformation reactions usually work together in a sequential way to convert xenobiotics to more easily excreted metabolites. The different phase I and II enzymes may also compete with each other for the parent xenobiotic or its metabolites. Xenobiotics, therefore, generally undergo several types of biotransformation reactions simultaneously, often resulting in the formation of a large number of metabolites or conjugates (Buhler and Williams, 1988). Compared to phase I enzymes the induction reaction of phase II enzymes is generally less pronounced (George, 1994). They may be more useful in an integrated biomarker approach using a combination of biomarkers such as the biotransformation index (BTI, reflecting the ratio between phase I and II activities), as this reflects a balance between bioactivation and detoxification (van der Oost *et al.*, 1998).

Oxidant and Antioxidant Responses

All aerobic life has the potential to experience oxidative stress, when antioxidant defences are overwhelmed by activated oxygen species, also referred to as oxygen free-radicals, reactive oxygen species (ROS), reactive oxygen intermediates (ROIs) or oxyradicals (Winston, 1991). Many environmental contaminants or their metabolites have been shown to enhance the production of reactive oxygen species within cells (Andersen, 1994). There are many endogenous sources of oxyradical production, the MFO system, for example, in addition to metabolically activating/detoxifying polycyclic hydrocarbons and other xenobiotics, is also involved in oxyradical generation (Andersen, 1994), but from an environmental biomarker perspective the ability of a number of exogenous compounds, particularly metals, to enhance intracellular oxyradical production through the process of redox cycling is of particular interest (Stegeman *et al.*, 1992; van der Oost *et al.*, 2003). Oxidant-mediated effects with the potential for use as biomarkers include either adaptive responses through increased activities of antioxidant enzymes and concentrations of non-enzymatic compounds, or evidence of oxidant-mediated toxicity such as oxidation of proteins, lipids and nucleic acids, as well as perturbed tissue redox status (Stegeman *et al.*, 1992; van der Oost *et al.*, 2003; Winston and Di Giulio, 1991).

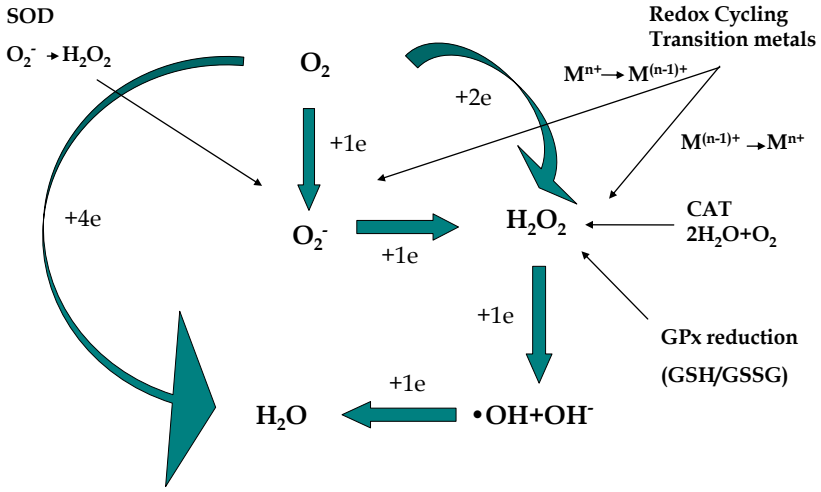

Figure 3. Oxygen reduction metabolism showing the 4 step electron-transfer reactions in the conversion of oxygen to water during energy transfer and the major enzymatic reduction mechanisms (modified from (Winston and Di Giulio, 1991)).

Oxygen reduction metabolism]

Molecular oxygen is required by all aerobic organisms for the provision of energy through the coupling of oxidation to energy transfer via the phosphorylation of adenosine diphosphate (ADP). In aquatic organisms this process is managed by the mitochondrial electron transport system; in which O_2 undergoes a concerted four electron reduction to water (Figure 3) (Winston and Di Giulio, 1991). The first reaction is a one electron reduction of O_2 to superoxide (O_2^-). Superoxide anions are then converted to hydrogen peroxide (H_2O_2) by a further one electron reduction. Superoxide and to a large extent hydrogen peroxide are highly reactive and toxic ROIs; H_2O_2 in conjunction with myeloperoxidase and a halide, forms the basis of a potent antibacterial system (Andersen, 1994). The reduction of H_2O_2 to the hydroxyl radical ($^{\cdot}OH + OH^-$) and then to H_2O is achieved by the addition of a further electron at each step (Figure 3).

Redox cycling

The hydroxyl radical is among the most potent oxidants known, capable of reacting kinetically indiscriminately with virtually all organic chemicals, including critical cellular macromolecules, possibly leading to protein degradation and enzyme inactivation, lipid peroxidation, DNA damage and ultimately cell death (Winston and Di Giulio, 1991). The production of $^{\cdot}OH + OH^-$ may be significantly enhanced through redox cycling, via the Fenton and Haber-Weiss reactions, using transition metal chelates such as iron, copper, chromium (III), (IV), (V), and (VI), vanadium (V) and cobalt (I) (Leonard *et al.*, 2004; Winston and Di Giulio, 1991) (Figure 3). Other redox-active compounds include aromatic diols and quinones, nitroaromatics, aromatic hydroxylamines and bipyridyls. In redox cycles where these organic xenobiotics are univalently reduced, the parent compound is typically first enzymatically reduced by a nicotinamide adenine dinucleotide phosphate (NADPH) dependent reductase, (such as cytochrome P450 reductase) to produce a xenobiotic radical. The radical donates its unshared electron to molecular O_2, producing O_2^- and the parent

compound. In this way at each turn of the cycle two potentially deleterious events have occurred: a reductant has been oxidised and an oxyradical has been produced (Winston and Di Giulio, 1991). These redox cycles produce O_2^- at the expense of cellular reducing equivalents such as NADPH (Winston and Di Giulio, 1991). In addition to the Fenton and Haber-Weiss mechanisms, redox inactive metal ions such as cadmium can indirectly influence the oxidative system by reacting directly with cellular molecules to generate ROIs, inducing cell signalling pathways (Leonard *et al.*, 2004) or depleting the cell's major sulfhydryl reserves (Ercal *et al.*, 2001). Free radicals are not always harmful, singly or collectively, ROIs can participate in the cell mediated destruction of bacteria, fungi and protozoa by specialised blood cells called phagocytes (Andersen, 1994; Langseth, 1995).

Antioxidant defence mechanisms

The proliferation of ROIs is mediated by a number of antioxidant defence mechanisms. These specially adapted enzymes tend to inhibit the formation of ROIs by scavenging and reducing them to non-reactive molecules. Defence systems include the cytoplasmic enzyme superoxide dismutase (SOD) which catalyses the conversion of O_2^- to H_2O_2. The reduction of H_2O_2 to molecular oxygen and water is catalysed by either; the antioxidant enzyme catalase (CAT); or via the glutathione peroxidase (GPx) enzyme system (Winston, 1991) (Figure 3).

It is likely that overproduction of ROIs via redox cycling, phagocytosis and general MFO activity could exhaust the inducible protective antioxidant defence system, contributing to pollutant-mediated toxicological responses (Andersen, 1994; Stegeman *et al.*, 1992; van der Oost *et al.*, 2003; Winston, 1991). Exposure of blood cells of a variety of aquatic animals to sub lethal levels of selected metals, pesticides and other organic compounds has also been shown to lead to a reduction in their production of ROIs. This form of immunosuppression may increase susceptibility to disease (Andersen, 1994).

Superoxide dismutase

Superoxide dismutases (SOD) are a group of metalloenzymes that catalyse the reaction where O_2^- is disproportioned to produce H_2O_2 (Figure 3). Three distinct types with different metal centres have been identified:

- CuZnSODs - typically associated with the cytosol of eukaryotes and chloroplasts of higher plants.
- MnSODs - found in bacteria and organelles such as mitochondria and chloroplasts of higher organisms.
- FeSODs - found in bacteria and a few higher plants.

They are considered to play a pivotal antioxidant role; their importance being indicated by their presence in all aerobic organisms examined. Further, the rate of O_2^- dismutation by SOD approximates the diffusion limit making it the most active of the antioxidant enzymes described (Stegeman *et al.*, 1992). The highly inducible nature of SODs is the basis for their potential as biomarkers (Stegeman *et al.*, 1992). Significant SOD induction has been noted in field surveys of exposed fish (van der Oost *et al.*, 2003). The study of responses of SOD isoenzymes associated with particular organelles may be of particular value for monitoring

oxidative responses at the subcellular level in organisms exposed invivo, including field studies (Stegeman *et al.*, 1992).

Catalases

Catalases (CAT) are haematin-containing enzymes that facilitate the removal of H_2O_2 by reducing it to water and free oxygen (Figure 3). Unlike other peroxidases which can also reduce various lipid peroxidases CAT can only reduce H_2O_2 (Stegeman *et al.*, 1992). CAT occurs in the peroxisomes of most cells where it scavenges the H_2O_2 produced during fatty acid metabolism.

It is also present in erythrocytes independent of peroxisomes in most vertebrates where is appears to act in concert with GPx and methemoglobin reductase to counter the oxidative stress to which these cells are prone (Stegeman *et al.*, 1992). CAT activities in these cells may have potential as a biomarker of oxidative stress (Stegeman *et al.*, 1992), however, as both induction and inhibition of CAT activity has been measured in fish after exposure to environmental contaminants its usefulness as a biomarker is not yet clear (van der Oost *et al.*, 2003).

Glutathione peroxidases and reductases

Peroxidases (GPx) reduce peroxides to their corresponding alcohols using a range of reductants. In animals the main peroxidase, which is a selenium-dependent tetrameric cytosolic enzyme, uses reduced glutathione (GSH) as a cofactor to reduce H_2O_2 to $2H_2O$ (Stegeman *et al.*, 1992) (Figure 3). Also of interest is the ability of GPx to reduce organic hydroperoxides to their corresponding alcohols, as this is considered an important mechanism for halting lipid peroxidation chain reactions. Reductases (GR) are not as active as GPx in antioxidant defences, however, they play an important role in maintaining appropriate GSH:GSSG ratios in response to oxidative stress (Winston and Di Giulio, 1991). GR catalyses the transformation of the GSSG to its reduced form, GSH, with the concomitant oxidation of NADPH to $NADP^+$ and can be measured spectrometrically by following the decrease in NADPH levels (van der Oost *et al.*, 2003).

Reduced and oxidised glutathione

Reduced glutathione (GSH), a tripeptide made up of glutamic acid, cystine and glycine (George, 1994), has two contrasting roles in detoxification; (i) as a key conjugate of electrophilic intermediates, principally by glutathione S-transferase (GST) activities in phase II metabolism, and (ii) as an important antioxidant enzyme (Stegeman *et al.*, 1992). In addition to its antioxidant function in the activities of GPx and GR already discussed, GSH can also act as a nonenzymatic scavenger of oxyradicals (Stegeman *et al.*, 1992). Increased fluxes of oxyradicals have been shown to alter GSH status with the most obvious direct effect being a decrease in the ratio of GSH to oxidised glutathione (GSSG), (Stegeman *et al.*, 1992) brought about by increased peroxidase and scavenging activities or indirectly due to reduced availability of NADPH following oxidations from the first step of the redox cycle (Figure 3). In healthy cells the GSH:GSSG ratio is typically high, greater than 10:1 (Stegeman *et al.*, 1992).

If GSSG accumulates, thiol-containing enzymes can be inactivated through the formation of mixed disulphides. GSSG has also been shown to inhibit protein synthesis through an interaction with one of the initiation factors for translation (Melancon *et al.*, 1992). Increased

synthesis of GSH in response to increased oxyradical generation might also result in the maintenance of the GSH:GSSG ratio and / or an increase in GSH levels (Stegeman *et al.*, 1992). The existence of effective feed back mechanisms for the maintenance of GSH levels in response to contaminant induced effects may mean that GSH levels alone are not useful as biomarkers of oxidative stress (Stegeman *et al.*, 1992). The measurement of elevated GSSG levels, however, suggest that the hepatic GSH:GSSG ratio may be a potential biomarker for oxidative stress (van der Oost *et al.*, 1996). The drain imposed on intracellular reducing equivalents such as NADPH by oxyradical-generating compounds can influence the redox status of cells with potentially profound consequences on a variety of metabolic processes (Stegeman *et al.*, 1992). Measurements of pyridine nucleotide ratios NAD(P):$NAD(P)^+$ may also be useful in assessing effects on redox status (Stegeman *et al.*, 1992).

Oxidative damage

A failure of the antioxidant defence system to prevent ROI proliferation may result in a variety of oxyradical induced perturbations, including; lipid peroxidation, DNA oxidation, methemoglobinemia and a reduced capacity to neutralise reactive oxygen species.

Total antioxidant capacity

The total antioxidant capacity (TAOC) assay provides an overall measure of the ability of the reactive oxygen species reduction system to neutralise reactive oxygen species (ROS). One specific assay developed for measuring and quantifying the capability of biological samples to neutralise ROS is the total oxygen scavenging capacity (TOSC) (Regoli, 2000; Winston *et al.*, 1998). The TOSC assay has been standardised for measuring the scavenging capacity of cellular antioxidants with respect to various ROS (Regoli and Winston, 1999). While this assay, like the TAOC assay, is not a specific measure of oxidative damage it provides information on the antioxidant capacity of specific chemical scavengers and their activities with different oxidants which is fundamental to understanding and predicting the susceptibility of biological tissues to oxidative stress (Regoli and Winston, 1999).

Lipid peroxidation

Lipid peroxidation is a widely recognised consequence of oxyradical production (Winston and Di Giulio, 1991). The process of lipid peroxidation proceeds in a chain reaction and like the redox cycle has the ability to propagate a number of deleterious biochemical reactions (Stegeman *et al.*, 1992). Lipid peroxidation has potential as a biomarker, however, it can occur due to cellular damage resulting from a range of insults other than chemically induced oxidative stress (Melancon *et al.*, 1992). A commonly used assay for lipid peroxidation is thiobarbituric acid reactive substances (TBARS) test for malonaldehyde (MDA), a byproduct of lipid peroxidation (Pedrajas *et al.*, 1995; Romeo *et al.*, 2003a).

DNA oxidation

The oxidation of DNA may produce hydroxylated DNA bases as result of alterations from $^{\cdot}OH$ attack at various DNA base sites (Stegeman *et al.*, 1992). Recently developed methods for measuring these products in biological samples, which show promise, use HPLC separation and electrochemical detection of hydroxylated bases, such as thymine glycol or 8-hydroxy deoxyguanosine. These methods are very sensitive but fairly involved, method refinements would enhance the feasibility of this promising biomarker (Stegeman *et al.*, 1992).

Micronuclei frequency

Micronuclei are small intracytoplasmic masses of chromatin resulting from chromosomal breakage or aneuploidy during cell division (Bolognesi *et al.*, 2004). They resemble the main nucleus and are easily observed in interphasic cells (Scarpato *et al.*, 1990). The micronucleus assay is one of the most promising techniques to identify genetic alterations in organisms exposed to toxicants (Bolognesi *et al.*, 2004). As an index of chromosomal damage the micronucleus test is based on the enumeration of downstream aberrations after DNA damage and shows a time-integrated response to pollutants. It is thought to be a fast and sensitive test since it is able to detect genomic damage due to both clastogenic effects and alterations of the mitotic spindle (Migliore *et al.*, 1987). The micronucleus test has proved suitable for application to aquatic invertebrates and is simpler and more rapid to perform than other measurements of chromosomal aberration (Burgeot *et al.*, 1996). Micronuclei frequency has been studied in fish, (Castano *et al.*, 1998; Williams and Metcalfe, 1992) and invertebrates, (Bolognesi *et al.*, 2004; Kalpaxis *et al.*, 2004; Majone *et al.*, 1987; Scarpato *et al.*, 1990; Wrisberg *et al.*, 1992).

Stress proteins

Stress proteins are a group of proteins which include two major groups of gene products: the 7–90 kDa *heat shock proteins* (hsp) induced by exposure to heat and a variety of other chemical and physical stressors; and the 78–100 kDa *glucose-regulated proteins*, (grp) synthesised in response to glucose and oxygen deprivation, and exposure to lead or agents which inhibit calcium and protein homeostasis (Locke, 2002). Each stress protein is made up of a multigene family in which some proteins are constitutively expressed and are present in cells under normal conditions, playing a role in basic cellular physiology while others are highly inducible in response to environmental stressors (Stegeman *et al.*, 1992). The term heat shock proteins was originally used to describe this family of proteins as they were originally studied in relation to heat shock response, it is now known that they can be induced by a number of environmental perturbations including heavy metals (Agell *et al.*, 2004; Bauman *et al.*, 1993; Del Razo *et al.*, 2001; Werner *et al.*, 2004) and organics (Ait-Aissa *et al.*, 2000; Werner *et al.*, 2004). In aquatic species members of the hsp70 and hsp60 groups are highly conserved and exhibit measurable increases in synthesis in response to environmental contaminants (Stegeman *et al.*, 1992). In particular the hsp72 is only synthesised in response to environmental stressors and is not found in most cells under normal conditions, making it an excellent candidate as an exposure biomarker for chemical contamination (Stegeman *et al.*, 1992).

Heme oxygenase

Heme oxygenase is a 32kDa stress protein which has been isolated and identified is inducible by metals (cadmium, zinc, copper and lead), sodium arsenite, oxidative stress and thiol-reactive agents (Sanders, 1990). It is described as a rate limiting enzyme which catabolises heme into three products: carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (which leads to the induction of ferritin, an iron-binding protein) (Otterbein *et al.*, 2003). It is thought that since these breakdown products of heme can react readily with peroxyl radicals they may play a significant role in protecting cells from oxidative damage as free radical scavengers in concert with glutathione (Otterbein *et al.*, 2003; Rivera and Zeng, 2005; Sanders, 1990; Stegeman *et al.*, 1992).

Jorgensen *et al*., (1998) examined the effect of a variety of stressors on heme oxygenase activity in atlantic salmon and mackerel liver and spleen and concluded that heme oxygenase may be suitable for developing as a biomarker for certain heavy metals and oxidative stress in fish but the application is reliant on the development of fish specific antibodies for the enzyme.

Metallothioneins

Metallothionein (MT) is a low molecular weight (≥ 10 kDa) cysteine rich metal binding protein synthesised in response to metal exposure (Roesijadi, 1996), which may have potential as biomarkers of exposure to toxic metals (Garvey, 1990; Petering *et al.*, 1990; Sanders, 1990; Stegeman *et al.*, 1992; van der Oost *et al.*, 2003). Its induction is slower than that of other "classic" stress proteins in response to transition metals, 24 hrs as opposed to 30 min (Sanders, 1990). Measurement of MTs does not necessarily reflect the degree of exposure to metals as physiological and environmental factors can affect mobilisation and partitioning of metals by MT (Roesijadi, 1996; Stegeman *et al.*, 1992). MTs may also be induced under many other conditions besides metal exposure, for example, glucocorticoid hormones (progesterone and glucagon) and peptide hormones (interleukin I and interferon) (Sanders, 1990; Stegeman *et al.*, 1992). The study of metal binding to MTs rather than measuring total tissue metal concentrations may be useful as it is increasingly clear that knowledge of intracellular compartmentalisation is essential to understanding mechanisms of metal-induced cell injury, as it aids in determining the extent to which organisms are able to sequester metals in forms which are not biologically reactive (Fowler, 1987; Vijver *et al.*, 2004). Since the normal physiological function of MT is presently not fully understood there is no way to determine if MT itself plays a direct role in the pathophysiology of cell injury. The current data suggest the reverse is true, the non MT bound fractions of these metals participate in the cell injury process, and MT induction appears to be a protective cellular response (Viarengo *et al.*, 1998). MT induction and metal binding appear to be a cellular defence mechanism against injury. Metal toxicity seems to occur only after this capacity has been exceeded (Roesijadi, 1996; Stegeman *et al.*, 1992). The use of MTs to assess organism health or fitness in response to toxic metal exposure requires extensive knowledge of their normal physiological function and the factors which control the levels of MT in selected organisms also needs to be established (Stegeman *et al.*, 1992).

Haematological Parameters

Haematological parameters provide a non-destructive method for effect assessment which are typically non-specific in their response to chemical stress (van der Oost *et al.*, 2003).

Serum enzymes

Increased serum enzyme concentrations can result from: enzyme leakage from a cell with a damaged cell membrane; increased enzyme production and leakage from the cell; or decreased enzyme clearance from the blood (Mayer *et al.*, 1992). *Serum transaminases*, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes that catalyse the inter-conversion of amino acids and α-ketoacids by transfer of amino groups

(van der Oost *et al.*, 2003). Increased levels of these enzymes in intracellular fluids may be a sensitive indicator of cellular damage as levels in cells usually exceed those in the intracellular fluids by more than three orders of magnitude (van der Oost *et al.*, 2003). Metals have been found to affect the activities of transaminases in fish. Fish exposed to acutely toxic concentrations of cadmium, copper or mercury had increased transaminase activities, alternatively chronic exposure to copper resulted in decreased AST activities and chronic exposure to cadmium had no effect on transaminase activity in fish serum (Mayer *et al.*, 1992). *Serum lysosomal enzymes* have been suggested as potential indicators of effect following exposure to organics, pesticides and metals. In particular N-acetyl-β-D-glucosaminidase (NAG) activity in spleen and liver dysfunction, and leucine amino naphthylamidase (LAN) enzyme activity for quantifying tissue damage in fish (Mayer *et al.*, 1992). The mechanism responsible for increased serum levels of lysosomal enzymes is not know but it is thought to differ from other serum enzymes. Lysosomes contain increased concentrations of metals and may be active in the degradation of metal binding proteins (Fowler and Nordberg, 1978). Metals have also been shown to increase lysosome numbers and reduce lysosomal membrane stability possibly leading to enzyme leakage (Castro *et al.*, 2004; Domouhtsidou *et al.*, 2004; Nicholson, 2003; Petrovic *et al.*, 2001; Versteeg and Giesy, 1986). Serum enzymes have been demonstrated as useful biomarkers of tissue damage, use of a suite of serum enzymes may prove useful for understanding population-level effects (Mayer *et al.*, 1992).

Heme / porphyrin pathway

The heme/porphyrin pathway is essential for synthesis of hemoproteins (e.g. haemoglobin) and various cytochromes (e.g. cytochrome P-450). A number of metals, metalloids and organics have been shown to induce enzymatic disturbances in this pathway, which correlate with overt cell injury (Stegeman *et al.*, 1992). In particular the activity of the δ-aminolevulinic acid hydratase (ALAD), a cytosolic enzyme found in many tissues and active in the synthesis of haemoglobin by catalysing the formation of porphobilinogen, a precursor of heme, has been shown to be inhibited by exposure to lead in mammals (Flora and Seth, 1999; Mayer *et al.*, 1992; Pande and Flora, 2002; Perottoni *et al.*, 2005). It has been suggested that the determination of ALAD activity in fish might be a useful biomarker of lead exposure and some studies have shown ALAD inhibition in fish blood and liver following water-borne exposure to lead (Conner and Fowler, 1994; Rodriguez *et al.*, 1989). Conner and Fowler (1994) found that although fish hepatic ALAD was inhibited by lead exposure the sensitivity of the fish reaction was lower than that reported for mammals, requiring a 40-fold increase in lead concentration exposure to produce the same IC_{50}. Further kinetic studies indicated major differences between fish and mammalian hepatic ALAD. They suggest the absence of a chelatable metal cofactor or greater binding affinity at the active binding site of the fish hepatic enzyme compared to that described for mammals may be responsible for the difference in sensitivity to lead. The use of this pathway may be applicable to marine bivalves like *Anadara trapezia* which have haemoglobin as a respiratory pigment (Sullivan, 1961).

Ion levels

Ion levels in aquatic organisms must be maintained through active regulation of water and ion influx and efflux. Exposure to metals can effect the ion regulatory organs, internal and external osmotic sensory receptors, endocrine system, metabolism or active transport processes, leading to alterations in the plasma ion levels of K^+, Na^+ and Cl^- ATPhase activity (Mayer *et al.*, 1992). Decreased levels of K^+, Na^+ ATPase activity have been measured in eel gills and intestines following exposure to cadmium (Lionetto *et al.*, 2000), fish (de la Torre *et al.*, 2000; Wong and Wong, 2000) and invertebrates exposed to silver (Bianchini *et al.*, 2005) and copper (Bianchini *et al.*, 2004).

However, other studies of fish exposed to elevated levels of cadmium (Benson *et al.*, 1988) and mercury (Jagoe *et al.*, 1996) have failed to show significant alterations in haemolymph ionic composition. Effects of stressors on osmoregulation have not been related conclusively to higher order and population level effects and this combined with difficulties of inherent variability, accessory factors and data interpretation, limits the potential for this technique as a biomarker for metal induced stress in field studies (Mayer *et al.*, 1992).

Neurotoxic measures

The principle neurotoxic enzyme identified in aquatic organisms is acetylcholinesterase (AchE), which is involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firings, which are vital for normal sensory and neuromuscular function. AchE activity is inhibited by organophosphate and carbamate pesticides and has been used in fish studies as an exposure biomarker for these xenobiotics (de Aguiar *et al.*, 2004; Eder *et al.*, 2004). It has been measured in mussels exposed to organophosphates but for these organisms it was not found to be a reliable indicator (Cajaraville *et al.*, 2000; Rickwood and Galloway, 2004), however, measurements in the clam and a polychaete worm showed it to be a sensitive biomarker along a pollution gradient (Perez *et al.*, 2004). It has been suggested that AchE may also be a sensitive biomarker in fish for a range of other chemicals including compounds in complex mixtures of combustion hydrocarbons and natural wood leachate (Payne *et al.*, 1996). Its use as a biomarker for metals has not so far been unequivocally established.

Endocrine system

Physiological and biochemical stress resulting from contaminant exposure must be compensated for in order to maintain homeostasis. The measurement of the synthesis, secretion, metabolism and clearance of hormonal concentrations in blood may be used to gauge the impact of contaminants on metabolism, growth and reproduction (Mayer *et al.*, 1992). Effective use of plasma concentrations of hormones as biomarkers requires knowledge of production, and clearance rates as well as seasonal, age, gender, reproductive and nutritional status influences. The following hormones have been considers as potential biomarkers: corticosteroids; catecholamines; thyroid hormones; reproductive steroids; insulin; glucagon and growth hormone (Mayer *et al*., 1992).

Other metabolic products

Reproductive hormone

Levels of reproductive hormones have been shown to be altered and reproductive function impaired in response to a variety of chemical stressors both organic and inorganic (Chen, 1988; Depledge and Billinghurst, 1999; Siah *et al.*, 2003; Thomas, 1988). The complexity of the interactions among various parts of the reproductive system is a limiting factor in their use as biomarkers (Melancon *et al.*, 1992).

Vitellogenin

Vitellogenin is a large molecular weight lipophosphoprotein synthesised by the liver in vertebrates regulated by β-estradiol. The analogous compound in invertebrates is lipoprotein which is synthesised by a variety of tissues. These molecules, which are precursors, of yolk proteins are released into the blood stream and sequestered in the developing oocyte in response to gonadotrophin and other hormones. Impaired reproductive function due to decreased plasma vitellogenin levels has been demonstrated in rainbow trout exposed to cadmium (Haux *et al.*, 1988), Florida largemouth bass exposed to organics and metals (Sepulveda *et al.*, 2002), and increased levels were measured in shore crabs exposed to cadmium (Martin-Diaz *et al.*, 2004). Riffeser and Hock (2002) found no significant induction (or supression) of haemolymph vitellogenin in mussels exposed to estrogenic compounds. Very little is known about the effect of chemicals on invertebrate vitellogenin. A better understanding is required before plasma vitellogenin concentrations can be reliably used as biomarkers in invertebrates (Melancon *et al.*, 1992).

Cellular Biomarkers

Cell and tissue perturbations may serve as biomarkers of both exposure and effect of environmental contaminants. While changes at the molecular level in the expression of the various enzymes involved in the conjugation, detoxification and excretion of toxins are sensitive first order measures of exposure and in some instances effect, measures of cell integrity and tissue morphology offer a second order measure of exposure and more particularly of effect. Being at a higher order of biological organisation the cellular and tissue effect response is less likely to be specific for a particular contaminant but rather a general response often indicative of chronic toxicity.

Linkage of molecular responses to damage at the cell and higher levels of organisation is only beginning to emerge, and more focus is being brought to it in an effort to determine the significance of molecular events to subsequent forms of cell and tissue injury and response (Hinton, 1994).

Immunological responses

A highly developed cell-mediated immune system, involving non-specific immune mechanisms and humoral antibody systems, has been demonstrated in teleosts (Weeks *et al.*, 1990). Assays of these immune responses, in their capacity to destroy foreign material and protect the host against disease can serve as useful sentinels of the health status of environmentally stressed organisms (Weeks *et al.*, 1990). Since immunocompetent cells are required for host resistance, measurement of increasing susceptibility to infectious agents or

tumour cells can provide insight into the biological significance of immune alterations induced by xenobiotics (Weeks *et al.*, 1992). Immunicological biomarkers in fish which have been found to react to experimental stress include:

- Decreased haemocyte count and viability (Liu *et al.*, 2009; Mirella da Silva *et al.*, 2008; Vijayavel *et al.*, 2009);
- Macrophage function (Zelikoff, 1998);
- Increased phagocytosis (Brousseau *et al.*, 1997; Luengen *et al.*, 2004) or decreased phagocytosis (Hannam *et al.*, 2009);
- Increased susceptibility to bacterial infections (Zelikoff, 1998);
- Enlarged lysosomes and the loss of lysosomal structures (Regoli *et al.*, 1998);
- Decreased respiratory burst (Rice *et al.*, 1996).

A three tiered approach to the application of immune system responses to the assessment of environmental impact is described in detail by Weeks *et al*., (1990). Briefly, the relatively simple Tier I assays provide a general effect screening while the more specific and sensitive Tier II assays provide a comprehensive evaluation of the various components of the immune response, finally a Tier III host resistance challenge study can be used to complete an immune function assessment.

Lysosomal membrane stability

Lysosomes play an important role in sequestration and detoxification of metals (Viarengo, 1989). High metal concentrations may induce synthesis of metallothioneins which enter lysosomes for degradation and normal protein turnover thus overloading their storage and detoxification capacity (Viarengo, 1989). Destabilisation of the lysosomal membrane following exposure to metals may be a result of direct interaction of metals with the lysosomal membrane and from metal induced oxidative stress (Regoli *et al.*, 1998). Lysosome membrane destabilisation has proven a useful effect biomarker of metal exposure in both field and laboratory exposures (Kalpaxis *et al.*, 2004; Nicholson, 1999a; b; Regoli *et al.*, 1998; Ringwood *et al.*, 2002; Romeo *et al.*, 2000; Werner *et al.*, 2004). Winston *et al* (1996) demonstrated that lysosomal destabilisation in mussels is affected by production of oxyradicals generated from contaminant exposure both internally and externally to the lysosomal membrane.

Histopathological Alterations

Histopathological alterations are effects measures of exposure to environmental stressors which can be used to examine organ, tissue or cellular perturbations of both acute and chronic exposure to contaminants (Hinton *et al.*, 1992). Histopathological lesions can result from exposure to a wide range of toxic agents and it is only possible to make broad generalisations about the toxin responsible for a particular lesion, however, they provide useful evidence of the magnitude of toxic impairment to the individual and in some cases the potential impact on the population (Hinton *et al.* 1992).

Physiological Biomarkers

An optimal assessment of the altered health of on organism or ecosystem requires the selection of a range of indicators from different levels of biological organisation. While measurements at the cellular level provide the greatest sensitivity the overall fitness can be better assessed using physiological responses since these represent an integration of individual cellular effects (Duquesne *et al.*, 2004). Physiological biomarkers are non-specific. They can be used as indicators of both exposure to and effect of xenobiotics and may be useful in integrating the effects of a number of stressors by quantifying organism health (Mayer *et al.*, 1992). Organism health has been defined by Bayne *et al.*, (1985) as the residual capacity to withstand stress; the more stressed the less capable the organism is of withstanding further stress.

General physiological biomarkers which are useful in assessing organism health and which can give some indication of the potential for population level effects are described below. These types of measurements can be highly variable, however, when linked with other evidence of perturbation at the subcellular and cellular level can provide further evidence for potential higher order effects (Figure 2).

Energetics

There are many energetic responses that may be initiated in response to toxicant exposure. Acute responses, which are generally controlled by the enzyme and hormone systems, are considered to be the initial response to a stressor and often involve an increase in energy related substrates in the haemolymph (Mayer *et al.*, 1992). Chronic stress can initiate compensatory physiological adjustments, so that energy metabolism changes may be needed to maintain normal physiological function, and this can result in reduced growth or reproduction (Mayer *et al.*, 1992). An assessment of the energy status of an organism can, therefore, give an indication of its overall condition.

Adenylate energy charge

Adenylate energy charge (AEC) is a measure of the metabolic energy available to an organism from the adenylate pool and is a direct calculation based on the concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Sokolova *et al.*, 2005b).

$$\text{Equation 1: EC} = \frac{\text{ATP} + \tfrac{1}{2}\,\text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

A decrease in the concentrations of ATP, the primary source of cellular energy, based on the increased use of energy by organisms under stress can result in reduced AEC values (Mayer *et al.*, 1992). Oysters *Crassostrea virginica* experimentally exposed to cadmium had reduced AEC values compared to control oysters (Cherkasov *et al.*, 2006; Sokolova *et al.*, 2005b) . Cellular metabolic supply and demand of ATP, ADP and AMP in a wide range of energy-stressed animals is reviewed by Staples and Buck (2009).

Cellular energy allocation

When organisms are exposed to suboptimal conditions there is a cost of dealing with stress in terms of metabolic resources. Energy available for growth based on energy budget analysis rather than direct measurements of growth itself may, therefore, provide a sensitive measure of stress in organisms (Smolders *et al.*, 2004). The cellular energy allocation (CEA) methodology was developed as a biomarker technique to assess the effect of toxic stress on the cellular energy budget of test organisms by (De Coen and Janssen, 1997). The assay is based on the biochemical measurement of changes in energy reserves (glucose, protein, and lipid content) and the energy consumption (electron transport system activity (ETS)). The difference between the energy reserves and energy consumption represents the net cellular energy budget, expressed as CEA, of the test organism (De Coen and Janssen, 1997).

Equation 2**:** Available energy (E_a) = glucose + lipid + protein (mJ mg^{-1} wet wt.)
Energy consumed (E_c) = ETS activity (mJ mg^{-1} wet wt. h^{-1})
Cellular energy allocation (CEA) = E_a/E_c

The utility of CEA as a stress biomarker, with links to higher order effects, has been shown for daphnids (De Coen and Janssen, 1997; 2003), mussels (Smolders *et al.*, 2004) and freshwater gastropods (Moolman *et al.*, 2007).

Scope for growth

Growth as a process represents an integration of major physiological responses and specifically the balance between processes of energy acquisition (feeding and assimilation) and energy expenditure (metabolism and excretion) (Widdows and Donkin, 1992). This physiological energetics-based approach is usually referred to as Scope for Growth (SFG).

Equation 3**:** SFG = $A – (R + U)$ (J mg^{-1} dry wt h^{-1})
A = energy absorbed, R = energy respired, and U = energy excreted

SFG has been used extensively and found to be a sensitive growth biomarker of stress in marine mussels (Goldberg and Bertine, 2000), cockles *Anadara trapezia* exposed to metals (Burt *et al*, 2007) and marine gastropods *Nassarius festivus* exposed to cadmium (Wo *et al.*, 1999).

Bayne *et al*., (1985) has reviewed the methods available for measuring the endpoints required to calculate SFG.

Condition index

A reduction in AEC, CEA or SFG values can ultimately lead to reduced growth and reproductive capability. Body condition index can provide information on the impact of pollutants on individual organism health through relatively simple growth measures, such as determining what proportion of bivalve internal shell volume is occupied by the organs (Mayer *et al.*, 1992). A widely used condition index (CI) measure for bivalves is based on the measurement of the ratio of soft tissue weight to valve weight.

Equation 4: CI = Tissue Mass (g) x 100
Valve Mass (g)

A high ratio indicates good physiological condition and reduced ratios indicate poor physiological condition. This CI has been used extensively with marine bivalves and gastropods exposed to metal and other contaminants and has proved to be a useful indicator of both severe and chronic stress (Andral *et al.*, 2004; Duquesne *et al.*, 2004; Leung and Furness, 2001a; Leung and Furness, 2001b; Li *et al.*, 2009; Lundebye *et al.*, 1997; Mubiana *et al.*, 2006; Romeo *et al.*, 2003b).

Integrated Assessment of Organism Metal Exposure – Dose – Response Framework

The preceding sections have dealt with the ways in which organisms are exposed to metals, the mechanisms they employ to manage their exposure, the consequent dose resulting from this and finally the myriad of molecular, cellular and physiological responses which may be measured as indicators that exposure to a toxicant has occurred and the dose has exceeded an organism's detoxification and repair capacity.

External exposure

Exposure under laboratory conditions with single contaminants enables potentially confounding factors to be controlled and the exposure concentration set. This approach is needed to establish relationships between exposure, dose and response of the test species to the contaminant of interest (McCarthy, 1990).

Exposure in the field can be assessed by measuring metals in the sediments and overlaying waters, this gives a measure of total metal availability but not of the bioavailable fraction. By relating the water and sediment total metal concentrations with that of exposed organisms' metal body burdens an estimate of the bioavailable metal fraction can be gained.

Internal dose

Further investigation of the partitioning of the metal in the various tissue subcellular fractions gives information on the proportion of the metal which is metabolically available, the biologically active metal (BAM), and therefore able to participate in effects and the proportion which is sequestered in the heat stable MT and MT like proteins and the metal rich granule fraction, the biologically detoxified metal (BDM), and therefore not metabolically available (Vijver *et al.*, 2004; Wallace *et al.*, 2003).

Response

A multi-biomarker approach at several levels of biological organisation has advantages over the use of a single biomarker and offers an effective early warning system of adverse effects in biomonitoring of aquatic environments (Adams *et al.*, 1988; Adams *et al.*, 1989; Brown *et al.*, 2004; Galloway *et al.*, 2004; Romeo *et al.*, 2003a; Smolders *et al.*, 2004). The biomarkers which derive from cytochemistry, cytophysiology, cytogenetics and pathology can detect early responses and prepathological alterations before other disturbances such as disease, mortality, or population changes occur (Adams *et al.*, 1989). Effects of environmental stressors such as the toxic metal cadmium on the health of aquatic organisms may involve a series of biological responses ranging from the molecular / biochemical to the

cellular and physiological levels. As suggested by McCarthy, (1990), development of biomarker protocols are best done under controlled laboratory conditions with single contaminants. Once processes are well understood assessments using mixed contaminants should be carried out to determine whether biomarker responses can be quantified under more realistic conditions.

Biomarker selection

It is important to select an appropriate *cascade* of biomarker measurements which demonstrate the relationship between exposure dose and response. Thus biomarkers must be selected which have a demonstrable link to exposure or dose (Figure 4). The oxidative system offers a range of general response and effect biomarkers which have been shown to be sensitive to metals through perturbations in the redox cycle and other oxidative pathways. The measurement of a suite of biomarkers within this system, from ROIs, through catalysing and phase II enzymes to oxidative damage indices offers a weight of evidence approach to assessing molecular level exposure and effects. As lysosomes are involved in metal management and are also susceptible to oxidative damage the measurement of their integrity offers a useful biomarker of effect at the cellular level. Impairment of the lysosomal membrane can be considered a second order effect which would follow perturbations at the molecular level. The frequency of micronuclei occurrence offers a measure of DNA damage which aids in completing the picture of a *cascade* of reactions resulting from exposure to toxic levels of metals for individual organisms. An interrelated *cascade* of biomarker measurements which demonstrate the relationship between exposure dose and response is necessary for the molecular perturbations measured to be interpretable and therefore able to be linked to potential higher order effects (Figure 4). By identifying and establishing relationships between exposure, dose and response to toxicants at these levels of biological organisation we should be better able to understand the mechanisms of stress responses in ecological systems that could ultimately result in improved predictive capability of ecological risk assessment and also allow for more informed decisions regarding remedial actions.

CADMIUM SPIKED SEDIMENT EXPOSURE – DOSE – RESPONSE CASE STUDY

Cadmium is a metal which is accumulated in high concentrations by a range of marine organisms and which has no known biological function. It is extremely toxic to aquatic organisms at high concentrations and even at low concentrations may adversely affect physiology (Ercal *et al.*, 2001; Sokolova, 2004; Sokolova *et al.*, 2004). To establish relationships between levels of exposure, organism dose and biological response to cadmium a benthic marine bivalve mollusc *Tellina deltoidalis* was exposed to different concentrations of single cadmium spiked sediments in laboratory aquaria and compared to unexposed organisms. Organism internal exposure was measured by total tissue cadmium burden. The internal dose was examined by subcellular fractionation of whole tissue to determine what fraction of the total cadmium taken up was in a metabolically available form. Marker enzymes specific for mitochondria and lysosomes were measured in subcellular fractions to further understand intracellular localisation of cadmium in these organelles (Sokolova *et al.*, 2005a). Molecular measurements of oxidative stress, which are

good general effects biomarkers for metal exposure (Stegeman *et al.*, 1992; van der Oost *et al.*, 2003; Winston and Di Giulio, 1991), selected were the total antioxidant scavenging capacity of cells, and the extent of lipid peroxidation. Lysosomal membrane stability was measured as a cellular effect biomarker, while the micronucleus assay was used to assess genotoxic damage.

Methods

Organism

Tellina deltoidalis (Figure 5) was selected as a test organisms for the cadmium exposure – dose - response study as it is a sediment dwelling bivalve which satisfies most of the basic requirements to be an effective biomonitor (Phillips and Rainbow, 1994). *Tellina deltoidalis* lie obliquely in the sediment at a depth several times their shell length of 20 - 30 mm with their siphons extending towards the sediment surface. The inhalant siphon ingests deposited organic material and sediment particles from the sediment surface, while the exhalent siphon expels faeces and water below the sediment surface (Beesley *et al.*, 1998).

Experimental design

The experimental design was as shown in (Figure 6). Procedures for conducting the exposures were adapted from the test method for conducting 28 day sediment bioaccumulation tests (Ingersoll *et al.*, 2000). Organisms were not given supplementary food and surface water was not changed during the 28 day exposure period.

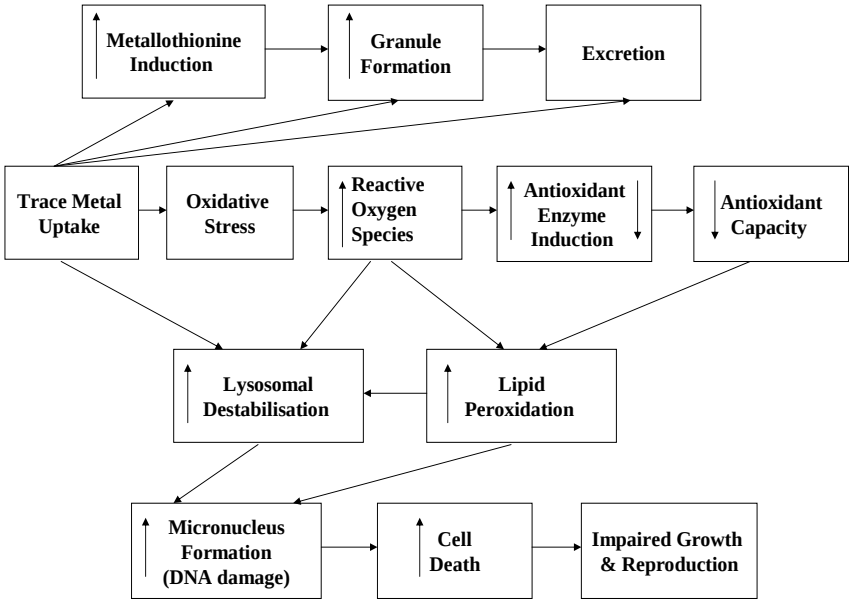

Figure 4. Flow diagram showing a *cascade* of interlinked cellular reactions which can occur in response to metal exposure.

Figure 5. *Tellina deltoidalis* (Linnaeus, 1758)

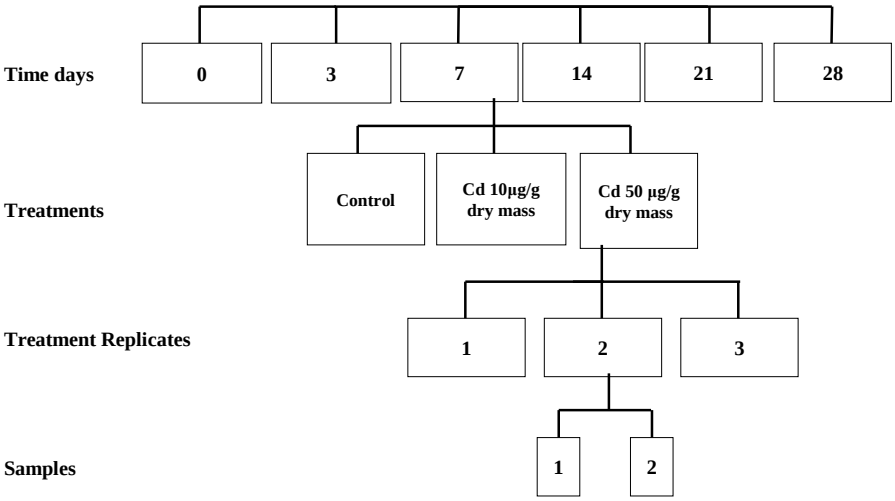

Figure 6. Experimental design for *T. deltoidalis* exposure to metal spiked sediments.

Sediments

Cadmium spiked sediments were prepared at concentrations of 10 mg/kg and 50 mg/kg dry mass of sediment with $CdCl_2$, (AR grade Merck) using a method developed by (Simpson *et al.*, 2004) modified by (Taylor, 2009).

Metal bioaccumulation

Tissue metal bioaccumulation was measured over time (Figure 6). Freeze-dried tissue was digested with nitric acid (Baldwin *et al.*, 1994) and cadmium measured on an ELAN® 6000 ICP-MS (PerkinElmer, SCIEX) (Maher *et al.*, 2001).

Subcellular metal distribution

Subcellular tissue metal distribution (Figure 7) was measured after 28 days using a procedure adapted from (Sokolova *et al.*, 2005a; Wallace *et al.*, 2003) by (Taylor, 2009). Mitochondrial and lysosomal content of fractions P1, P3 and P4 was established by the activity of enzymes specific for these organelles, Cytochrome c Oxidase and Acid Phosphatase (Sigma-Aldrich, USA), respectively. Final fractions were digested in nitric acid in a water bath at 80^{o}C for 4 hours. Metal analysis was by ICPMS as previously described.

Biomarkers

Total antioxidant capacity (TAOC) assay, based on the ability of antioxidants in tissue lysates to inhibit oxidation of ABTS® to ABTS®•+ by metmyoglobin in the presence of H_2O_2, was measured using a colourimetric assay (Cayman Chemicals). Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS), using a colourimetric assay based on the specificity of a lipid peroxidation by-product, malondialdehyde (MDA), for TBARS (Zepometrix Corporation). Enzyme concentrations are expressed as mg^{-1} of protein, measured with a FluoroProfile™ epiccoconone fluorescence assay (Sigma-Aldrich, USA). Lysosomal stability was assessed using a method developed for oysters (Ringwood *et al.*, 2003). Cells incubated in neutral red accumulate the lipophilic dye in the lysosomes. Healthy cells retain the dye in the lysosomes (Figure 8a) whereas in cells with damaged lysosomal membranes it leaks out into the cytoplasm (Figure 8b).

The micronuclei assay was based on a nuclei specific fluorescent dye (DAPI), technique developed on mussels (Gorbi *et al.*, 2008). Micronuclei are small round structures less than one third the diameter and in the same optical plan as the main nucleus, with a boundary distinct from the nuclear boundary (Figure 9).

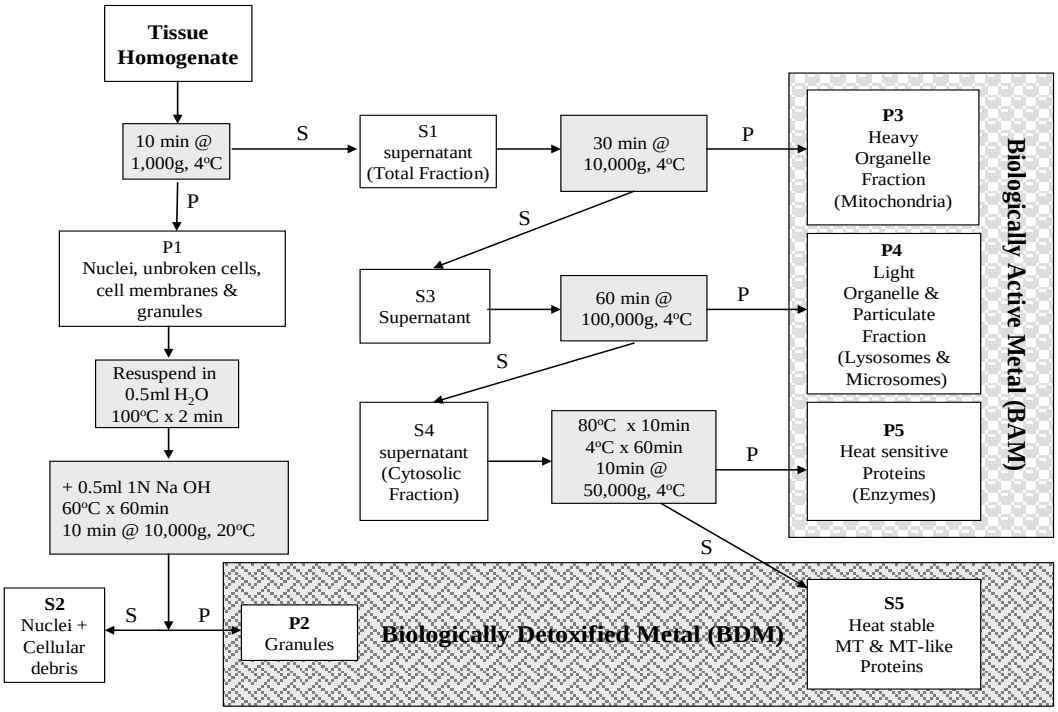

Figure 7. Procedure for subcellular fractionation of bivalve tissues by differential centrifugation. The shaded boxes show details of the centrifugation and digestion / heating steps used to obtain the specific fractions. The final fractions, four pellets P2, P3, P4 & P5 and two supernatants S2 & S5 are grouped as: biologically detoxified (BDM) P2 & S5; biologically active (BAM) P3, P4 & P5 metals or S2 which contains metal associated with nuclei and cellular debris.

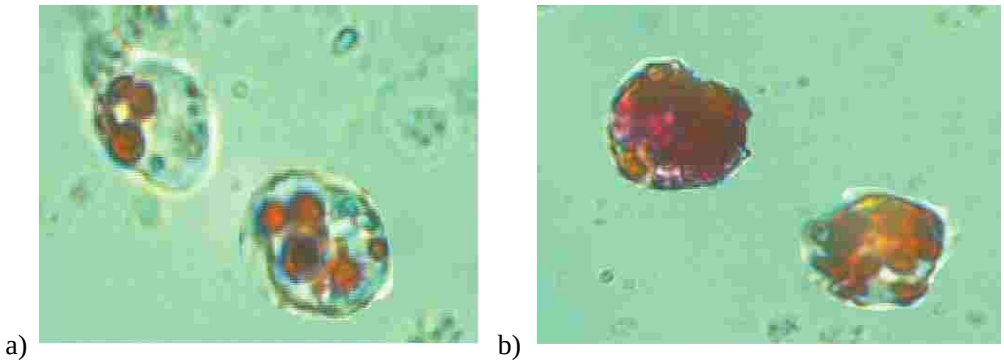

Figure 8. *T. deltoidalis* cells scored as; a) Stable - dye present in the lysosome; b) Destabilised - dye present in the cytosol. Magnification x100.

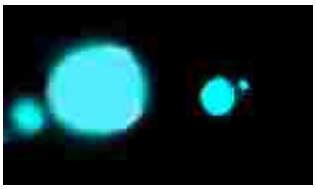

Figure 9. Examples of DAPI stained nuclei with associated micronuclei from tissue of *T. deltoidalis*. Magnification x 1000.

Results and Discussion

Cadmium bioaccumulation

The *T. deltoidalis* exposed to 10 and 50 µg/g of cadmium both showed an increase in tissue cadmium over 28 days of exposure with final tissue concentrations equal to that of the spiked sediments (Figure 10a). Most bivalve molluscs accumulate both essential and non-essential metals in direct proportion to their ambient concentrations and are generally classed as partial-regulators (Phillips and Rainbow, 1989). *T .deltoidalis* cadmium uptake in these experiments fits this pattern and the day 28 tissue cadmium concentration of exposed organisms shows they reached equilibrium with their exposure environment in this time. This is at odds with modelling work on cadmium accumulation in *T. deltoidalis* tissues by King *et al* (2005) which concluded that > 40 days would be required for *T. deltoidalis* to reach a steady-state concentration with their cadmium exposure environment. Bioturbation of sediments by deposit feeders leads to dispersal of metal oxides by mechanical action through burrowing as well as via bioresuspension when fluid faecal pellets are ejected into the water column (Meysman *et al.*, 2006). Atkinson (2007) showed that *T. deltoidalis* caused bioturbation of sediments which increased metal release compared to unmixed sediments. Surprisingly their study, which exposed *T. deltoidalis* to sediment containing concentrations of 25 µg/g of cadmium for 21 days, achieved a final cadmium tissue concentration of only 5 µg/g dry mass. The sediment used was highly silty < 63 µm = 73 % compared to this experiment < 63 µm = 30 % which may have affected feeding and burrowing behaviour. Their sediment also had significant concentrations of copper, lead and zinc so metal interactions may have affected the individual metal bioavailability (Moolman *et al.*, 2007). In

estuarine and marine environments cadmium is relatively soluble due to strong complexation by chloride (Stumm and Morgan, 1996). The positive linear sediment tissue cadmium concentration relationship for the two treatments (Figure 10a) suggests the sediment bound cadmium was readily resuspended and bioavailable and the major exposure route remained the same over the course of the experiment. King *et al* (2005) concluded this was largely via sediment ingestion, although, the model developed, using *T. deltoidalis*, was based on 24 hour exposure and depuration experiments and the authors felt that sediment retained in the gut and shell may have resulted in an overestimation of the tissue concentrations from sediment exposure compared to water and food exposures used.

Cadmium subcellular distribution

Once accumulated, around 50 % of the cadmium was detoxified (BDM) in organisms from all treatments (Figure 11). The control and 10 µg/g exposed organisms only converted a quarter of their total BDM into metal rich granules (MRG) with the remaining 75 % in the metallothionein like proteins (MTLP) fraction. The 50 µg/g exposed organisms, in contrast, converted 60 % of BDM cadmium to MRG with 40 % in the MTLP fraction (Figure 11). The formation of MRG has been associated with increased metal tolerance in marine organisms (Wallace *et al.*, 1998). George, (1983b) found that granules of cadmium exposed *Mytilus edulis* contained high concentrations of protein, calcium and sulphur which he postulated may be due to an increase in lysosomal protein degradation, due to enzyme inactivation by intracellular cadmium, causing an increase in intracellular protein turnover. The reduction in the percentage of cadmium in the lysosomal fraction of the exposed versus control organisms and the increase in cadmium in the MRG fraction of the 50 µg/g expose *T. deltoidalis* tends to support the route of cadmium transfer from the BAM lysosomal fraction to the MRG fraction (Figure 11). Metallothionein (MT) plays a key role in metal detoxification and the relationship between MT and transport of metal to sites of MRG production is also likely to be important (Wallace *et al.*, 1998). Cadmium bound to MT in the kidney of the mussel *Mytilus edulis* became incorporated in the granules (George, 1983a). Cadmium bound to MT in the digestive gland of *Mytilus galloprovincialis* becomes incorporated into lysosomes and is transformed into insoluble thionein polymers, a likely precursor of MRG (Viarengo *et al.*, 1987). It is likely that a combination of these two transfer routes are operating in *T. deltoidalis* as seen in the change in the percentage distribution of cadmium in the lysosomal, MTLP and MRG fractions with increasing cadmium exposure (Figure 11). The activity of marker enzymes cytochrome c oxidase (CcO), for mitochondria and acid phosphatase (AP), for lysosomes in the total homogenates (P1) showed an increase in activity of both organelles with increased cadmium exposure, particularly in the mitochondria. This is also seen in the mitochondria and lysosomal subcellular fractions (P3 & P4) and suggests that both organelles are responding to the accumulation of cadmium within the cells. The majority of cadmium in the BAM fractions of the cadmium exposed *T. deltoidalis* was in the mitochondrial fraction (Figure 11). Cadmium burdens in the freshwater bivalve *Pyganodon grandis* were also found to be higher in the mitochondria than the lysosome+microsome fraction with increased cadmium exposure (Bonneris *et al.*, 2005).

Li *et al*, (2003) showed that cadmium could directly lead to dysfunction of mitochondria including inhibition of respiration, loss of transmembrane potential and the release of CcO. In the BAM portion of the cadmium exposed *T. deltoidalis*, the percentage of cadmium in the mitochondrial fraction of the 10 µg/g and 50 µg/g organisms was up to 72 % compared to only 3 % in the controls (Figure 11). In terms of the total cadmium recovered in the mitochondrial

fractions of the 10 µg/g and 50 µg/g organisms, this represents a 2000 and 7200, respectively, fold increase in mitochondrial cadmium, compared to the control organisms. Extensive Cd^{2+} accumulation in mitochondria mediated by Ca^{2+} voltage dependant channels has previously been reported by Li *et al.* (2000; 2003). The increased mitochondrial cadmium observed in *T. deltoidalis* is also in agreement with studies on cadmium subcellular distribution following increased exposure in oysters *Crassostrea virginica* (Sokolova *et al.*, 2005a). Mitochondrial function was also found to be highly sensitive to cadmium at physiological and environmentally relevant low concentrations in oysters (Sokolova, 2004). Cadmium has been shown to have a high affinity for mitochondria, it is capable of inhibiting respiration and oxidative phosphorylation and interfering with the 1-hydroxylation of vitamin D (Fowler and Mahaffey, 1978). Lysosomes are involved in numerous functions including, nutrition, tissue repair, cellular defence, turnover of membranes, organelles and proteins as well as in the sequestration and metabolism of toxins, such as organic xenobiotics and metals and are an important target organelle for metal toxicity (Ringwood *et al.*, 1998).

While the percentage of cadmium in the lysosomal+microsomal fraction of the 10 µg/g and 50 µg/g cadmium exposed organisms was slightly less than half that of the controls (Figure 11), the cadmium associated with them was 46 and 100, respectively, times greater than that of the controls. Lysosomes have been identified as the metal cation homeostasis mechanism which sits between soluble binding ligands such as MT and the formation of insoluble precipitates such as MRG (Viarengo and Nott, 1993). While lysosomal cadmium uptake may reflect sequestration and detoxification of the metal, it can also lead to adverse effects when the handling capacity of the lysosomes is overwhelmed (Sokolova *et al.*, 2005a; Viarengo *et al.*, 1987). This fraction also contained the microsomal component of the cell. Since this includes fragmented endoplasmic reticulum, which is generally responsible for protein synthesis and transport; if cadmium in this fraction was associated with microsomes rather than lysosomes then this could be indicative of toxicity (Bonneris *et al.*, 2005). Similar amounts of cadmium were also associated with the heat sensitive proteins (HSP) of the 10 µg/g and 50 µg/g cadmium exposed organisms compared to the controls as those found in the lysosome+microsome fraction (Figure 11). The HSP fraction contains enzymes, high and low molecular weight proteins and other target molecules which are sensitive to metals. (Wallace *et al.*, 2003). The increased binding of cadmium to this fraction may therefore have implications for toxicity. The percentage of cadmium within the nuclei+cellular debris fraction of 10 µg/g cadmium exposed organisms was double, and in the 50 µg/g two and a half times the control cadmium. This fraction was not included in the BAM and BDM compartments as it contains tissue fragments, cell membranes and other cellular components of unknown consequence in terms of function, as well as the nucleic material (Wallace *et al.*, 2003). Binding of cadmium to cell membranes could result in toxicity, however, if the cadmium in this fraction was bound to less sensitive fractions within the cellular debris this could reduce binding to more sensitive cell components (Lucu and Obersnel, 1996). MT has been shown to be present in the nucleus of gill, digestive gland, gonad and posterior adductor muscle of *Mytilus edulis* in equal or lower concentrations than the corresponding tissue cytosol (del Castillo and Robinson, 2008). If this is also the case for *T. deltoidalis*, cadmium in the nuclei+cellular debris fraction may be associated with nuclear MT and so detoxified. The effectiveness of cadmium detoxification relies on binding to MTs transfer across cell membranes, compartmentalisation within lysosomes and the formation of MRG.

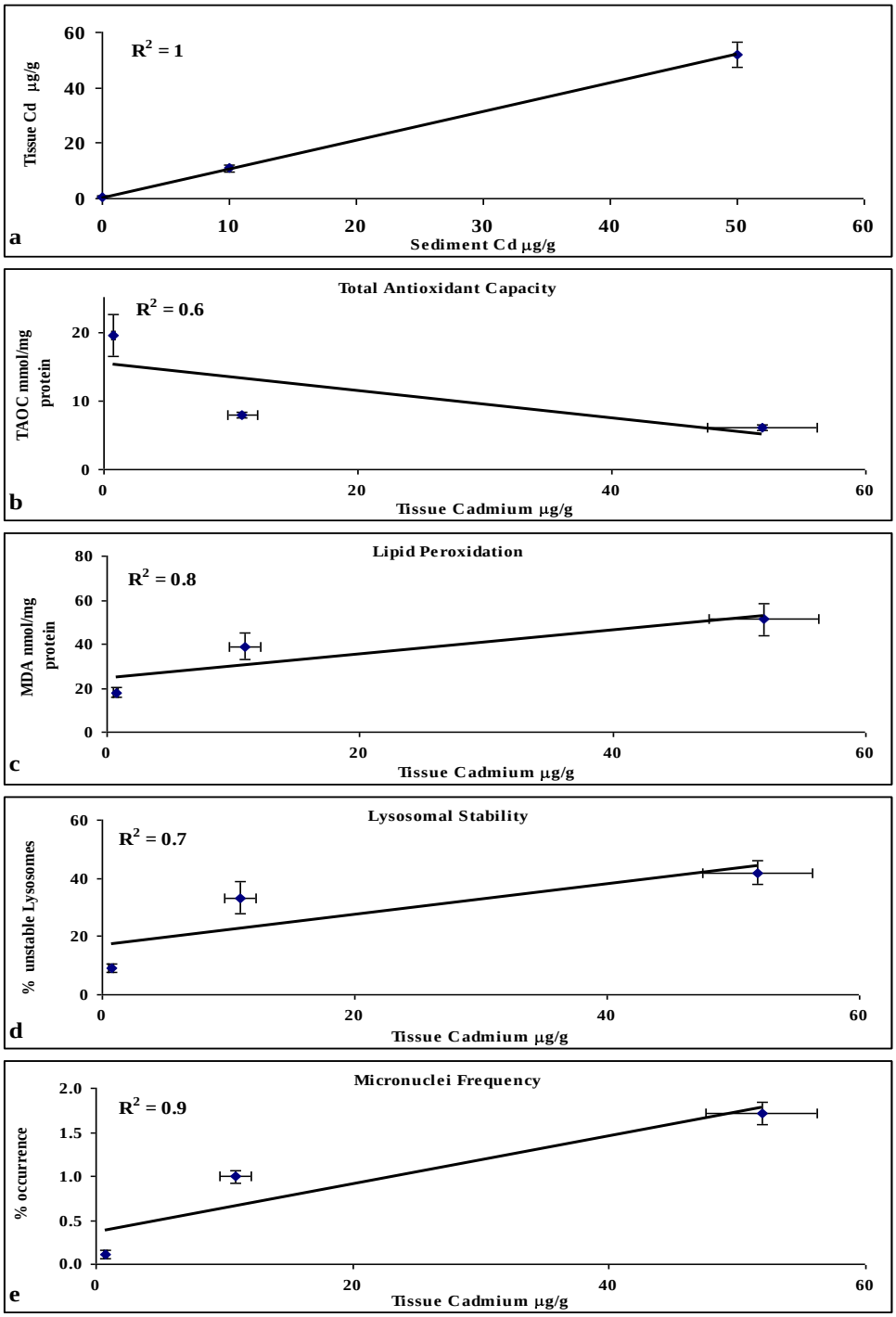

Figure 10. Regressions of *T. deltoidalis* tissue cadmium accumulation against sediment cadmium (a) and biomarkers total antioxidant capacity, lipid peroxidation, lysosomal stability and micronuclei frequency against tissue cadmium (b-e) after 28 days of exposure to cadmium spiked sediments. Mean ± SE, n = 12.

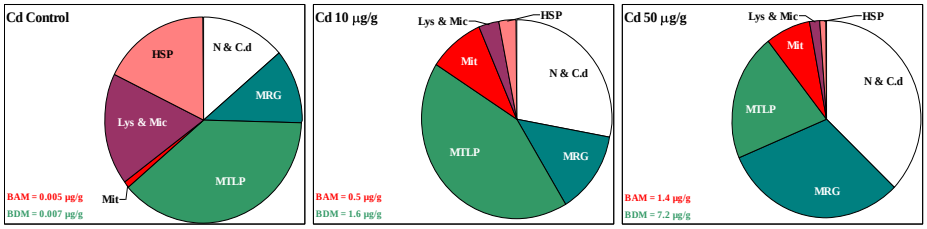

Figure 11. Distribution of cadmium in each of the subcellular tissue fractions of T. deltoidalis after 28 days exposure to cadmium spiked sediments. N & C.d = nuclei+cellular debris, MRG = metal rich granules, MTLP = heat stable metallothionein like proteins, Mit = mitochondria, Lys & Mic = lysosomes+microsomes, HSP = heat sensitive proteins. Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM), n = 2.

The presence of increased cadmium associated with these fractions shows that these processes were occurring, however, increases in cadmium within the mitochondrial fraction and to a lesser extent the HSP fraction indicates the detoxification and storage mechanisms were overwhelmed and unable to process the excess cadmium suggesting the potential for cadmium toxicity.

Biomarkers

Total antioxidant capacity

Marine invertebrates exposed to elevated concentrations of metals are susceptible to intracellular fluxes of reactive oxygen species (ROS) mainly produced by the Fenton reaction in the redox cycling of transition metals (Winston, 1991) or by redox inactive metal ions, such as cadmium, which can indirectly influence the oxidative system by reacting directly with cellular molecules to generate ROS, inducing cell signalling pathways (Leonard *et al.*, 2004) or depleting the cell's major sulfhydryl reserves (Ercal *et al.*, 2001). The capacity to reduce reactive oxygen species (TAOC) was significantly reduced in *T. deltoidalis* from both cadmium treatments compared to the control organisms (Figure 10b). A reduced oxyradical scavenging capacity in mussels exposed to a range of contaminants has also been reported: *Mytilus galloprovincialis*: sewage, agricultural, industrial and oil tanker effluents (Camus *et al.*, 2004), metals and PAHs (Frenzilli *et al.*, 2004; Regoli, 2000; Regoli *et al.*, 2004); *Modiolus modiolus*: cadmium (Dovzhenko *et al.*, 2005). A TAOC reduction in cadmium exposed *T. deltoidalis* indicates a breakdown in the reactive oxygen species detoxification pathway with the potential for higher order effects.

Lipid peroxidation

Thiobarbituric acid reactive substances TBARS are a measure of lipid peroxidation, which is a widely recognised consequence of excess oxyradical production (Winston and Di Giulio, 1991). Like the oxygen reduction system, the lipid production process is a complex sequence of biochemical reactions, broadly defined as oxidative deterioration of polyunsaturated fatty acids, which results in the production of highly reactive and unstable lipid radicals and a variety of lipid degradation products, the most abundant of which is malondialdehyde, that can alter the structure of cell membranes (Viarengo, 1989). The

process of lipid peroxidation destabilises cell membranes which can lead to loss of lysosomal integrity and the leaking of the lysosomal contents into the cytoplasm (Winston *et al.*, 1991). The TBARS concentration was increased in *T. deltoidalis* from both cadmium treatments compared to the control organisms (Figure 10c). Other cadmium exposed marine bivalves have also shown reduced antioxidant enzyme activity and a consequent increase in lipid peroxidation (Chelomin *et al.*, 2005; Company *et al.*, 2004; de Almeida *et al.*, 2004; Legeay *et al.*, 2005). The TBARS concentration was highly negatively correlated with the TAOC (Figure 12a), indicating that the progressive reduction in the capacity to reduce ROS and their subsequent increase directly influenced the build up of lipid peroxidation by-products.

Lysosomal stability

Lysosomes are intracellular organelles that contain acid hydrolases for the digestion of cellular waste: including excess or damaged organelles; food particles; viruses and bacteria. The lysosomal interior is more acidic (pH ≈ 4.8) than the cytosol (pH ≈ 7.2) and it is enclosed in a single membrane which stabilises the low pH by pumping protons from the cytosol via proton pumps and ion channels. Metals can also enter lysosomes via these channels, or more usually as protein complexes with metallothioneins, and it is thought that from here they are then formed into granules for storage or excretion (Marigómez *et al.*, 2002). Metal accumulation in the lysosomes can induce lipid peroxidation through redox cycling or by direct reaction with cellular molecules to generate ROS. This can destabilise the lysosomal membrane causing the contents to leak out into the cytosol thereby reducing the cell's capacity to remove waste which will ultimately lead to cell death (Viarengo *et al.*, 1987).

The cadmium exposed *T. deltoidalis* had significantly higher lysosomal destabilisation than the control organisms which had less than 10 % of lysosomes destabilised (Figure 10d), which is in the concern / stressed range of the criteria proposed by Ringwood *et al*., (2003) for lysosomal destabilisation organism health effects. Cadmium accumulation has been linked to lysosomal destabilisation in other marine bivalves: mussels *Mytilus galloprovincialis* (Regoli et al., 2004; Viarengo and Nott, 1993) and oysters *Crassostrea virginica* Ringwood *et al*., (2004; 2002). The lysosomal destabilisation was strongly negatively correlated with TAOC (Figure 12b) and positively correlated with TBARS (Figure 12d). This suggests that increased cadmium accumulation in metal sensitive tissue fractions initiated a reduction in the capacity to reduce ROS which may have both directly damaged lysosomal membrane but also induced lipid peroxidation which also induced lysosomal membrane destabilisation.

Micronuclei frequency

Micronuclei (MN) are small, intracytoplasmic masses of chromatin resulting from chromosomal breakage or aneuploidy during cell division. As an index of chromosomal damage the MN test is based on the enumeration of downstream aberrations after DNA damage and gives a time-integrated response to toxic exposure. The MN test is a sensitive test to detect genomic damage due to both clastogenic effects and alterations to the mitotic spindle (Migliore *et al.*, 1987). It has been used in bivalves to examine the genotoxicity of a range of chemicals including metals (Bolognesi *et al*., 2004; Burgeot *et al.*, 1996; Scarpato *et al.*, 1990; Williams and Metcalfe, 1992). The *T. deltoidalis* MN frequency increased with cadmium exposure (Figure 10e). Increased MN frequency has been observed in wild and, after 30 days, in caged mussels *Mytilus galloprovincialis* exposed to PAHs, cadmium and mercury along a pollution gradient on the Ligurian coast of Italy (Bolognesi *et al.*, 2004) and to a mix of metals near an offshore platform in

the Adriatic sea, Italy (Gorbi *et al.*, 2008). The frequency of MN in the cadmium exposed *T. deltoidalis* was negatively correlated with TAOC (Figure 12c) indicating that an increase in ROS resulted in an increase in genotoxic damage. DNA in cellular nuclei is a key cellular component that is particularly susceptible to oxidative damage by ROS (Cerutti, 1985). The frequency of MN in the cadmium exposed *T. deltoidalis* was positively correlated with TBARS (Figure 12e) suggesting that an increase in lipid peroxidation products may have contributed to an increase in genotoxic damage.

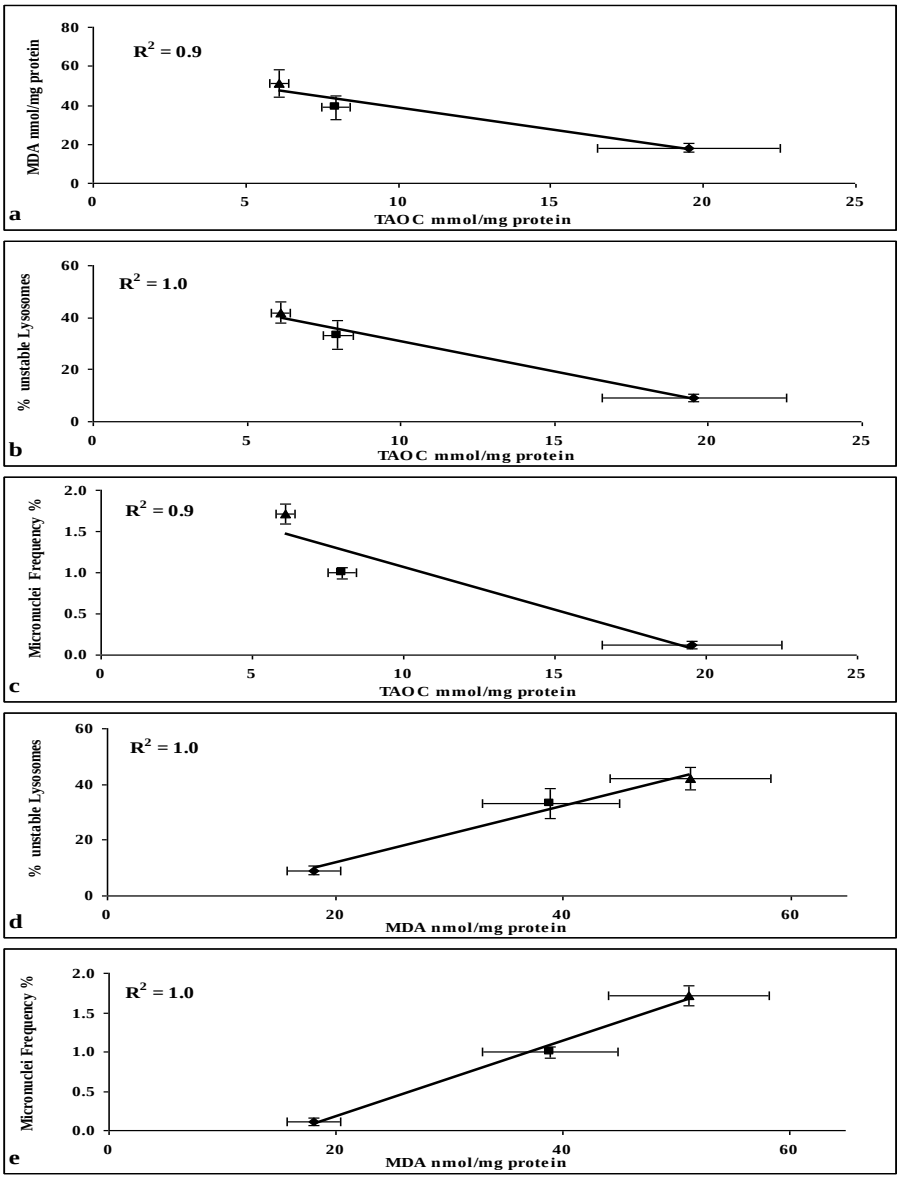

Figure 12. Regressions of total antioxidant capacity against lipid peroxidation, lysosomal stability and micronuclei frequency (a-c) and lipid peroxidation against lysosomal stability and micronuclei frequency (d-e) for control (◆), 10 (■)and 50 (▲)µg/g sediment cadmium treatments. Mean ± SE n = 12.

Gorbi *et al*., (2008) found zinc and cadmium bioaccumulation only slightly increased oxidative stress, intracellular accumulation of neural lipids and lysosomal destabilisation but MN frequency increased significantly, particularly during winter. Micronuclei frequency appears to be a sensitive indicator of cadmium toxicity in *T. deltoidalis*.

SUMMARY

A significant exposure – dose – response relationship for cadmium in *T. deltoidalis* was demonstrated in this study. Cadmium exposed *T. deltoidalis* accumulated cadmium over 28 days and reached equilibrium tissue concentrations which were equal to that of the sediment cadmium exposure concentrations. Approximately 50 % of accumulated cadmium was detoxified. *T. deltoidalis* exposed to 10 µg/g of cadmium converted 25 % and those exposed to 50 µg/g of cadmium converted 60 % of BDM to MRG with the remainder in the MTLP fraction. The majority of BAM cadmium in exposed organisms was in the mitochondrial fraction. Mitochondrial cadmium burdens of the 10 and 50 µg/g cadmium exposed *T. deltoidalis* were 2000 and 7200, respectively, greater than the control organisms and this was associated with an increase in the activity of the mitochondrial cytochrome c oxidase enzyme. The TAOC of cadmium exposed *T. deltoidalis* was significantly reduced compared to control organisms. The impairment of the oxidative system initiated a cascade of cellular damage. Lipid peroxidation increased, contributing to significant lysosomal destabilisation and increased frequency of micronuclei. The suite of biomarkers used in this study are good general biomarkers of exposure and effect and may be suitable for measuring responses to other contaminants. The biomarkers used show a cascade of metal exposure effects which are inter-linked, providing a more powerful picture of response to metals than a series of unrelated biomarker measurements. The significant exposure – dose – response relationships for cadmium established in this study indicate that sediment cadmium at these concentrations has the potential to lead to increased BAM burdens and impairment of individual *T. deltoidalis* at a cellular and subcellular level. This has implications for higher order effects which may impact on population viability in the long term.

CONCLUSION

A structured approach is required to establish links between organism metal exposure, dose and response. Specific measurable indicators that clearly demonstrate the interrelationships between each of the links need to be used. *Exposure* measures include sediment, water and dietary inputs, *dose* measures include total tissue metal burden, however, this does not give information on the metabolically available metal burden. The use of subcellular tissue fractionation allows the metabolically available burden, which has the potential to cause toxic effects, to be established. *Response* measures require the selection of a suite of biomarkers that both relate specifically to the effects of the toxicant of interest and are interlinked with each other in a *cascade* of effects from metabolic/enzymatic to cellular and genotoxic, which can ultimately be shown to lead to physiological and population level responses. Future development of this approach to sediment metal toxicity should attempt to

relate cellular damage to organism energy status to aid in linking these effects measures with overall organism condition and ultimately population health.

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