STUDIES ON THE METAL-CONTAINING GRANULES IN THE MUSSELS, *MYTILUS GALLOPROVINCIALIS* AND *VELESUNIO ANGASI*

This thesis is presented for the degree of Doctor of Philosophy at Murdoch University

2003

Submitted by

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I declare that this thesis is my own account of my own research and contains, as its main content, work that has not previously been submitted for a degree at any tertiary education institution.

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SIR WALTER MURDOCH

ON SITTING STILL

“Thinking is the hardest work in the world; most of us are too lazy to attempt it. We prefer what we call the Strenuous Life, which means being busy and fussy, and joining a dozen committees, imagining that we are doing a great deal of good in the world, and blinding ourselves to the fact that we are all suffering from St Vitus’ Dance – a disease which we can cure only by shaking off our laziness and acquiring the difficult art of sitting still.”
ACKNOWLEDGEMENTS

There is a long list of people without whom this document would never have reached completion. Firstly, I would like to thank my supervisors Associate Professor David Macey and Professor John Webb, both of Murdoch University, for their support, encouragement and patience.

I am very grateful to all those people who assisted me with sample collection, as I was unable to do this personally (although I would have liked to!). Thanks to the people at West Coast Mussels, particularly Mr Murray Campbell for allowing me to use his mussels free of charge, and to Mark Pieper and Peter Cross and their crews for collecting the mussels. Thanks also to Dr Rob Dineen for collecting the mussels from sites around the Derwent River and to Dr Barry Noller and Daphne, Rachel, Karen and Monica together with several children from the Kybrook Farm community, near Pine Creek, for the collection of *Velesunio* from creeks in the Northern Territory. Extra thanks also to both Barry and Rob for providing information on water quality and metal levels at the collection sites.

Huge thanks must go to Lesley Brooker for all her support, assistance with microscopy and for being a good friend. Thanks also to Gordon Thomson and Peter Fallon for assistance and advice on both light and electron microscopy, particularly to Gordon for keeping me sane during many months of sectioning.

A whole series of people were invaluable in assisting with analysis of samples. Many thanks to David Faithfull for carrying out the inductively-coupled plasma spectroscopy and helping me with the analysis of these results, to Tom Osborne and Andrew Foreman for assistance with anodic stripping voltametry and to Dr David Ralph for help with the analysis of these results.
Many thanks to Dr Shirley Slack-Smith of the WA Museum for unravelling the mysteries of freshwater mussel anatomy. Thanks also to Associate Professor Michael Borowitzka for providing the dried *Spirulina* to feed the mussels and assisting with culturing *Phaeodactylum* algae, and to Camilla Maltas for translating papers written inconveniently in French. Thanks to Stephen van Leeuwen for assistance with statistical analysis, and to the staff at CALM, Pilbara for continual harassment and encouragement.

Finally, a very big thankyou to all the other people who have supported and encouraged me during the years. These include my parents (Merilyn and Andrew) and parents-in-law (Kay and Mike) and the many friends and other students at Murdoch who provided a shoulder to cry on. Last but definitely not least, thankyou to my husband Jayson for all his love and support and for keeping the spark alive, and my son Tim, who provided many diversions, which had the unfortunate consequence of distracting me from the main job.

The work for this thesis was carried out with the financial support of Murdoch University and the provision of a Commonwealth of Australia APRA scholarship.
There is a continuing need to develop a simple and reliable biomonitoring technique for pollutants in aquatic systems. Mytilid mussels have been used extensively around the world for monitoring a range of pollutants. However the analysis of soft tissues in such programmes has some drawbacks, one of which is the large amount of variation between individuals in levels of specific pollutant. In order to assess the suitability of lysosomal kidney granules for such a role, it must be established that they are involved in the accumulation and detoxification of pollutants, and the range of change within the granules must also be assessed.

The structure and composition of intracellular metal-containing granules extracted from the kidney of the marine mussel *Mytilus galloprovincialis* from a non-polluted site have been examined using a variety of techniques. Comparisons were made between granules extracted from these mussels and those loaded with zinc in the laboratory, and those exposed to excess levels of zinc in the field. In addition, intracellular and extracellular metal-containing granules have been extracted from the freshwater mussel, *Velesunio angasi* and examined using similar techniques.

Granules isolated from the kidney tissue of fifty *M. galloprovincialis* (using a centrifugation process) were pooled to produce a single sample. Inductively-coupled plasma spectroscopy (ICP) revealed that the concentration of zinc in the kidney granules increased with the level of zinc-loading from a mean value of 5 260 µg.g\(^{-1}\) in freshly harvested animals to 12 487 µg.g\(^{-1}\) in animals loaded with 2.5 µg.g\(^{-1}\) zinc for 28 days. The other main elements present in the granules were phosphorus, sulfur, sodium, calcium, iron and copper. Granules from mussels exposed to excess zinc in the field had a greater concentration of zinc (13 570 µg.g\(^{-1}\)) than both field control animals (1 424 µg.g\(^{-1}\)) and the mussels loaded at the highest level of zinc in the laboratory. Silicon was also present in the granules extracted from the field-contaminated mussels.
Both light and transmission electron microscopy (TEM) of kidney tissue from *M. galloprovincialis* loaded with zinc in the laboratory showed signs of increasing degeneration as the level of zinc-loading increased. All cells examined contained membrane-bound granules. The kidney tissue of the field-contaminated mussels, despite containing high levels of zinc and many granules, showed no signs of degeneration.

Scanning electron microscopy (SEM) of the granules revealed that they were spherical in shape, while energy dispersive spectroscopy (EDS) of the isolated granules confirmed the results of the ICP analysis with regard to elemental composition. TEM also revealed that the granules themselves underwent structural changes as the level of zinc-loading increased. Thus, granules extracted from freshly harvested animals were electron-dense and regular in shape while zinc-loading produced granules with a concentric ring formation. In kidney tissue from mussels that were maintained in clean seawater for 14 days following 28 days of zinc-loading, the majority of the granules visible had the concentric ring formation. Granules with concentric rings were not found in kidney cells of the field-contaminated mussels. The number of multivesicular bodies in individual kidney cells also increased during zinc-loading and a positive relationship exists between the number of these bodies and the mean diameter of the granules in the cells.

The lack of degeneration in the cells of the kidney tissue in the field-contaminated mussels is almost certainly due to the fact that these animals were subjected to a chronic excess of zinc, rather than an acute dose as occurred in the laboratory-loaded animals. Presumably, this longer time period allows the animal to adapt and establish defence mechanisms, including the production of granules. The acute exposure to zinc, combined with the stress of being kept in the laboratory, may also have resulted in the appearance of granules with concentric rings in the laboratory zinc-loaded mussels.
In the freshwater mussel *Velesunio angasi*, extracellular granules were found mainly on the labial palps, mantle edge, gills and the junction between the foot and the visceral mass. ICP of pooled granules and EDS analysis of isolated granules showed that calcium, phosphorus, iron, manganese and barium were the major elements present.

Two types of intracellular granules were isolated from the kidney cells of *V. angasi*, the first time this has been recorded. The first was similar in composition to the extracellular granules and it was hypothesised that these two groups of granules are actually insoluble deposits of metal phosphates, despite being membrane-bound in the kidney cells. The second type of intracellular granule was mainly composed of phosphorus and sulphur and was possibly lysosomal.

It appears that difficulties with extraction of the granules and the analysis of the samples preclude the use of the metal-containing kidney granules of either *M. galloprovincialis* or *V. angasi* as biomarkers. The use of other biomarkers for pollutant contamination in mussels is discussed.
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

In recent decades, there have been increasing concerns about pollutants entering the aquatic environment. These have ranged from a general unease about damage to the environment to specific anxieties regarding pollutants entering foods for human consumption. A need to discover simple and reliable ways to monitor the levels of particular chemicals, heavy metals or other pollutants in the aquatic environment, and to elucidate the mechanisms of pollutant uptake and storage in organisms, has resulted in a proliferation of studies into the use of particular aquatic organisms as indicators of pollution.

1.1 MUSSELS AS BIOMONITORS

Some organisms have the ability to concentrate pollutants in their tissues and, as such, can be used to give a time-integrated indication of the environmental metal levels. These organisms have been dubbed “bioindicators” or “biomonitors” (Phillips, 1977). The characteristics of an ideal bioindicator were outlined by Phillips, (1977) from the descriptions of other authors. These characteristics are listed as follows:

1. The organism should accumulate the pollutant without being killed by the levels encountered.
2. The organism should be sedentary in order to be representative of the area of collection.
3. The organism should be abundant in the study region.
4. The organism should be sufficiently long-lived to allow the sampling of more than one year class.
5. The organism should be of reasonable size, giving adequate tissue for analysis.
6. The organism should be easy to sample and hardy enough to survive in the laboratory, allowing defeacation before analysis (if desired) and laboratory studies of the uptake of pollutants.

7. The organism should tolerate brackish water.

8. The organism should exhibit a high concentration factor for pollutants allowing simple direct analysis without pre-concentration.

9. A simple correlation should exist between the pollutant content of the organism and the average pollutant concentration in the surrounding water.

10. All organisms in a survey should exhibit the same correlation between their pollutant contents and those in the surrounding water, at all locations studied, under all conditions.

Bivalve molluscs have been recognised as being particularly useful bio-indicator organisms and, as such, have been used in many studies of the effects of pollutants such as metals (see for example Bryan, 1971; Elliott et al., 1985; McLusky et al., 1986; Fabris et al., 1994; Richardson et al., 1994; and Regoli, 1998). Mussels, in particular species of the genus *Mytilus*, have been used most extensively as they fulfil all the requirements of an ideal bio-indicator. Hence, as a group, they have worldwide distribution and where found, are usually abundant (Farrington et al., 1983). They are sedentary filter-feeders and appear to accumulate metals at levels representative of their environment. They also accumulate large, and thus, measurable quantities of metals/elements (Phillips, 1976b; Phillips, 1977) and quickly grow to a size which yields enough tissue for analysis. Probably most importantly, the concentration of many pollutants in the tissues of mussels appears to be proportional to the concentration of pollutant in the surrounding water (Amiard et al., 1987; Ritz et al., 1982). Finally, mussels are easy to collect from the environment and survive well under laboratory conditions, for as long as five months (Thompson et al., 1974).

The general goal of Mussel Watch programs is to determine the status and trends of chemical contamination of the coastal and estuarine environment. A number of
programs have operated throughout the world, including the United States (Goldberg, 1975; Goldberg et al., 1978; Goldberg, 1986; O'Connor, 1998; Tripp et al., 1992; O'Connor et al., 1994; Lauenstein & Daskalakis, 1998), France (Claisse, 1989; Beliaeff et al., 1998) the Mediterranean, Caribbean, South Pacific and Persian Gulf (Kistner, 1984), and throughout Asia (Tanabe, 2000). Trends have emerged for some contaminants but not for others. However, no adjustment is made for the potential impacts of physiological or external natural factors on the chemical concentrations. As time passes trend detection improves and it is expected that trends that are currently obscured by natural factors will eventually emerge (O'Connor et al., 1994).

The Mussel Watch programme conducted in the United States of America between 1976 and 1978 used whole body analysis of mussels (M. edulis and M. californianus) to determine background and pollutant levels of heavy metals, transuranic elements, halogenated hydrocarbons and petroleum-based hydrocarbons (Goldberg et al., 1978). Some reservations were raised in this study over the analysis of frozen whole animals, both with regard to the sediment trapped in the gut, and the variability between the two species of mussel. It was suggested that the shell may be a better indicator of pollutant levels than the frozen tissue (Goldberg, 1986). The major problem with using whole body analysis lies in the inherent variability of individual mussels to absorb and store pollutants. One particular case is the kidney of Mytilus edulis where zinc concentrations between individuals could range from 3% to 53% of the total body zinc load (Lobel, 1986). There are also variations in metal concentrations in mussels taken from different sites and at different times of the year. These type of problems led Luoma (1983) to declare that "realistic studies of metal uptake [in aquatic organisms] cannot be conducted without further understanding the role of membrane-bound vesicles and synthesis of metal-specific binding proteins in that process". The argument has also arisen that using mussels alone is that they cannot provide a complete picture of chemical contamination, and so other species such as birds, cetaceans and even humans may also need to be sampled (Kistner, 1984; Tanabe, 2000). In addition,
(Tanabe, 2000) has suggested analysis of air, water and sediments be incorporated into future monitoring programs.

In recent years there has been increasing interest in the use of mussel shells, rather than soft body tissues, as indicators of metal pollution. In order for this to be effective, a shell structural component that has not been exposed to metals, either dissolved or particulate, in the water must be used (Puente et al., 1996). Bivalve shells are made of crystalline calcium carbonate structured by an organic matrix, which are deposited from the pallial fluid (Lingard et al., 1992). Mytilus shell has two structural components, an outer prismatic calcite layer and an inner nacreous aragonite layer (Bourgoin, 1990), while freshwater bivalve shells have five layers, an outer proteinaceous layer, the periostracum, and four aragonite layers, with the innermost being the nacre (Imlay, 1982). The pallial fluid contains components involved in biomineralization and other constituents from the surrounding water, such as metals. Thus, any metals incorporated into the shell from the pallial fluid must have been assimilated by the mussel (Bourgoin, 1990; Lingard et al., 1992). In Mytilus, the only shell layer not exposed to the environment is the nacreous layer (Bourgoin, 1990; Lingard et al., 1992; Puente et al., 1996). However, the inner aragonite layers in freshwater mussels are neither exposed to the environment nor the pallial fluid and so reflect the metal levels in the environment at the actual time of deposition (Dermott & Lum, 1986).

The use of shells for biomonitoring has some advantages over the analysis of soft tissues. Shell nacre is far easier to handle and store and requires no depuration or freezing (Lingard et al., 1992; Puente et al., 1996). The process of metal incorporation into the shell is slow compared to metal uptake and release in soft tissues resulting in less variability in metal concentrations (Lingard et al., 1992) and higher consistency with levels in the surrounding water (Puente et al., 1996). In addition, shell nacre appears to be more sensitive as the metal is deposited over a longer period of time (Lingard et al., 1992; Puente et al., 1996). As the nacre consists of both a crystalline and an organic portion, there are potentially two sites of metal-binding. Firstly, metals
may be passively adsorbed from the pallial fluid into the organic matrix. Alternatively, metal ions may be actively substituted for calcium ions and incorporated into the crystal structure as part of the annual growth layers (Sturesson, 1976; Lingard et al., 1992). These growth layers provide the ability to use the shells of dead animals as environmental indicators. Carell et al. (1987) and Mutvei & Westermark (2001) analysed these layers in the shells of freshwater bivalves. These authors suggested that by using the shells of living mussels, together with museum or subfossil shell material, such studies could be used to determine the pollution history of a number of industries, including processing, mining, energy production, and the nuclear industry, as well as natural events such as volcanic emissions and the eutrophication of freshwaters. Specialised techniques such as µ-PIXE can show growth increments a short as a few hours, and seasonal variations with a single year (Carell et al., 1987).

Lingard et al., 1992 showed that the majority of lead and cadmium in the bivalve Elliptio complanata were bound in the crystal lattice and that the shell nacre had the potential to provide a reliable chronological index of metal exposure. However, these authors cautioned that species with a high organic matrix component in their shells should not be used as bioindicators. The shell of Mytilus galloprovincialis has been shown to be a good indicator of lead (Puente et al., 1996; Sturesson, 1976; Bourgoin, 1990) and cadmium (Sturesson, 1978). However, it does not appear to be useful for determining levels of zinc relative to levels in the environment, as the majority of this metal is taken up by the soft tissues. In addition, much of the zinc in the shell of the bivalve Elliptio complanata was found in the organic periostracum, an outer layer that is exposed to the environment (Dermott & Lum, 1986), while up to 50% of the zinc in shells of Scrobicularia was speculated to be adsorbed onto the outside of the shell directly from solution (Bryan & Uysal, 1978).

Levels of some metals may be many orders of magnitude lower or higher in shells than in soft tissues. In freshwater mussels, Imlay, (1982) found that, when compared to soft tissue concentrations, levels of cadmium were nearly 100 times higher, copper six
times, lead 450 times and manganese one time higher in the shell. In contrast, zinc was two to four times lower, magnesium was 10 times lower and iron was approximately the same in shell and soft tissues (Imlay, 1982). Goldberg (1986) also noted that there was not a strong relationship between metal levels in shells and soft tissues in mussels analysed during the US Mussel Watch. Thus, monitoring programs in less contaminated sites may be restricted to using soft tissues (Bourgoin, 1990). In addition, levels of metals in shells may not directly reflect levels in the environment, but rather availability and physiological exchange rates during periods of growth (Dermott & Lum, 1986).

1.2 MUSSEL TAXONOMY

Until relatively recently, the taxonomy of the genus *Mytilus* has been somewhat confused (Koehn, 1991; McDonald et al., 1991; Seed, 1992). While a number of factors have contributed, this was mainly due to the widespread location of these animals throughout the temperate waters of the world, plasticity of the shape of the shell especially with regard to environmental factors, and the fact that morphological characters were mainly used in the identification of species. The greatest confusion and controversy has arisen through attempts to delineate *Mytilus edulis* and *M. galloprovincialis* as separate species or alternatively, to determine whether the latter is merely a subspecies.

The *Mytilus* lineage dates back to the Jurassic, between 200 and 300 million years ago, when shell characteristics enabling more powerful attachment to the substrate evolved (Skibinski et al., 1980). Amongst the smooth-shelled mytilids, *Mytilus edulis* appears to be the ancestral species (Seed, 1992). *Mytilus galloprovincialis* is thought to have arisen in the Mediterranean from *M. edulis* (Seed, 1992). This differentiation could have been favoured by warmer conditions in the Mediterranean and reduced contact with the Atlantic during one of the Pleistocene ice ages. This places the time of
divergence of \textit{M. edulis} and \textit{M. galloprovincialis} within the last one or two million years (Skibinski \textit{et al.}, 1980).

Historically, smooth-shelled taxa considered to be distinct species included \textit{M. edulis} Linnaeus 1758 from north temperate waters, \textit{M. galloprovincialis} Lamarck 1819 from the Mediterranean Sea, \textit{M. trossulus} Gould 1850 from the Pacific coast of North America, \textit{M. chilensis} Hupe 1854 from Chile, \textit{M. platensis} Orbigny 1846 from Argentina, \textit{M. planulatus} Lamarck 1819 from Australia and New Zealand, and \textit{M. desolationis} Lamy 1936 from the Kerguelen Islands in the southern Indian Ocean (Lamy, 1936). However, Soot-Ryen (1955) considered most of these to be subspecies of the \textit{M. edulis} complex. Koehn (1991) also considered \textit{M. desolationis} to actually be synonymous with \textit{M. edulis}. \textit{M. aoteanus} Powell 1958 was described from New Zealand, but was reduced to a subspecies of \textit{M. edulis} shortly afterwards. \textit{M. californianus} Conrad 1837 from the Pacific coast of North America and \textit{M. coruscus} Gould 1861 from the Pacific coast of Asia both have rough shells and hence, are easily distinguished from the other species (Soot-Ryen, 1955). DNA sequence divergence has separated these two species (Milyutina & Petrov, 1989, (in Russian) cited in McDonald \textit{et al.}, 1991).

When reviewing the taxonomic status of \textit{Mytilus galloprovincialis} in Western Europe, Gosling (1984) concluded that differences between geographically isolated populations of \textit{M. edulis} and \textit{M. galloprovincialis} were not great enough for them to remain as a distinct species, and that they should be regarded as a subspecies, or race, of \textit{Mytilus edulis} and that the name \textit{Mytilus edulis} var. \textit{galloprovincialis} be adopted. However, this suggestion seems to have been largely ignored by subsequent publications. This is likely to be due mainly to the advent of techniques such as allozyme analysis that have greatly clarified the status of the genus. Indeed, three major groupings have been identified within this complex using these techniques - \textit{M. edulis}, \textit{M. galloprovincialis} and \textit{M. trossulus} (Koehn, 1991; McDonald \textit{et al.}, 1991).
Only *M. edulis* and *M. galloprovincialis* occur in the Southern Hemisphere (McDonald *et al.*, 1991). Hybridisation occurs between these taxa where their distributions overlap (Sarver & Foltz, 1993), although only two species are ever found at the one site (McDonald *et al.*, 1991), and this has caused some speculation on the validity of their taxonomic status. However, many authors feel that because each of the three taxa maintains a distinct set of alleles, with fairly homogenous allele frequencies, over vast distances, in the face of massive migration of planktonic larvae, they should be regarded as distinct species (see for example Koehn, 1991; McDonald *et al.*, 1991; Seed, 1992).

A number of studies using Australian mytilid mussels have added to the confusion regarding the taxonomic status of the animal by using different species names. For example the name *M. edulis planulatus* was used by Ritz *et al.* (1982), Elliott *et al.* (1985), Fabris *et al.* (1994) and Richardson *et al.* (1994) while *M. edulis* was used by Talbot (1985), Bootsma *et al.* (1988), (1990), and Burbidge *et al.* (1994).

In Australia, McDonald *et al.* (1991) provide concrete evidence for the taxonomic status of the *Mytilus* group of mussels. Mussels collected from both Tasmania and the south of Western Australia had allele frequencies and shell characters similar to those of *M. galloprovincialis* from the Northern Hemisphere. The authors thus concluded that the mussels found in Australia were, in fact, *M. galloprovincialis* despite some differences in the allele frequencies between these mussels and those from the Northern Hemisphere. Alleles at two of the eight loci investigated, which are found only in *M. edulis* in the Northern Hemisphere, were seen in the Australian mussels. One of these was found to occur at low frequency in Tasmanian mussels while the second was common in mussels from both areas. Statistical analysis of both the allele frequencies and shell characters provided clear evidence that the Australian mussels were far more similar to *M. galloprovincialis* than *M. edulis* (McDonald *et al.*, 1991). The same study also indicated that *M. galloprovincialis* was indigenous to Australia and had not been introduced in recent years through the movements of man, as it has to areas such as Japan (Wilkins *et al.*, 1983) and South Africa (Grant & Cherry, 1985).
1.3 ZINC

1.3.1 Zinc requirements

All living organisms require zinc (Galdes & Vallee, 1983). While the majority of the effects of zinc have been elucidated in mammals, the fact that it is involved in fundamental biological and biochemical processes suggests that it could be assumed to have the same or very similar functions throughout the Animal Kingdom. Zinc plays an important role in nucleic acid synthesis and protein metabolism and so, in cell replication (Underwood, 1977). It is also required for gene activation (Underwood, 1977). Zinc is an integral part of many membranes, enzymatic and non-enzymatic proteins, with at least thirty mammalian enzymes containing zinc (Bettger & O'Dell, 1981; Galdes & Vallee, 1983). Examples include copper/zinc superoxide dismutase, which acts to prevent the accumulation of the superoxide radical in the tissues (Fridovich, 1975; Brock & Harris, 1979), aspartate transcarbamylase which catalyses the first step in pyrimidine biosynthesis (Stryer, 1988), metallothionein which acts as a scavenger of heavy metal ions (Viarengo et al., 1999), and the zinc-finger proteins. This last group have no enzymatic activity but many bind to DNA promoter sites or are part of critical enzymes and appear to have a structural, or control, role (daSilva & Williams, 1991).

Optimum concentrations of zinc are presumed to be those normally found in the environment (Bryan, 1971). Excess levels of an essential metal may become inhibitory (Viarengo, 1985) and the effects of excess zinc on growth are thought to be due to inhibition of enzymes involved in protein synthesis and cell division (Bryan, 1971). On the other hand, zinc deficiency in mammals may cause symptoms ranging from loss of appetite and growth retardation to effects on reproduction, nucleic acid and protein synthesis and decreased activities of numerous enzymes in the tissues (Spivey Fox, 1970; Underwood, 1977). Utilisation of amino acids in protein synthesis is impaired under conditions of zinc deficiency, possibly due to an abnormality in synthesis and/or

1.3.2 Zinc in the Environment

Zinc is a classic example of a metal which has become relatively more abundant in the marine environment in recent times. It may enter the oceans either from sacrificial anodes used on ships and jetties or from industries using processes such as metal stripping, galvanising or synthetic fibre production (Bryan, 1971). In most marine environments, zinc is the major trace metal and natural levels in inshore seawater range from approximately 0.1 to 20 $\mu$g.l$^{-1}$ (George & Pirie, 1980). One study on the Indian Ocean found zinc concentrations ranging from 0.2-13.0 $\mu$g.l$^{-1}$ (Danielsson, 1980). However, these values may be slightly high due to the possibility of contamination.

Studies on the speciation of zinc in seawater have shown that the majority is complexed to both organic and inorganic ligands (Bruland, 1989; Lewis & Landing, 1992; Morel et al., 1991; Zirino & Yamamoto, 1972). Modelling of dissolved zinc speciation carried out by Zirino & Yamamoto (1972) showed that at pH 8.1, 17% of the zinc remained uncomplexed, while Turner et al., (1981) showed that at pH 8.2, 46% of the dissolved zinc was present as the free ion. These findings are substantially higher than estimates of 1-2 % made from studies of actual seawater (Bruland, 1989). In the former studies, the complexed zinc was present as Zn(OH)$_2$ (62%), ZnCl$^+$ (6.4%), ZnCO$_3$ (5.8%), ZnSO$_4$ (4%) and ZnCl$_2$ (4%) (Zirino & Yamamoto, 1972) or ZnCl$_2$ (65%), Zn(OH)$_2$ (22%), ZnSO$_4$ (7%), ZnCO$_3$ (5%) and ZnF (<1%) Turner et al., (1981). However, these models do not account for the particulate fraction of the zinc, that is zinc absorbed onto particulates in the water or taken up by plankton. This is due to the limitations of the techniques used which can only measure dissolved free metal ions (Florence & Batley, 1988).
Unpolluted waters contain very little free metal ion as most of the metal is present as non-toxic complexes or adsorbed on colloidal particles (Florence & Batley, 1988). There is considerable metal speciation of class b metals as they have a strong tendency for covalent binding (Simkiss & Taylor, 1989). Studies by Bruland (1989) found that in the surface water of the central North Pacific, 80-90% of the zinc was dissolved and that organic ligands, which were present in low nanomolar concentrations, formed strong complexes with the zinc, and, as such, controlled speciation. In the Black Sea, a similar situation was found where the majority of the zinc in the surface water (more than 95%) was dissolved and present as organo-zinc complexes (Lewis & Landing, 1992). It would appear that trace metal content of the particulate material in coastal marine waters is governed by numerous factors, of which adsorption, desorption, coprecipitation, and concentration by marine organisms are the most important (Abdullah & Royle, 1974).

The fraction of total metal in a solution that is available to animals is dependent on the relative affinity of the metal for solution ligands and the number and type of receptor molecules on the cell’s surface (Florence & Batley, 1988). Previous studies have shown that the free (hydrated) metal ion is the most toxic form and that toxicity is related to the activity of the free metal ion rather than the total metal concentration (Turner, 1984). The free metal ion is also the most bioavailable form. Bioavailability depends strongly on the chemical speciation and it has been shown that uptake of soluble zinc in M. edulis decreased when organic ligands were present to reduce the amount of bioavailable free metal ion (Vercauteren & Blust, 1996). Lipid-soluble complexes are also very toxic due to their ability to pass quickly and easily across cell membranes (Turner, 1984; Florence & Batley, 1988). The availability of the various metal species to organisms could result in differences occurring between uptake after acute and chronic exposure and between studies which measure initial uptake and those which take measurements over a significant part of the organism's life.
1.3.3 Zinc Uptake

The level of zinc in individual mussels has been found to be affected by the size, and hence age and weight, with smaller individuals in a population usually having a higher concentration of zinc within their tissues (Boalch et al., 1981; Boyden, 1974; Boyden, 1977; Jones & Walker, 1979). Salinity and temperature do not seem to affect the uptake of zinc by mussels, although zinc becomes lethal more quickly at higher salinities and temperatures (Phillips, 1976b; Cotter et al., 1982; McLusky et al., 1986).

Levels of zinc in mussels change both with the season and the reproductive state of the animal. Thus, in both *M. edulis* (Amiard et al., 1986) and *M. galloprovincialis* (Regoli & Orlando, 1994), maximum zinc levels have been recorded in late winter and early spring with minimum values occurring in late spring and early summer. These variations, however, reciprocate the seasonal changes in body weight (Dare & Edwards, 1975; Regoli & Orlando, 1994) and so the total metal content of individual mussels remains relatively constant throughout the year (Phillips, 1976a).

Very little is known about the uptake of particulate zinc in *Mytilus*, or the differences between this and the uptake of soluble zinc, including its radioactive forms. It has been suggested that food particles provide the predominant source of zinc to mussels (Bryan, 1971; Wang & Fisher, 1997), making the study of particulate zinc uptake vital to the understanding of the accumulation of this metal.

The uptake of soluble zinc by *M. edulis* has been shown to be linear and in direct proportion to the time of exposure (Myint & Tyler, 1982; Ritz et al., 1982; Elliott et al., 1985) and the concentration in seawater (Wang et al., 1996). In bivalves, the gills and digestive system are the most important organs for uptake of soluble zinc (Pentreath, 1973; Carpene & George, 1981; Amiard et al., 1986; Vercauteren & Blust, 1996). Vercauteren & Blust, (1996) showed that the uptake of free zinc increased in the digestive system, gills and haemolymph with increasing free zinc ion activity.
In contrast to soluble zinc, particulate zinc needs to be digested and solubilized in order to be available to the mussel. There are two distinct phases of digestion in bivalve molluscs (Morton, 1973). Extracellular digestion occurs in the stomach where the crystalline style releases extracellular enzymes which degrade whole food particles. Intracellular digestion occurs in the digestive tract and involves cells phagocytosing fine particles released from the stomach following extracellular digestion. These fine particles are then digested by the action of intracellular enzymes (Morton, 1973). Intracellular digestion is more efficient than the extracellular process. The uptake of particulate zinc has been shown to be partially managed via a reduction in the efficiency of assimilation of radio-labelled food particles when food quantity increases (Wang et al., 1995). In fact, these authors showed that a 1 mg.h\(^{-1}\) increase in ingestion rate, a measure of food availability, produced a 12% reduction in the assimilation efficiency of *M. edulis*. The observation of a higher assimilation efficiency when food quantity was lower is due to a longer residence time in the gut and a higher proportion of the food being digested intracellularly (Wang et al., 1995).

Regardless of their initial source, one process by which heavy metal ions enter the cell is thought to be transport across the cell membrane via a "shuttle". Indeed, zinc uptake into mammalian hepatocytes is via a membrane-transport protein that shuttles the metal back and forth across the membrane (Simkiss & Taylor, 1989). However, the question remained as to whether the transport molecule diffused across the membrane, acting as a carrier, or if the protein instead acts as a channel. Carpene & George (1981) have shown that the transport of zinc in the gills of *M. edulis* proceeds by initial binding to membrane-bound proteins, followed by a lag during which the zinc ions become bound to metallothionein. The results obtained by George (1980); Vercauteren & Blust (1996); and Wang & Fisher (1999) also suggest that zinc uptake in *Mytilus* is a carrier-mediated process involving intracellular proteins and low molecular weight SH containing compounds, such as metallothionein. (Vercauteren & Blust, 1996) concluded that zinc is released from complexes when it enters the gut, as the availability of zinc was higher in the gut than in the gills while the free zinc ion activity was the
same. Studies on the impacts of calcium channel blockers on zinc uptake indicated that, in *Mytilus edulis*, zinc is preferentially taken up through different gateways than calcium, but that cadmium and zinc share a common uptake pathway (Vercauteren & Blust, 1999). These authors also showed that different channels may be used in different organs.

### 1.4 Detoxification of Metals

Three metal cation detoxification systems have been shown to act in the cells of marine invertebrates (Viarengo & Nott, 1993). The first system utilises specific, soluble ligands which bind to the metal ions, the most important of which is metallothionein. The second mechanism involves compartmentalization of metals within membrane-limited vesicles, now recognised as lysosomal. Thirdly, the animal can form insoluble precipitates such as Ca/Mg or Ca/S granules into which the metal ions are incorporated. These three systems show different degrees of effectiveness in detoxification of metals both in different organisms, and in different cell types in the same organism (Viarengo & Nott, 1993).

#### 1.4.1 Soluble Ligands

1.4.1.1 Metallothionein

Metallothioneins are a superfamily of metal-binding proteins. Metallothionein was first isolated from equine kidney by Margoshes & Vallee (1957) and it has since been isolated from a number of vertebrates, including a range of mammals and fish, and many invertebrates. Noel-Lambot (1976) was the first to isolate metallothoinien-like proteins in an invertebrate, the mussel *Mytilus edulis*. It has subsequently been isolated and characterised in a number of aquatic invertebrates, and it has been further characterised by Talbot & Magee (1978); George & Pirie (1979); Frankenne *et al.* (1980); and Nolan & Duke (1983).
Amongst the crustaceans, species shown to have metallothionein include the crabs \textit{Scylla serrata}, \textit{Cancer magister} (Olafson et al., 1979), and \textit{Carcinus maenas} (Rainbow & Scott, 1979), and the shrimp \textit{Acetes sibogae} (Olafson et al., 1979). Molluscs shown to have metallothionein include the chiton \textit{Cryptochiton stelleri} (Olafson et al., 1979), the winkle \textit{Littorina littorea} (Bebianno et al., 1992), the clam \textit{Macoma balthica} (Bordin et al., 1994), oysters (unspecified species) (Casterline & Yip, 1975) and \textit{Crassostrea virginicus} (Roesijadi, 1994), the marine mussel \textit{M. galloprovincialis} (Bebianno & Langston, 1992) and the freshwater mussels \textit{Anodonta} (now \textit{Pyganodon}) \textit{grandis} (Couillard et al., 1993) and \textit{Hyridella depressa} (Vesk & Byrne, 1999).

Metallothioneins are low molecular weight, soluble, heat stable proteins that are rich in sulphhydryl (SH) groups and have an unusual amino acid content, characterised by a high number of cysteine residues (nearly one-third in mammalian metallothioneins) and a virtual absence of histidine, methionine and aromatic amino acids (Viarengo & Nott, 1993). Metallothioneins have a strong metal-binding capacity and are able to chelate both essential (for example zinc and copper) and non-essential (for example cadmium, mercury, silver, gold and bismuth) metals (Olafson et al., 1979; Bremner & Beattie, 1990; Viarengo et al., 1999a). They also show different affinities for different metals (Viarengo, 1989).

In mussels, the production of metallothioneins is strongly induced by metals and to a lesser extent by oxidants (Viarengo et al., 1999ab). The level of these metal-binding proteins is also influenced by strong changes in environmental parameters such as temperature and oxygen levels in the water (Viarengo et al., 1988) and with the seasons, following zinc levels in the tissues (Harrison et al., 1988). However, organic pollutants seem to be unable to stimulate the production of metallothionein in mussels, as they do in vertebrate animals such as fish (Viarengo et al., 1999a). The movement of metal ions into the cell induces the synthesis of apothioneins, which then chelate the metal ions (Brady, 1982; Viarengo & Nott, 1993). There is, however, a lag period observed in both mussels and other organisms, between exposure of the animal to excess metal and
the production of new metallothionein (Brady, 1982). Metallothionein is thus able to protect cell structures from non-specific binding with metal cations, and to detoxify excess metal (Viarengo et al., 1999a).

This role in detoxification has been suggested for non-essential metals such as cadmium and mercury. However, metallothionein has been implicated in the regulation of the essential metals copper and zinc in mussels and oysters (Bebianno & Langston, 1993; Roesijadi, 1994). In mammals, metallothionein is believed to be involved in macromolecular synthesis, and is implicated in the transfer of zinc and copper to apoenzymes (Brady, 1982). This may be linked to the fact that the rate of turnover of metallothionein is much higher in mammals than in mussels (Bebianno & Langston, 1993). The fact that metallothionein synthesis is inducible by oxidants also indicates a role for this protein in the cellular antioxidant defence system (Viarengo et al., 1999b). In fact, metallothionein clearly protects cells from oxygen free radicals, and it is speculated that this is due to scavenging of hydroxyl radicals (OH\(^\cdot\)) (Viarengo et al., 1999b) and through metal binding/release dynamics, as the protein acts to reduce levels of the prooxidant metals copper and iron, and release zinc which helps to stabilise membranes (Viarengo et al., 2000).

Metallothionein can be considered as an important specific biomarker to detect organism response to inorganic pollutants such as cadmium, mercury, zinc and copper (Viarengo et al., 1999a). Reducing the bias in biomonitoring data, introduced by changes in environmental parameters effecting metallothionein levels, can be done by using caged mussels for short periods (up to one month) in sites where these parameters are comparable (Viarengo et al., 1999a). In addition, metallothionein should be used in conjunction with other biomarkers of pollutant exposure such as mixed-function oxygenase activity and acetylcholinesterase activity, and biomarkers of stress such as destabilisation of lysosomal membranes, lipofuscin accumulation and DNA damage (Viarengo et al., 1999a).
1.4.1.2 Other Metal-Binding Proteins

Other soluble cytosolic proteins may also be involved in the detoxification of excess metals. In *M. edulis* exposed to normal environmental levels of soluble zinc, the majority of the zinc is weakly associated with very low molecular weight proteins, although these have not been identified (George, 1980). However, in the oyster, *Ostrea edulis*, 40% of the cellular zinc was shown to be weakly bound to taurine, lysine, ATP and possibly homarine, all of which weigh less than 5000 Da (Coombs, 1974). In contrast, brief exposure of *M. edulis* to metals resulted in the zinc being bound to high molecular weight macromolecules (George & Viarengo, 1985).

Specific transport and storage proteins can be used in the control of intracellular concentrations of metal ions through varying degrees of metal saturation. The metal storage proteins tend to contribute to a greater proportion of the total metal burden due to their ability to bind a much larger number of metal atoms (George, 1982). Most of these proteins are continually degraded and synthesised so that the buffering of the metal concentration can be achieved by inducing the synthesis of more protein (George, 1982). Of these proteins, the major transporter of zinc is thioalbumin, a variant of serum albumin that contains an SH group to bind the zinc. Some of the zinc is also transported by α2-macroglobulin, which carries 3-8 zinc atoms (George, 1982). In addition, proteins not normally associated with a particular metal may be utilised. For example, oysters (*Ostrea edulis*) with elevated levels of tissue zinc utilise the iron storage protein ferritin to bind and store up to 40% of the zinc (Webb et al., 1985), although the majority of the zinc was bound to proteins with a molecular weight of less than 15 000 Da. These authors suggested that ferritin may also be involved in the zinc metabolism of other bivalves. The iron transport protein transferrin may also be involved in the transport of zinc (George, 1982). A low molecular weight ligand of about 300 Da that binds zinc and has been putatively identified as dicarboxylic acid, has been isolated from crab hepatopancreas (Viarengo & Nott, 1993).
1.4.2 Metal-containing Granules

1.4.2.1 Types of Granule

While metal-containing granules can be found in all of the major invertebrate phyla, they are mainly present in organs with digestive, excretory and/or storage functions (for example the hepatopancreas, kidney, digestive gland and Malphigian tubules) (Brown, 1982). The majority of these granules are intracellular, with the occurrence of extracellular granules being less frequent. However, freshwater bivalves contain extensive deposits of extracellular granules, with the study of intracellular granules in this group being very limited. Generally, metal-containing granules appear to come in two forms: those which involve the lysosomal system in their formation, and those which are formed from insoluble metal precipitates (Viarengo & Nott, 1993). Granules are commonly amorphous rather than crystalline in structure, are often heavily hydrated and usually contain some organic component (Taylor & Simkiss, 1989).

The type of anion present can determine which metal ions are found in the granules. Metal ions can be divided into three classes (Ahrland et al., 1958, Figure 1.1). Class 'a' metals are those which form their most stable complexes with the first ligand atom in each Group of the periodic table. This class includes most metals in their common valency state and they are more commonly found associated with oxygen donor ligands, such as carbonate, oxalate, phosphate and sulphate. Examples of class 'a' metal ions are aluminium $3^+$, magnesium $2^+$ and calcium $2^+$. Class 'b' metals form their most stable complex with the second or subsequent ligand in each Group (Ahrland et al., 1958). Metal ions in this class include copper$^+$, silver$^+$, gold$^+$, plutonium $3^+$ and mercury $^+\text{ and } 2^+$. These metals usually associate with sulfur or occasionally nitrogen ligands (Ahrland et al., 1958; Taylor & Simkiss, 1989). There are also a range of metal ions which are classed as borderline, including manganese $2^+$, iron $2^+$ nickel $2^+$, cadmium $2^+$, copper $2^+$ and lead $3^+$ (Ahrland et al., 1958). While zinc $2^+$ is strictly classified as a
‘class a’ metal ion, it behaves more like a borderline element with considerable ‘class b’ characteristics (Niebor & Richardson, 1980).

Figure 1.1: Periodic table showing the position of class ’a’, ’b’ and borderline elements (after Ahrland et al., 1958).

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LEGEND:
- Class ’a’ elements
- Class 'b' elements
- Borderline elements
Granules that contain the carbonate anion are usually found in the connective tissues, and are generally involved in the storage and remobilisation of calcium (Taylor & Simkiss, 1989). They are surrounded by a vacuole membrane, although they are not lysosomal, and show characteristic concentric layering. The major cation may be calcium, or magnesium as in *Littorina littorea* (Mason & Nott, 1981). These granules are spherical or ovoid, 0.5-40 µm in diameter (Taylor & Simkiss, 1989), crystallographically well-organised and form in the Golgi apparatus (Simkiss, 1976), and their composition appears not to be affected by the environment (Taylor & Simkiss, 1989).

A more complex system exists in granules containing phosphate as the major anion. These granules may be intra- or extracellular and contain metal ions as well as the phosphate (Taylor & Simkiss, 1989). Again, calcium is usually the most abundant cation. Some granules may be predominantly calcium phosphate, while others may also contain magnesium, potassium, manganese, iron and/or zinc. The phosphate may be present either as polyphosphate or pyrophosphate. Phosphate granules are also membrane bound and usually show concentric rings. The water content of these granules ranges from 18 to 31% while the organic component may be between 2 and 60% wet weight and their composition may be environmentally dependant (Taylor & Simkiss, 1989).

Granules which contain more than one anion are also quite common. These types of granules fall into two groups, those which contain carbonate or oxalate as well as phosphate, and those containing mainly phosphorus, sulfur and chlorine (Taylor & Simkiss, 1989).

Class 'b' and borderline metals can be found in sulfur granules which appear to be involved in the regulation and detoxification of these metals. This may be related to the binding of these metals by proteins such as metallothionein (Taylor & Simkiss, 1989). Sulfur granules have a heterogeneous appearance without concentric rings and are
membrane-bound. The most common cations found in the sulfur granules are copper, zinc, cadmium and mercury (Taylor & Simkiss, 1989). These are also the metal ions which bind most frequently to metallothionein (Viarengo et al., 1999a). Sulfur granules appear to have a different origin from the carbonate or phosphate granules described above (Taylor & Simkiss, 1989). It has been hypothesised that sulfur granules contain metallothionein degradation products, presumably as a result of lysosomal activity. Granules that contain copper as their major metal component may also function as a copper store (Brown, 1982).

1.4.2.2 Formation of lysosomal metal-containing granules

Many of the intracellular granules that have been studied from a wide variety of invertebrate animals appear to be lysosomal in origin. The granules themselves are thought to be either tertiary lysosomes or residual bodies, with the majority containing lipofuschin as part of the organic component. Lipofuschin has been suggested to be the product of peroxidation of proteins and lipids in membranes, and is accumulated by the lysosomes as a waste product (Porta, 1991).

Primary lysosomes, which contain digestive enzymes, are pinched off from the endoplasmic reticulum from which lysosomes arise, in the region of the Golgi (Pitt, 1975). Soon after their formation, they merge with phagosomes, pinocytotic vesicles or autophagic vacuoles. At this stage they are known as secondary lysosomes. Shortly after this fusion, the pH of the secondary lysosomes drops to the pH optima for the digestive enzymes, which is approximately pH 5 (Reid & Leech, 1980). Presumably this fall in pH then activates the digestive enzymes and the process of digesting cellular macromolecules and organelles begins. Repeated fusions between phagosomes, pinocytotic vesicles, primary and secondary lysosomes may result in the formation of a multivesicular body which retains digestive capacity. These bodies are also known as secondary lysosomes. The enzyme activity of secondary lysosomes may be "topped up" by merging with primary lysosomes (Pitt, 1975). Once the enzyme activity stops, these
organelles become known as tertiary lysosomes. Tertiary lysosomes contain primarily the indigestible remains of membranes, proteins and lipids which may include lipofuscin and excess metals (George & Viarengo, 1985). Tertiary lysosomes retain some internal structure and degenerate further into residual bodies (Reid and Leech, 1980).

Lipofuscin accumulates in the lysosomes and is recognised as the end product of the physiological decay of the cell's own constituents (Porta, 1991). It is made up of highly cross-linked lipoproteins (Zs.-Nagy, 1988). Thus, lipofuscin is present as part of the normal aging processes occurring within the cell. In fact, lipofuscin granules have been isolated from a variety of mammalian tissues (Porta, 1991). The main constituents of lipofuscin are protein (30-70%), lipid (20-50%), carbohydrate (4-7%) and traces of some metals, including iron, copper, aluminium and zinc (Porta, 1991).

There are two main theories to explain the formation of lipofuscin. The first is the proteolytic decline theory. Protein synthesis and degradation have been shown to decline with age (Porta, 1991) resulting in less proteolytic activity. Lysosomes appear to be the main site of proteolysis within the cell and, as such, are responsible for the degradation of membranes, organelles and long-lived cytosolic proteins. Since the administration of cysteine protease inhibitors to man and other animals induces the production of a lysosomal lipofuscin-like pigment, it has been hypothesised that the production of lipofuscin is due to a decline in the activity of lysosomal proteases that occurs with aging (Porta, 1991). The major flaw in this theory is that the nature of the pigment produced experimentally does not share all the characteristics of lipofuscin, in particular its slow formation, and that it remains in the cell and accumulates with age. In contrast, the experimental pigment forms rapidly and is only transient in nature.

The second proposal, the peroxidative theory, proposes that the peroxidation of both lipids and proteins is involved in the production of the pigment. Lipid peroxidation is a set of chain reactions which are free radical-mediated and once started, are self-
perpetuating. The process is initiated by the abstraction of a hydrogen atom from carbon in a polyunsaturated fatty acyl chain (PUFA). The resultant carbon-centred fatty acid radical immediately rearranges forming conjugated diene structures. Under aerobic conditions, oxygen is rapidly added to form lipid peroxyl radicals. This compound continues to abstract hydrogen atoms from other unsaturated fatty acids (Halliwell & Gutteridge, 1984; Slater, 1984; Pacifici & Davies, 1991; Cheeseman, 1993) (see equations below).

\[
LH + R\cdot \rightarrow L\cdot + RH
\]

\[
L\cdot + O_2 \rightarrow LOO\cdot
\]

\[
LOO\cdot + LH' \rightarrow LOOH + L' \text{ etc}
\]

where LH is the target PUFA

R\cdot the initiating radical

L\cdot the carbon-centred fatty acid radical

LOO\cdot a lipid peroxyl radical and

LOOH a lipid hydroperoxide.

Lipid hydroperoxides are not very stable compounds and readily break down to alkoxy and peroxyl radicals. This process can be catalysed by the presence of some transition metal ions, particularly iron and copper and the resultant radicals are capable of initiating new chains thereby perpetuating lipid peroxidation (Cheeseman, 1993).

\[
LOOH + Fe^{2+} \rightarrow LO\cdot + OH^- + Fe^{3+}
\]

\[
LOOH + Fe^{3+} \rightarrow LOO\cdot + H^+ + Fe^{2+}
\]

Hydrogen peroxide, produced from the dismutation of the superoxide radical (O$_2$\textsuperscript{-*}), can also be a source of radicals in the presence of iron and copper ions (Cheeseman, 1993).

\[
O_2^{-} + O_2^{-} \rightarrow H_2O_2 + O_2
\]

\[
H_2O_2 + Fe^{2+} \rightarrow \cdotOH + OH^- + Fe^{3+}
\]

The fact that PUFAs are a major constituent of cell membranes, makes them very prone to lipid peroxidation. Peroxidised membranes become rigid, lose selective permeability
and may lose their integrity (Pacifici & Davies, 1991). They become incorporated into the lysosomes where cross-linking occurs. This is thought to be the basis for the formation of lipofuscin (George, 1990).

Oxygen free radicals can also damage the primary, secondary and tertiary structure of proteins (Davies, 1988). The actual process of oxidation is similar to that undergone by lipids, that is a protein radical is formed which then reacts with oxygen to form a peroxyl radical. The peroxyl radical decomposes causing peptide chain scission and production of protein fragments (Davies, 1988). These damaged proteins are normally degraded by the proteolytic systems in the cell, for example proteases and peptidases. However, when the cell is under high oxidative stress or a decrease in proteolytic activity occurs, for example during aging, the rate of damage to proteins may exceed the capacity of these proteolytic systems (Davies, 1988). Protein radicals may also react with each other to form covalent cross-links (Davies, 1988). Accumulation of the damaged proteins begins to occur as the tendency for cross-linking increases and they become increasingly insoluble and resistant to proteolysis. Such cross-linked proteins may then react with the products of lipid peroxidation and formation of highly insoluble lipid-protein aggregates, such as lipofuscin, follows. The role that the oxidation of proteins plays in the formation of lipofuscin has only been recognised in recent years (Davies, 1988) but it may be of more importance than the peroxidation of lipids, since \( \text{OH}^- \) radicals form in the aqueous phase and, therefore, have a more direct impact on proteins than on lipids (Zs.-Nagy, 1988).

1.4.2.3 Occurrence of metal-containing granules

Although granules have been isolated from all the major invertebrate phyla including Protozoa, Cnidaria, Platyhelminthes, Nematoda, Annelida, Echinodermata, Mollusca and Arthropoda, this review will concentrate only on some examples of molluscs and arthropods in order to demonstrate the widespread occurrence and variability of
granules (Table 1) (see for example Brown, 1982; George, 1982; Mason & Nott, 1981 and references therein).

Barnacles have been shown to contain three types of granules. The parenchyma cells surrounding the midgut of Balanus balanoides contain two kinds of membrane bound granules, a zinc phosphate type and a copper-rich type (Walker et al., 1975; Walker, 1977). The zinc-rich type has also been isolated from the midgut epithelial cells of Lepas anatifera (Rainbow, 1975). Calcium phosphate granules, which also contain either iron and phosphorus, or zinc and sulfur, as their major constituents, have been isolated from Elminius modestus and Semibalanus balanoides (Pullen & Rainbow, 1991). Phosphate granules have also been found in the hepatopancreas of the crab, Carcinus maenus (Simkiss et al., 1990). These granules are amorphous, with calcium, magnesium and phosphorus being the major elements present with smaller amounts of potassium, iron and zinc. The amphipod Corophium volutator also contains granules in the hepatopancreas, although they are made up of mainly copper and sulfur, and have been suggested to function as a copper store (Icely & Nott, 1980).

Granules have been isolated from a range of gastropod species. The slug, Arion ater, contains zinc and calcium in lipofuscin-containing granules in the digestive gland (Recio et al., 1988). Granules from the digestive gland of the dog whelk, Nucella lapillus, contain phosphorus, magnesium, zinc, copper and calcium with zinc and copper being higher in male animals (Ireland, 1979). The abalone, Haliotis rubra, contains granules in vacuoles in the digestive gland and cells of the gill and kidney. These granules are irregular and contain calcium, phosphorus, iron, copper, sodium, potassium and sulfur (Hyne et al., 1992). A second type of granule, present only in the kidney of this animal, is spherical and contains predominantly silica (Hyne et al., 1992).

Extensive work has been carried out on the hepatopancreatic granules of the snail, Helix aspersa. In this species, granules found in the calcium cells of the hepatopancreas can be up to 100 µm in diameter and consist of calcium magnesium pyrophosphate (Howard
et al., 1981). They have been shown to accumulate both zinc and iron if exposed to these metals (Taylor et al., 1990). Another well-studied animal is the marine prosobranch *Littorina littorea* which contains two types of granules. A phosphate-rich type occurs in the basophil cells of the digestive tubules. These granules are approximately 1-2 µm in diameter, membrane-bound and have a concentric ring structure. They may also contain magnesium and potassium, and any other metals, including zinc, that the animal is exposed to (Mason & Nott, 1981, Nott & Langston, 1993). The granules are released into the lumen where they are eliminated in the faecal pellets. The second type of granule is found in the foot and other organs in the connective tissue. These carbonate-rich granules are not membrane bound and contain more calcium than the phosphate granules. They occur in vacuoles within the calcium cells. Discontinuities in the plasma membrane of these cells connect with the vacuoles resulting in the granules being in contact with the blood (Mason & Nott, 1981). There is evidence that materials within these granules are mobilized into the blood in response to physiological stimuli such as acidosis and shell repair (Mason & Nott, 1981 and references therein).

The most studied group of molluscs with regard to the occurrence of metal-containing granules is the bivalves. In marine bivalves, the major site of granule occurrence is the kidney. Oysters have been widely studied and contain granules, not only in the kidney but also in circulating amoebocytes, thus enabling them to detoxify metals from all tissues of the body. There are three types of granules in amoebocytes in *Ostrea edulis* that contain either zinc and phosphorus, copper and sulfur, or zinc and copper (George et al., 1978). In contrast, *O. angasi* and *Crassostrea gigas* only possess zinc and copper granules (Pirie et al., 1984). Lysosomal granules also occur in the gills, the outer edge of the mantle and the kidney of the oyster, *O. edulis* (Thomson et al., 1985).
Table 1: Occurrence of metal-containing granules in some crustaceans and molluscs.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>COMPOSITION</th>
<th>ORGAN/CENTRAL PARTS</th>
<th>CHARACTERISTICS</th>
<th>REFERENCE(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRUSTACEA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Balanus balanoides</td>
<td>1. mainly ZnPO₄; Mg, K, Ca, Fe</td>
<td>parenchyma cells</td>
<td>1. membrane-bound; concentric rings</td>
<td>1. Walker et al., 1975 2. Walker, 1977</td>
</tr>
<tr>
<td>(barnacle)</td>
<td>2. Cu, S</td>
<td>surrounding midgut</td>
<td>2. no rings</td>
<td></td>
</tr>
<tr>
<td>Lepas anatifera</td>
<td>Zn</td>
<td>midgut epithelial</td>
<td></td>
<td>Rainbow, 1975</td>
</tr>
<tr>
<td>(barnacle)</td>
<td></td>
<td>cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elminius modestus</td>
<td>Fe, P or Zn, S; also Ca, Mg, K</td>
<td>analysed whole body</td>
<td></td>
<td>Pullen and Rainbow, 1991</td>
</tr>
<tr>
<td>Semibalanus balanoides</td>
<td>(barnacles)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinus maenas (crab)</td>
<td>Ca, Mg, P, K, Mg, Fe, Zn</td>
<td>hepatopancreas</td>
<td>calcium phosphate; concentric rings</td>
<td>Simkiss et al., 1990</td>
</tr>
<tr>
<td>Corophium volutator</td>
<td>Cu, S, Ca</td>
<td>hepatopancreatic ceaca</td>
<td>possibly copper storage for the blood</td>
<td>Iceley and Nott, 1980</td>
</tr>
<tr>
<td>(amphipod)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GASTROPODA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucella lapillus</td>
<td>P, Mg, Zn, Cu, Ca</td>
<td>digestive gland</td>
<td>Zn and Cu higher in males</td>
<td>Ireland, 1979</td>
</tr>
<tr>
<td>(dog whelk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arion ater (slug)</td>
<td>Zn, Ca</td>
<td>Digestive gland</td>
<td>contain lipofuscin under high zinc</td>
<td>Recio et al., 1988</td>
</tr>
<tr>
<td>Helix aspersa (snail)</td>
<td>Ca, Mg, PO₄; also other metals when exposed to</td>
<td>Hepatopancreas</td>
<td>membrane-bound; concentric rings; 100 μm diameter; associated with the Golgi or ER</td>
<td>Howard et al., 1981  Taylor et al., 1990</td>
</tr>
<tr>
<td>Littorina littorea</td>
<td>1. phosphate - Ca, Mg, K; other metals accumulated on exposure 2. carbonate</td>
<td>digestive gland 1. gland and gills 2. foot and other regions</td>
<td>1. membrane-bound; spherical; concentric rings; 1-2 μm diameter; released into lumen and eliminated in faecal pellets 2. in contact with blood; no membrane</td>
<td>Mason and Nott, 1981 Nott and Langston, 1993</td>
</tr>
<tr>
<td>(marine gastropod)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### BIVALVIA - MARINE

<table>
<thead>
<tr>
<th>Species</th>
<th>Composition</th>
<th>Localization</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Donacilla cornea</em></td>
<td>mainly CaPO$_4$, also Zn, Mg, Fe</td>
<td>kidney - main vacuole of columnar epithelial cells and in the lumen</td>
<td>more granules in animals exposed to Cu and Cd</td>
<td>Regoli <em>et al.</em>, 1992</td>
</tr>
<tr>
<td><em>Pecten maximus</em> (scallop)</td>
<td>mainly Ca, Mn, Zn, P; also Mg, Cu, Fe, Cd, K, S, Cl</td>
<td>kidney - vacuole in columnar epithelial cells</td>
<td>lysosomal; excreted into lumen; 5-15 µm diameter; spheroidal</td>
<td>George <em>et al.</em>, 1980</td>
</tr>
<tr>
<td><em>Ostrea edulis</em> (oyster)</td>
<td>1. Zn, P 2. Cu, S</td>
<td>amoebocytes, mantle, gill, digestive gland, kidney</td>
<td>membrane-bound; the two types of granule occur in two types of cell; granules in mantle, kidney and gills are lysosomal</td>
<td>George <em>et al.</em>, 1978; Thompson <em>et al.</em>, 1985</td>
</tr>
<tr>
<td><em>Ostrea angasi</em> Crassostrea gigas (oysters)</td>
<td>Cu, Zn</td>
<td>amoebocytes</td>
<td></td>
<td>Pirie <em>et al.</em>, 1984</td>
</tr>
</tbody>
</table>

### BIVALVIA - FRESHWATER

<table>
<thead>
<tr>
<th>Species</th>
<th>Composition</th>
<th>Localization</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Margaritifera margaritifera</em></td>
<td>Ca, P, Cl, Mn, Ba, S, Si, Zn, Mg, carbonic anhydrase</td>
<td>mantle</td>
<td>extracellular; Zn associated with carbonic anhydrase</td>
<td>Roinel <em>et al.</em>, 1973</td>
</tr>
<tr>
<td><em>Anodonta cygnea</em></td>
<td>1. PO$_4$ 2. CO$_3$</td>
<td>gills</td>
<td>extracellular</td>
<td>Moura <em>et al.</em>, 1999</td>
</tr>
<tr>
<td><em>Anodonta anatina</em> Anodonta cygnea <em>Unio pictorum</em></td>
<td>CaPO$_4$; also Mn, Fe, Zn</td>
<td>mostly gill, also mantle, midgut gland and granulocytes</td>
<td>1-7 µm diameter; clusters may form; pyrophosphate</td>
<td>Pynnonen <em>et al.</em>, 1987</td>
</tr>
<tr>
<td><em>Anodonta grandis</em> Ligumia subrostrata</td>
<td>Ca, Mn, Fe; also Zn</td>
<td>gills</td>
<td>extracellular; source of Ca for reproduction; membrane-bound; formed in phagocytic cells</td>
<td>Silverman <em>et al.</em>, 1987; Silverman <em>et al.</em>, 1989</td>
</tr>
<tr>
<td><em>Velesunio angasi</em> Velesunio ambiguus</td>
<td>insoluble phosphates of Ca, Fe, Mg; also radionuclides eg Ra, Po, U</td>
<td>mantle, gills, labial palps, visceral mass</td>
<td>extracellular; accumulation of metals relies on solubility of their phosphate</td>
<td>Jones and Walker, 1979; Jeffree and Simpson, 1984; Jeffree, 1985, 1988; Jeffree and Brown, 1992; Jeffree <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><em>Hyridella depressa</em></td>
<td>CaP granules, with Fe, Mn, Cu, Zn; S containing granules, with P, Fe, Ca</td>
<td>Mantle, gonad, palps, gills, visceral mass</td>
<td>Both types appear to be extracellular and intracellular</td>
<td>Adam <em>et al.</em>, 1997; Adams and Shorey, 1998; Vesk and Byrne, 1999; Byrne and Vesk, 2000</td>
</tr>
</tbody>
</table>
The clam, *Mercenaria mercenaria*, contains both extracellular and intracellular granules in the kidney (Sullivan et al., 1988). The intracellular granules, which are less than 10 µm in diameter, are lysosomal and made up mainly of calcium phosphate. They are excreted into the lumen of the kidney and are then defined as extracellular. They continue to accumulate metals in the kidney lumen and grow in size until they are finally excreted from the body (Sullivan et al., 1988). Lysosomal granules are also found within vacuoles of the kidney epithelial cells of the scallops *Argopecten irradians*, *A. gibbus* (Carmichael et al., 1979) and *Pecten maximus* (George et al., 1980) and the bivalve *Donacilla cornea* (Regoli et al., 1992). In the scallop species, they are approximately 5-15 µm in diameter and contain mainly calcium, phosphorus, manganese and zinc with smaller amounts of magnesium, copper, iron, cadmium, potassium, sulfur and chlorine (George et al., 1980) while those in *D. cornea* contain predominantly calcium phosphate with zinc, magnesium and iron (Regoli et al., 1992).

1.4.2.4 Metal-containing granules in *Mytilus*

In *M. edulis* metal-containing granules have been isolated from the mantle (Bubel, 1973; Lowe & Moore, 1979), oocytes (Lowe & Moore, 1979), gills, (George et al., 1976), digestive gland (Lowe and Moore, 1979; George et al., 1976; Viarengo et al., 1985) and the kidney (George et al., 1976); (George et al., 1982); (George & Pirie, 1979); (George & Pirie, 1980); (George, 1983). In this species, the kidney granules are spherical, electron-dense, membrane-limited and contain lipofuscin and acid hydrolases indicating that they are lysosomal in origin. The granules can make up some 20% of the kidney cell volume (George et al., 1982). They contain mostly calcium and phosphate, but a variety of other metals may also be incorporated into their structure. They contain relatively little inorganic material with this accounting for only 10% of the dry weight of the granules (George et al., 1982). George & Pirie (1980) have also demonstrated that the granules are excreted into the lumen of the kidney and can be voided in the urine.
1.4.2.5 Metal-containing granules in freshwater bivalves

Metal-containing granules have also been isolated from a number of freshwater bivalves. Despite some of the granules being described as forming intracellularly, either in specialised cells or in lysosomes, they are considered here to be extracellular, since their metal-binding functions are performed outside the cell. True intracellular granules have only been described from *Hyridella depressa* (Adams et al., 1997).

In unionid mussels, such as *Anodonta anatina, A. cygnaea, A. grandis, Unio pictorum* and *Ligumia subrostrata*, extracellular granules occur in the gills, mantle, midgut gland and around the granulocytes (Pynnonen et al., 1987; Silverman et al., 1987; Silverman et al., 1989). They are predominantly composed of calcium phosphate with smaller amounts of manganese, iron and zinc. The likely site of formation of the granules is in the phagocytic cells in the gills (Silverman et al., 1989). These 'concretion-forming cells' appear to be related to amoebocytic blood cells. The concretions initially form inside these cells and are then released and accumulate in the connective tissue of the gill (Silverman et al., 1989). The granules will preferentially bind calcium and are used as a source of calcium during reproduction. However, if calcium is not sequestered by the granules they will take up other metals such as zinc (Silverman et al., 1987).

The mantle edge, labial palps and visceral mass of *Velesunio angasi* (Jeffree & Simpson, 1984) and *V. ambiguus* (Ch'ng-Tan, 1968) are also rich in extracellular calcium granules. Again, they are mostly calcium phosphate but will also accumulate other metals, such as magnesium, barium, radium, lead, zinc, manganese and iron (see Chapter 6 for further information). Extracellular calcium granules have also been isolated from the mantle of *Margaritifera margaritifera* (Roinel et al., 1973) and *Amblema plicata perplicata* (Davis et al., 1982). Granules from both animals were smooth, spherical and contained mainly calcium phosphate, together with chlorine, manganese, barium, sulfur, silicon (as silica, the form found in biological systems), magnesium and zinc. In *M. margaritifera*, the calcium in the granules was found to be
exchangeable at a slow rate, while the zinc was thought to be associated with a peripheral layer of the zinc-containing enzyme carbonic anhydrase (Roinel et al., 1973). Davis et al., (1982) established that the granules from A. plicata perplicata possessed an organic glycoprotein matrix and speculated that they were originally lysosomal.

*Hyridella depressa* contains two types of metal-containing granules, both of which appear to be extracellular and intracellular (Adams et al., 1997). The first type are calcium phosphate granules which occur in many different tissues (Adams & Shorey, 1998; Vesk & Byrne, 1999). In addition to calcium and phosphate, they also contain iron, barium and magnesium. The second type are sulphur-containing granules, which also contain phosphorus, iron, calcium and sometimes copper and zinc (Adams et al., 1997; Byrne & Vesk, 2000).

1.5 INVOLVEMENT OF METAL-CONTAINING GRANULES IN METAL DETOXIFICATION

Both metallothionein and lysosomal lipofuscin granules have been implicated in the detoxification of excess metals. Although they have generally been regarded as two separate mechanisms, it can be shown that they are linked. The uptake and degradation of metalloproteins, including metallothioneins, may have an effect on the production of lipofuscin. Uptake of metalloproteins containing iron and copper ions increases peroxidation once the metals are released (by proteolysis or acid pH inside the lysosome) (George, 1990). Metals bound to metallothionein (MT) cannot be released outside the lysosomes. However, zinc/copper MT may be taken in by the lysosome and then degraded (Viarengo & Nott, 1993). The acid pH in the secondary (active) lysosome releases the zinc from the zinc/copper MT. The Zn$^{2+}$ ions will not stimulate peroxidation and subsequent lipofuscin formation but are incorporated into the existing matrix of lipofuscin and other indigestible remains (Viarengo & Nott, 1993). Under oxidative conditions, the remaining copper-thionein polymerises to form an insoluble complex. Thus, copper may also be sequestered in this way. The presence of free Cu$^{2+}$
ions not only leads to the induction of more thionein but increases production of oxygen free radicals which perpetuate the process of lipid peroxidation and the production of lipofuscin. The insoluble copper-thionein will form cross-links with other proteins and lipids (from peroxidation) and eventually result in the production of lipofuscin in the lysosome (Davies, 1988).

If the low pH inside the lysosomes is generated by a proton pump then metal ions could be co-transported into these organelles. Alternatively, the metals could be sequestered by acidic molecules within the lysosomes (George, 1982). This author also suggests that primary lysosomes have a low number of metal binding sites and a low capacity for metal sequestration. Therefore, metals accumulate via a combination of autophagy and the generation of acidic groups within secondary and tertiary lysosomes. Lipids become more acidic after peroxidation and these acidic groups could be involved in the sequestration of metals. It appears that lysosomal granules can preferentially bind metals from metalloproteins since zinc was taken up from Zn-thionein, while cadmium was not removed from its metallothionein (George, 1983). However, this may also be due to cadmium having a higher stability constant for binding to the protein (George, 1983). Incubation of isolated granules with $^{65}\text{Zn}$ and $^{109}\text{Cd}$ has shown that mussel kidney granules can take up metals rapidly via a passive adsorption process (George, 1983). However, since zinc uptake across the cell membrane is carrier-mediated and the granules are membrane-bound, it is likely that some facilitated uptake also occurs. Metal-binding sites are randomly distributed throughout the matrix of the granule. Which metal ion binds to each site depends on the size of the ion, the geometry of the binding site and the distance between the binding ligands. Metals bound to "outer" sites can be exchanged, while metals bound to sites further in become trapped as peroxidation continues (George, 1983 - Fig 9 therein).

Due to their ability to permanently bind metal ions, one of the main functions of metal-containing granules is thought to be in the detoxification of excess metals and other pollutants. Lipofuscin granules appear to have a dual role, both thermodynamic and
kinetic. They are involved thermodynamically by modulating the equilibration of granule metal concentrations with cytoplasmic metal. Some of the metal ions within the lysosome are exchangeable with the cytoplasm and can therefore, maintain the necessary levels of ions in the cytoplasm (George, 1983). Their second function is kinetic whereby the granules act as a 'sink' for excess metals. This occurs via irreversible binding of the metal ions as they are trapped in the matrix of peroxidised proteins and lipids (George, 1983). Since lipofuscin is made up of lipid and protein, it contains an excess number of metal binding sites so that any metal which is taken up into the lysosome is readily bound to the lipofuscin. It has been suggested that metals other than calcium are sequestered via pathways originally evolved for the control of excess calcium (Simkiss, 1976).
1.6 AIMS

There continues to be a need to monitor aquatic systems worldwide for a wide variety of pollutants. A simple and reliable method is required to enable such monitoring to be carried out in any area and to allow comparisons between different sites. Despite the widespread use of mytilid mussels as biomonitors there remains some doubt over the validity of using whole soft tissues to determine pollutant levels (Goldberg et al., 1978; Lingard et al., 1992). A number of more specific biomarkers have been suggested including the shell (Dermott & Lum, 1986; Bourgoin, 1990; Lingard et al., 1992; Puente et al., 1996), metallothionein (George & Olsson, 1994; Viarengo et al., 1999a) and the stability of lysosomal membranes (Lin & Steichen, 1994; Lowe & Pipe, 1994). Zinc is one pollutant metal that shows marked variation in concentration amongst individual mussels (Lobel, 1987a; Lobel, 1987b). Using isolated pooled granules in *Mytilus* as a biomarker of zinc pollution may reduce this variation.

This project was designed to test the hypothesis that metal-containing granules in the kidney of *Mytilus galloprovincialis* are used as a detoxification mechanism for excess zinc. In addition, the validity of using the granules as a biomarker for excess zinc was explored. More specifically, the aims were:

- to ascertain the involvement of the intracellular kidney granules of *Mytilus galloprovincialis* in the accumulation of excess particulate zinc;
- to identify any differences in the structure or composition of both the kidney tissue, and any granules that the cells may contain, under different conditions of zinc-loading in apristine environment.
- To evaluate the composition of granules extracted from mussels at pristine and impacted sites.

To this end, the mussels were loaded with particulate zinc in the laboratory or collected from a zinc-polluted field site and the kidney granules extracted and analysed.
Granules were also isolated from a freshwater species, *Velesunio angasi* from impacted sites in the Northern Territory:

- to determine whether intracellular granules exist in the kidney of *V. angasi*; and
- if so, to determine their elemental composition and structure by analysis of both the kidney tissue and the granules.

Thus, microscopy studies were undertaken to determine the position of the granules in the cells and how the excess metals may affect their structure.
CHAPTER 2

MATERIALS AND METHODS

The information, materials and methods contained in this chapter are common to more than one, but not necessarily all, parts of this study. More specific information is included in each chapter to complete the description of the materials and methods used for the entire study.

2.1 SPECIES OF *MYTILUS* INVESTIGATED

The commercially grown mussels used in this study (Chapters 3 and 4) were young, being approximately 10 months old, and were living in conditions ideal for growth, that is at low density and with plentiful food. They had dark coloured, purple to red mantles. The shells of these mussels were almost symmetrical in shape and smoothly rounded (Figure 2.1).

The shells of the species of mussel used in Chapters 3 and 4 exhibited characteristics that initially suggested that all of them could be assigned to the species *Mytilus edulis*. However, Seed (1968) noted that shell morphology in *Mytilus* is greatly influenced by growth rate, density and the age of the animal and thus, is not a good taxonomic indicator. Beaumont *et al.* (1989) used a combination of shell measurement ratios and mantle colour to distinguish between *M. edulis* and *M. galloprovincialis*. In *M. galloprovincialis*, the mantle is typically dark coloured, blue, purple or violet while in *M. edulis* it is a lighter straw colour (Hepper, 1957).
Figure 2.1: Specimen of *Mytilus galloprovincialis* showing the rounded shape of the shell, dark purple colour of mantle and location of the kidney. Scale bar = 20 mm.
Shell measurements carried out on individuals used in Chapters 3 and 4 of this study were compared to the results described in Beaumont et al. (1989). The mantle of these animals was generally a deep purple colour. When the data obtained from observation of the mantle colour and shell measurements were combined, the results indicated that the mussels used in this study were *Mytilus galloprovincialis*.

The mussels used in Chapter 5 of this study were considered to be the same species as those used in Chapters 3 and 4, despite differences in shell morphology. These mussels also had mantles of a deep red to purple colour. Genetic studies have previously indicated that *Mytilus* from both coasts of Australia are the same species (McDonald et al., 1991).

The use of genetic information is another tool useful for distinguishing between species in the genus *Mytilus*. This type of analysis was considered to be beyond the scope of this study and thus, there is no specific information for the animals used. The only study regarding the speciation of *Mytilus* in Australia concluded that mussels from Tasmania and the south coast of Western Australia (Albany) were very similar to *M. galloprovincialis*, and that they were distinct from *M. edulis* (McDonald et al., 1991). Thus, it was decided to refer to the mussels used in this study as *Mytilus galloprovincialis*.

### 2.2 Collection of Mytilus

Mussels (*Mytilus galloprovincialis*) were obtained from a commercial mussel farm, located in Cockburn Sound just to the north-east of Garden Island, Western Australia (32°10'S, 115°40'E) (Figure 2.2). This location is well flushed by ocean currents and is well away from any industrial influences. As such, this was considered to be a control site, lacking any zinc load. Following collection, the shells were cleaned of any adhering algae, barnacles and other epiphytes and placed in aquaria containing full-strength seawater at a maximum density of 4 animals per litre.
Figure 2.2: Map of Garden Island area showing the location of the mussel farm from which mussels were obtained.
All animals were maintained for the duration of the experiments under continuous aeration and at temperature (16±2°C) and light conditions paralleling those found in the field. The pH of the seawater in the aquaria did not change more than 0.2 pH units in any one 48 hour period. Prior to experimentation, the water was filtered continuously.

2.3 Feeding Trials

The main digestive organ, the hepatopancreas, contains the stomach and large numbers of digestive tubules which are made up of two main types of cell. The digestive cells are mostly circular in shape and easily distinguished from the smaller, triangular pyrimidal cells (Lowe, 1988). Healthy digestive tubules have a relatively small lumen and intact cells (Burbidge, 1990). However, after the mussels have been kept in the laboratory for a number of days (as described above) some of the digestive tubules had larger lumens but no evidence of necrosis or cell damage (c.f. Chapter 4). These tubules are in what is termed ‘resting phase’, a state which appears to be triggered by nutritional deprivation (Lowe, 1988).

Three food sources were assessed in order to determine how best to meet the mussels’ nutritional requirements. The foods tested were a commercial fish food (Carnation products, Sydney), dried green algae \( \text{Spirulina sp.} \) and live diatoms \( \text{Phaeodactylum tricornutum} \). The fish food was obtained as flakes that were finely ground and kept in an airtight container. The dried algae were also in powder format. \( \text{P. tricornutum} \) were cultured using the technique described in (Chrismada & Borowitzka, 1994).

Aquaria (800 mm length, 300 mm width, 400 mm height) were set up each containing 50 mussels and maintained as described above. One food source was provided to each aquarium daily at a dose of either 20 µg.g\(^{-1}\) fish food, 8 µg.g\(^{-1}\) \text{Spirulina} or 1 ml per mussel (equivalent of approximately 2.49 x 10\(^{-6}\) cells per mussel) cultured \( \text{P. tricornutum} \). Mussels were maintained in the aquaria as described above.
Mussels were removed after 12 and 20 days of feeding respectively. The hepatopancreas was removed and fixed overnight in 2.5% gluteraldehyde in filtered seawater. The fixed tissue was then processed for routine light microscopy (full details are described in Chapter 4). Sections (6 µm) were cut and stained with hematoxylin and eosin (Pearse, 1972). They were viewed and photographed using a Zeiss Photomicroscope III stereo light microscope.

The condition of the digestive tubules was noted for each tissue section. After 12 days of feeding, the majority of the digestive tubules in hepatopancreatic tissue in mussels from all three treatments were in the normal phase.

After 20 days of feeding, there were many more digestive tubules in the resting phase and there was some evidence of necrosis, or cell death, in the tissue from mussels in all three treatments. Based on the premise that a lower number of tubules in resting phase and less necrosis indicates that the mussel is less nutritionally deprived, the results of this trial indicated that fish food was the most nutritious food given, followed by *P. tricornutum* and *Spirulina*. This may partly be due to the fish food being given at a higher dose than the other foods. In order to provide the best possible conditions, it was decided that for future experiments, the mussels would be fed fish food, supplemented with dried *Spirulina* to provide added nutrients, at a rate of 20 µg·g⁻¹ daily.

### 2.4 Zinc-Loading

Zinc-loading (Chapters 3 and 4) was achieved using particulate (elemental) zinc as spheres <5 µm in diameter (BDH Chemicals, AnalaR Grade) at doses of either 0.5 µg·g⁻¹, 1.0 µg·g⁻¹ or 2.5 µg·g⁻¹ administered daily to the aquaria for up to 28 days after allowing two days for acclimation. To provide a consistent period of zinc exposure, all aquaria filters were turned off daily for six hours immediately prior to dosing. Seawater was analysed using Anodic Stripping Voltammetry, which allows the analysis of trace amounts of elements in solution with reasonable accuracy (Skoog *et al.*, 1996). This
technique involves the analyte being deposited on a microelectrode, usually from a stirred solution. A potential, that is a few tenths of a volt more negative than the half-wave potential of the ion of interest, is then applied to the microelectrode. After an accurately measured period, electrolysis is discontinued, stirring is stopped and the deposited analyte is determined by a voltammetric procedure. During this second step, the analyte is stripped, or redissolved, from the microelectrode, and measured. In anodic stripping, the microelectrode acts as a cathode during the deposition step and an anode during the stripping step, with the analyte being oxidized back to its original form.

Analysis of the seawater after the addition of particulate zinc at 2.5 µg.g⁻¹ revealed that, after 6 hours, a maximum soluble zinc concentration of 0.7 µg.g⁻¹ was attained. All animals were also fed either ground commercial fish flakes (Carnation products, Sydney) or a mixture of the fish flakes and dried algae (Spirulina) at a dose of 20 µg.g⁻¹. Analysis revealed that the concentration of zinc added in the food was approximately 0.007 µg.g⁻¹, and thus minimal compared to that given during the experimental period. The bottom of each aquarium was cleaned daily using the filter intake hose as a vacuum cleaner. In addition, in order to remove any material not taken up either by the mussels or the filters, and to ensure a consistent level of zinc exposure, the aquaria water was completely replaced with fresh seawater at least twice a week for the duration of the experiment.

2.5 Kidney Structure

Most molluscan kidneys take the form of a coelemic duct draining from the pericardium to the exterior (Potts, 1967). The walls of the kidney are generally deeply folded and consist predominantly of epithelial cells with a brush border. Urine is usually formed by ultrafiltration and then modified by secretion and resorption (Potts, 1967).
The kidney of *Mytilus* is a simple organ, a dark brown spongy tissue extending from the labial palps to the posterior adductor muscle (Pirie & George, 1979) (Figure 2.1). It follows the general pattern outlined above, being made up of a convoluted tube or duct with the entrance (renopericardial funnel) and exit (excretory pore) of the kidney only a very short distance apart (approximately 1.5 mm apart in a 5 cm long animal). The remainder of the kidney tubules branch off this duct. Thus, the ultrafiltrate may not necessarily pass through the entire length of the kidney (Pirie & George, 1979), but may be diverted via one of the branching tubules. The blood is ultrafiltered in the pericardial cavity surrounding the heart to produce the urine. The urine then enters the kidney via the renopericardial canal and funnel.

As would be expected in an osmoconformer, that is an organism that does not change the osmolarity of its urine, there is no cell differentiation along the length of the kidney tubules. The tubules are made up of a single layer of columnar cells in which both the nucleus and the many mitochondria are located basally. The surface area of individual cells is increased by the presence of microvilli on the upper surface and by infolding of the basement membrane. These cells are characteristically packed with granules, which make up approximately 20% of the cell volume (Pirie & George, 1979), and which are typically found in the apical region.

**2.6 Removal of the Kidney**

The diffuse nature of the kidney of *Mytilus galloprovincialis* initially lead to difficulty in removing this tissue without also removing at least part of the surrounding gonad, gill and/or hepatopancreas. In order to determine the most successful method of removing the kidney cleanly, both dissection and aspiration techniques were trialled. For microscopy, dissection was found to be the best method as the organ remained intact after removal and contaminating tissue could be identified during subsequent microscopic examination (Chapters 4, 5 and 6). However, dissection proved to be an unreliable method of removing kidneys for subsequent granule extraction (Chapters 3, 5
and 6) due to the difficulty of avoiding contamination of the tissue by the surrounding gonad and gills. As such, aspiration was the preferred method used for kidneys from which granules were to be subsequently extracted. This technique relies on the presence of a vacuum to ‘suck’ the tissue out of the mussel (P.B. Lobel, personal communication). The design of the aspirator is shown in Figure 2.3.

![Diagram of the aspirator used to extract kidney tissue from mussels](image)

Figure 2.3: Diagram of the aspirator used to extract kidney tissue from mussels (design after P.B. Lobel, personal communication).

### 2.7 Granule Extraction

Kidneys from both *Mytilus galloprovincialis* (Chapters 3 and 5) and *Velesunio angasi* (Chapter 6) were removed using the aspiration technique into chilled, distilled, deionised water (DDI H₂O). Kidneys from 50 mussels (that is 100 kidneys) were pooled for each sample. All subsequent procedures were carried out on ice or at 4°C.
Granule extraction was then carried out on the pooled sample using a method modified from George et al. (1982) (see Figure 2.4). The combined tissue was homogenised with a tissue grinder (Kinematica Polytron) at speed setting 6, and then centrifuged at 720g for 2 min (Beckman GPR). The supernatant was removed and set aside, while the pellet was resuspended and centrifuged again at 720g for 2 min. The pellet was then discarded and the second supernatant was combined with that from the first spin and then centrifuged at 4500g for 10 min (Beckman J221/ME). The resulting final supernatant was discarded and 5 ml DDI H2O added to the pellet. The tube containing the pellet was gently swirled to release an upper light brown layer which was then discarded, leaving a lower dark brown layer which contained the granules. The lower layer was resuspended, recentrifuged at 4500g for 10 min and divided as before. The remaining pellet was transferred to an Eppendorf tube, spun at 9500 g and dried under vacuum in a desiccator.
Figure 2.4: Flow chart indicating the granule extraction process.
2.8 Transmission Electron Microscopy

The transmission electron microscope (TEM) uses a beam of electrons which passes through a thin section of a specimen, transmitting an image onto a screen. It is used to elucidate the morphology of specimens.

An electron gun at the top of the microscope produces a stream of monochromatic electrons. This stream is focused to a small, thin, coherent beam by the use of two condenser lenses. The first lens largely determines the "spot size", or the general size range of the final spot that strikes the sample. The second lens actually changes the size of the spot on the sample, changing it from a wide dispersed spot to a pinpoint beam. The beam is restricted by the condenser aperture, knocking out electrons far from the optic axis. The beam strikes the specimen and parts of it are transmitted, then focussed by the objective lens into an image. The image is passed down the column through the intermediate and projector lenses, being enlarged all the way. It then strikes a phosphor screen and light is generated, allowing the user to see the image. The darker areas of the image represent those areas of the sample that fewer electrons were transmitted through, that is thicker or denser areas. The lighter areas of the image represent those areas of the sample that more electrons passed through (University of Nebraska, 1995a).

Specimens are prepared by fixing and embedding tissues in resin. Very thin slices, approximately 80 nm, are taken and placed on a very fine grid of metal, usually copper. The tissue slices may be stained using heavy metals to increase the contrast between elements of the cell.

In this study, the Phillips 301 transmission electron microscope was used to view and photograph specimens.
2.9 SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy enables the user to determine topography and morphology of a sample. An electron gun produces a stream of monochromatic electrons, that is condensed by the first condenser lens. This lens is used to both form the beam and limit the amount of current in the beam. It works in conjunction with the condenser aperture to eliminate the high-angle electrons from the beam. The second condenser lens forms the electrons into a thin, tight, coherent beam. A set of coils then "scan" or "sweep" the beam in a grid fashion, dwelling on points for a period of time determined by the scan speed. The final lens, the objective, focuses the scanning beam onto the part of the specimen desired. When the beam strikes the sample interactions occur inside the sample and are detected with various instruments. Before the beam moves to its next dwell point these instruments count the number of interactions and display a pixel on a screen, the intensity of which is determined by this number (the more reactions the brighter the pixel). This process is repeated until the grid scan is finished and then repeated, with the entire pattern being scanned around 30 times per second (University of Nebraska, 1995b).

A number of different interactions can occur when the energized electrons in the microscope strike the sample. The production of backscattered electrons is relative to the atomic weight of the sample, with higher atomic number elements appearing brighter than those with a lower atomic number. Secondary electrons assist with building a picture of the specimen’s topography. Auger electrons and X-rays are emitted after secondary electrons are produced. Both have characteristic energies unique to the element from which they are emitted, allowing analysis of the elemental composition of a sample (University of Nebraska, 1995b).

Specimens are prepared by fixation, dehydration and coating with a very thin layer of either gold or carbon. They are mounted on a purpose-built stub, generally made of aluminium.
The Philips XL20 scanning electron microscope was used to view specimens in this study. While the age of this machine presented some limitations, many of the problems associated with SEM encountered in this study were more to do with the samples themselves (see Chapter 4).

2.10 ENERGY DISPERSIVE SPECTROSCOPY

Energy dispersive spectroscopy (EDS) is a technique that is based on the characteristic X-ray peaks that are generated when an energetic electron beam interacts with a specimen in an electron microscope. It can provide qualitative or semi-quantitative data (Segrave, 2000). Each element produces characteristic x-rays that may be used to identify the presence of that element in the region of the specimen being examined. Comparison of the relative intensities of x-ray peaks may be used to determine the relative concentrations of each element in the specimen (University of Oklahoma, 1995-99).

2.11 STATISTICAL ANALYSIS

Where necessary, statistical analysis was undertaken using SigmaStat®. Student’s t-tests were carried out on individual data sets to determine if significant differences existed between treatments and at what level of significance. Analysis of varience (ANOVA), followed by Tukey’s Test of Multiple Comparison was carried out on both the number and types of granules found in kidney cells from different treatments to determine if significant differences were present. The text of each chapter indicates which data statistical analysis was performed on.
CHAPTER 3

THE EFFECT OF PARTICULATE ZINC-LOADING ON THE COMPOSITION OF GRANULES FROM THE KIDNEY OF *MYTILUS GALLOPROVINCIALIS* IN ACUTE LABORATORY TESTS

3.1 INTRODUCTION

The majority of the work on zinc in *Mytilus* sp. has been undertaken with regard to the mussels’ ability to act as a bio-indicator organism (Phillips, 1980). In general, these studies include the effects of both environmental and biotic factors; uptake and loss of zinc from the tissues of *M. edulis*; and the effects of other metals on the uptake and levels of zinc in the mussel (see for example McLusky *et al.*, 1986; Regoli & Orlando, 1994). However, while such studies have provided a wealth of information, (Phillips, 1976a) notes that caution must be taken to avoid atypical uptake of metals, including zinc, by mussels due to environmental and biotic factors. In this regard, environmental factors include the position of the animal in the water column or intertidal zone, the salinity and temperature of the water, and the season, while the biotic variables include the size, age, sex and reproductive state of the individual. Fortunately, a careful sampling regime can eliminate the effects of most of these factors.

The presence of other metals in the environment is also thought to affect the uptake of a particular metal by indicator organisms, including mussels. The results of Jackim *et al.* (1977), who noticed that cadmium uptake was influenced by the presence of zinc, seem to confirm this, although Phillips (1980) suggests that this effect only occurs when the levels of zinc in the environment are in excess of several hundred µg.L⁻¹. In contrast, the uptake of soluble zinc by *M. edulis* was found to be independent of initial zinc levels in either the mussel or the environment (Amiard *et al.*, 1987).

While many of the studies into the effects of environmental variables have been undertaken on mussels collected from the field without any additional exposure to the
metals in question, *M. edulis* will accumulate soluble zinc in the laboratory (see for example Keckes *et al.*, 1969; Bayne, 1973; Pentreath, 1973; Phillips, 1976b; George & Pirie, 1980; Myint & Tyler, 1982; Ritz *et al.* , 1982; Elliott *et al.*, 1985; Amiard *et al.*, 1987). The uptake of soluble zinc in *M. edulis planulatus* has been shown to be linear and in direct proportion to the time of exposure (Myint & Tyler, 1982; Ritz *et al.*, 1982; Elliott *et al.*, 1985). *M. edulis* will also accumulate particulate zinc in the laboratory (Burbidge *et al.*, 1994).

Following exposure to zinc in both soluble and particulate forms, the highest concentration of this metal is normally found in the kidney. In addition, George & Pirie (1980) found that in *M. edulis* exposed to radiolabelled zinc (\(^{65}\)Zn) the kidney contained approximately 30% of the total body load. In mussels fed particulate zinc, the kidney again accumulated the majority (19%) of the body zinc (Burbidge *et al.*, 1994). Zinc accumulation in the kidney occurs rapidly, with the majority being stored in lysosomal granules in the kidney cells (George & Pirie, 1980). It has been suggested that the granules function as a detoxification system for excess metals, by binding the metal ions and isolating them “from the tissue fluids that are in contact with cellular processes essential to the continued life of the whole animal” (George & Pirie, 1980). A variety of metals, including cadmium, copper, lead, mercury and zinc, have been shown to be accumulated by these lysosomal granules (see for example George *et al.*, 1976; Lowe & Moore, 1979; George & Pirie, 1980; Viarengo *et al.*, 1985b). If the granules do indeed act in this way, it would be reasonable to assume that not only do the granules accumulate metal ions, but that their composition will also change in response to an excess of metal ions.

The aim of the present section of this study was:

- to characterise the zinc content of any granules present under normal conditions and following zinc loading; and
• to ascertain the involvement of the intracellular kidney granules of *Mytilus galloprovincialis* in the accumulation of excess particulate zinc.

In order to determine whether granules are produced in response to excess zinc and whether they will accumulate and sequester the metal, young, commercially grown *M. galloprovincialis* were loaded with particulate zinc at 0.5, 1.0 or 2.5 μg.g⁻¹ in the laboratory, the kidney tissue removed and composition of the granules chemically analysed.
3.2 MATERIALS AND METHODS

3.2.1 Zinc-Loading and Granule Extraction

Mussels (*Mytilus galloprovincialis*) obtained from a commercial farm at Garden Island, Western Australia were treated as described in Chapter 2, transferred to seawater aquaria and exposed to particulate zinc at daily doses of 0.5, 1.0 or 2.5 µg.g⁻¹. In addition, mussels were also sampled on the day of collection and were denoted “freshly harvested mussels”. Control animals were kept in another aquarium under the same conditions, with the exception of the addition of zinc, and were denoted “fed controls”. While mussels were usually sampled after either 21 or 28 days of exposure, some samples were taken at additional times. Kidney granules were extracted as detailed in Chapter 2.

3.2.2 Inductively-Coupled Plasma Spectroscopy Analysis

Following granule extraction, a portion of the final pellet was weighed and digested using a nitric/perchloric acid digest (Reuter *et al.*, 1981). Briefly, this involved adding 70% HNO₃ to the dried granule sample and heating gently until the brown nitric oxide fumes disappeared. Perchloric acid (70%) was then added and the resulting mixture vigorously heated. Digestion continued until white perchloric fumes were no longer evident. Samples were made up to 10 ml with distilled deionised water (DDI H₂O). At least one reagent blank and a standard were included in each digestion batch. The standard used was either National Institute for Environmental Studies (NIES) mussel standard (certified reference material No. 6, Environmental Agency of Japan, Tsukuba) or a secondary mussel standard. The secondary mussel standard was obtained by shucking 200 mussels which had been previously depurated for 48 hours. The resultant tissue was oven-dried at 90°C, freeze-dried, ground to a fine powder, which also ensured thorough mixing, and then stored in an airtight jar at 60°C. The zinc levels in the secondary standard were checked by analysis against a primary standard of Citrus
Leaves (NBS standard material 1572, National Bureau of Standards, Washington, D.C.). All glassware and other equipment used during the digests were thoroughly cleaned by soaking sequentially in each of 0.5% Decon-90, 0.02M EDTA and 12% nitric acid for at least two hours prior to use.

Metals were assayed using an ARL 5320 inductively coupled plasma spectrometer. Plasma sources yield significantly better quantitative analytical data than other emission sources (Skoog et al., 1996). This stems from the high stability, low noise, low background and freedom from interference of the sources when operated under appropriate experimental conditions. Argon plasma is employed for emission analyses, with argon ions and electrons being the principal conducting species. The inductively-coupled plasma (ICP) source appears to offer the greatest advantage in terms of sensitivity and freedom from interference (Skoog et al., 1996).

The inductively-coupled plasma torch consists of three concentric quartz tubes through which streams of argon flow. Surrounding the top of the largest tube is a water-cooled induction coil powered by a radio-frequency generator. The argon is ionised, and the resulting ions and their associated electrons interact with the fluctuating magnetic field produced by the induction coil. The sample is carried into the hot plasma at the head of the tubes, as either an aerosol, a thermally generated vapour or a fine powder (Skoog et al., 1996).

3.2.3 Analysis of Data

Statistical analysis was carried out as outlined in Chapter 2, on extracted granule weights, all element concentrations and the amount of zinc in the kidney granules.
3.3 RESULTS

3.3.1 Mussel Mortality

While under the original experimental design it was intended to load the mussels for 28 days, in many cases, mussels were unable to tolerate the high levels of zinc they were exposed to in individual experiments. Aquaria were checked daily for any dead mussels which were removed. Death was defined as a lack of response to external stimuli. Deaths were observed relatively earlier in mussels exposed to higher levels of zinc-loading. In all cases, the mussels were loaded for as long as possible, prior to large scale mortality, which resulted in the mussels loaded at 1.0 µg.g⁻¹ being exposed for up to 25 days, while those loaded at 0.5 µg.g⁻¹ and the fed controls were maintained for either 21 or 28 days. Unfortunately, mortality was such that it was only possible to get one data set for mussels loaded at 2.5 µg.g⁻¹, with only 13 days of exposure (Table 3.1). In addition, in the 2.5 µg.g⁻¹ and one of the 1.0 µg.g⁻¹ experiments less than 50 mussels were available for granule extraction, and thus in these cases the amount of granules obtained was limited.

In general, the total amount of kidney tissue in individual mussels decreased as zinc-loading increased. However, this cannot be quantified as, due to the extraction technique, an accurate determination of the weight of kidney tissue could not be made prior to granule extraction. In addition, the colour of the kidney changed from the reddish-brown colour found in control mussels to a yellowish-brown in zinc-loaded animals.

3.3.2 Granule extraction

The weight of granules extracted from fifty freshly harvested mussels ranged from 95 mg to 115 mg, with a mean value of 105 mg (Table 3.1). Fed controls yielded a significantly (p < 0.05) higher weight of granules, with up to 193 mg of granules being
obtained after 28 days of feeding. With the experimental zinc-loaded animals, a significantly ($p < 0.05$) higher yield of granules resulted from longer periods of exposure to zinc when mussels were loaded with 0.5 $\mu$g.g$^{-1}$ (Table 3.1). Thus, mussels loaded for 21 days yielded a mean weight of 22.8 mg (range 20 mg to 25 mg), while after 28 days of loading at 0.5 $\mu$g.g$^{-1}$, the mean weight had increased to 84.2 mg (range 53 mg to 102 mg). When mussels were loaded at 1.0 $\mu$g.g$^{-1}$, the weight of granules ranged from 52 mg after 14 days of loading to 99 mg after 25 days of loading.

The only discrepancy in these results was seen in a group of mussels loaded at 1.0 $\mu$g.g$^{-1}$ for 21 days which yielded a granule weight of only 8 mg. However, this result was obtained from only 35 mussels, not the usual 50. Loading mussels at 2.5 $\mu$g.g$^{-1}$ for 13 days yielded 54 mg of granules, but from only 36 mussels (Table 3.1).

With one exception, when mussels spawned they were discarded, as spawning significantly reduces body weight and puts undue stress on the mussels, making them unsuitable experimental candidates. One group of freshly harvested mussels to be used for granule extraction spawned just prior to the beginning of the experimental period (the “spawned” group in Table 3.1). In this case it was decided to extract the granules in order to determine whether spawning had any effect on granule abundance and composition. The weight of granules was substantially lower in this group when compared to other control mussels, with just 28 mg being extracted (Table 3.1). This weight can be compared to a mean weight of 105 mg extracted from the remaining groups of freshly harvested mussels. The amount of zinc in the granules from the spawned mussels was also lower than that in the other freshly harvested mussels, with only 177 $\mu$g compared to a mean of 535 $\mu$g in the remainder.
Table 3.1: The number of days mussels were exposed to each treatment, weight (mg) of granules, concentration (µg·g⁻¹) and amount (µg) of zinc in the granules extracted from *M. gallovincialis* under different conditions of zinc-loading.

<table>
<thead>
<tr>
<th>Loading conditions</th>
<th>No. of days fed</th>
<th>Weight (mg)</th>
<th>Zinc concentration (µg·g⁻¹)</th>
<th>Amount of zinc (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly harvested</td>
<td>0</td>
<td>115</td>
<td>3 252</td>
<td>374</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>112</td>
<td>3 179</td>
<td>356</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>95</td>
<td>6 757</td>
<td>642</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>98</td>
<td>7 855</td>
<td>770</td>
</tr>
<tr>
<td>Spawned freshly harvested</td>
<td>0</td>
<td>28</td>
<td>6 329</td>
<td>177</td>
</tr>
<tr>
<td>Fed control</td>
<td>28</td>
<td>153</td>
<td>4 233</td>
<td>647</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>193</td>
<td>6 591</td>
<td>1272</td>
</tr>
<tr>
<td>0.5 µg·g⁻¹ zinc-loaded</td>
<td>21</td>
<td>25</td>
<td>8 220</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>20</td>
<td>8 920</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>25</td>
<td>8 516</td>
<td>213</td>
</tr>
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<td></td>
<td>21</td>
<td>21</td>
<td>16 009</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>53</td>
<td>4 344</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>102</td>
<td>4 776</td>
<td>487</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>80</td>
<td>6 611</td>
<td>529</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>102</td>
<td>6 326</td>
<td>645</td>
</tr>
<tr>
<td>1.0 µg·g⁻¹ zinc-loaded</td>
<td>14</td>
<td>52</td>
<td>14 171</td>
<td>737</td>
</tr>
<tr>
<td></td>
<td>21*³</td>
<td>8</td>
<td>16 675</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>99</td>
<td>5 695</td>
<td>564</td>
</tr>
<tr>
<td>2.5 µg·g⁻¹ zinc-loaded</td>
<td>13*⁶</td>
<td>54</td>
<td>12 487</td>
<td>674</td>
</tr>
</tbody>
</table>

NOTE: Unless indicated, each sample is the result of pooling kidneys from 50 mussels.

* denotes that less than 50 mussels were used for granule extraction while the superscript number indicates the number of mussels used.
3.3.3 Inductively-Coupled Plasma Spectroscopy Analysis

Inductively-coupled plasma spectroscopy (ICP) revealed that the major constituents of the granules, in order of abundance, were sodium, sulfur, phosphorus, zinc, calcium, iron and copper (Table 3.2). The concentrations of sodium, sulfur and phosphorus did not change significantly (p < 0.05) between the freshly harvested animals and those found in the zinc-loaded mussels, or between the mussels loaded at 0.5 µg.g\(^{-1}\) zinc for different periods of time.

In dramatic contrast, the concentration of zinc in the granules increased as the level of zinc-loading increased (Tables 3.1 and 3.2). Thus, while the mean concentration of zinc in the granules of freshly harvested mussels was 5 260 µg.g\(^{-1}\), this increased to 12 487 µg.g\(^{-1}\) in mussels loaded at 2.5 µg.g\(^{-1}\). Surprisingly, concentrations of zinc were generally lower after longer experimental periods. For example, the mean zinc concentration was significantly (p < 0.05) lower after 28 days of loading at 0.5 µg.g\(^{-1}\) at 5 514 µg.g\(^{-1}\) zinc, while after only 21 days of loading the mean zinc concentration was 10 416 µg.g\(^{-1}\). Interestingly, while there was actually a slight increase in zinc concentration in mussels loaded at 1.0 µg.g\(^{-1}\) after 21 days compared to the zinc concentration after 14 days (16 675 µg.g\(^{-1}\) compared to 14 171 µg.g\(^{-1}\)), a dramatic decrease was seen between the concentration after 21 days and that after 25 days of loading at this level of zinc (16 675 µg.g\(^{-1}\) compared to 5 695 µg.g\(^{-1}\)).

When viewed in terms of amount, the difference in the increase in zinc between the control mussels and the fed controls was greater than that seen in the concentration data (Table 3.1). Significant differences (p < 0.05) were observed in the mean amount of zinc between granules extracted from freshly harvested mussels (536 µg) and those loaded with 0.5 µg.g\(^{-1}\) particulate zinc for 21 days (233 µg), and between those loaded for 21 days and those loaded for 28 days at 0.5 µg.g\(^{-1}\) zinc (473 µg). However, the amount of zinc increased significantly between day 21 and day 28, while the concentration decreased significantly (Table 3.1).
The concentration of both iron and copper decreased dramatically from a mean concentration of 7474 µg.g⁻¹ and 374 µg.g⁻¹ respectively in the freshly harvested mussels controls to 484 µg.g⁻¹ and 52 µg.g⁻¹ respectively in mussels loaded at 2.5 µg.g⁻¹ (Table 3.2). In contrast to the situation with zinc, when mussels were loaded with 0.5 µg.g⁻¹ particulate zinc, the mean iron concentration increased throughout the loading period. Thus, iron levels rose from a mean of 727 µg.g⁻¹ after 21 days of loading at 0.5 µg.g⁻¹ to a mean of 979 µg.g⁻¹ after 28 days of loading, although the difference was not significant (p < 0.05), and one of the results in this latter data set is substantially higher than the others. Paralleling the situation with zinc, the copper concentration actually decreased during the loading period, from a mean of 184 µg.g⁻¹ after 21 days of loading to 37 µg.g⁻¹ after 28 days of exposure to 0.5 µg.g⁻¹ zinc (Table 3.2). Once again, this difference was not significant (p < 0.05).

The mean concentration of calcium decreased significantly (p < 0.05) from 1610 µg.g⁻¹ in granules from freshly harvested mussels to 786 µg.g⁻¹ after 21 days of loading with 0.5 µg.g⁻¹ particulate zinc and to 900 µg.g⁻¹ after 28 days of loading at this level. There were no significant differences seen in the calcium concentration in other treatments, with the mean concentration of the fed controls being 1068 µg.g⁻¹ while that in granules extracted from mussels loaded at 2.5 µg.g⁻¹ zinc was 1074 µg.g⁻¹.

The results from the freshly harvested mussels have some outlying points. In particular, the concentrations of zinc, iron, copper, sulfur and sodium in the third and fourth data sets are quite different from the concentrations for these elements in the first two data sets. The results indicate that as the zinc, sodium and sulfur concentration rose, the iron and copper concentrations dropped. Mussels from which the first two data sets were derived were collected in spring prior to spawning, while the second two data sets were derived from mussels collected in spring and summer after spawning had occurred. Fluctuation in zinc levels in *Mytilus edulis* (Amiard et al., 1986) and *M. galloprovincialis* (Regoli & Orlando, 1994) occur throughout the year and it is likely that other elements undergo similar fluctuations. Spawning results in changes in metal
Table 3.2: Concentrations of elements in granules extracted from control and zinc-loaded *Mytilus galloprovincialis*.

<table>
<thead>
<tr>
<th>Loading Conditions</th>
<th>Concentration of elements (µg g⁻¹)</th>
<th>No. days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>S</td>
</tr>
<tr>
<td>Freshly harvested</td>
<td>8 939</td>
<td>8 278</td>
</tr>
<tr>
<td></td>
<td>8 732</td>
<td>8 072</td>
</tr>
<tr>
<td></td>
<td>8 280</td>
<td>13 010</td>
</tr>
<tr>
<td></td>
<td>10 663</td>
<td>10 805</td>
</tr>
<tr>
<td>spawned freshly</td>
<td>7 857</td>
<td>9 929</td>
</tr>
<tr>
<td>harvested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed control</td>
<td>4 572</td>
<td>9 879</td>
</tr>
<tr>
<td></td>
<td>11 984</td>
<td>9 093</td>
</tr>
<tr>
<td>0.5 µg.g⁻¹ zinc</td>
<td>9 688</td>
<td>5 356</td>
</tr>
<tr>
<td></td>
<td>8 185</td>
<td>5 790</td>
</tr>
<tr>
<td></td>
<td>4 415</td>
<td>9 050</td>
</tr>
<tr>
<td></td>
<td>6 442</td>
<td>9 345</td>
</tr>
<tr>
<td></td>
<td>5 384</td>
<td>8 962</td>
</tr>
<tr>
<td></td>
<td>5 851</td>
<td>9 875</td>
</tr>
<tr>
<td></td>
<td>10 224</td>
<td>10 201</td>
</tr>
<tr>
<td></td>
<td>8 918</td>
<td>7 997</td>
</tr>
<tr>
<td>1.0 µg.g⁻¹ zinc</td>
<td>12 538</td>
<td>11 721</td>
</tr>
<tr>
<td></td>
<td>5 811</td>
<td>7 663</td>
</tr>
<tr>
<td></td>
<td>4 343</td>
<td>9 643</td>
</tr>
<tr>
<td></td>
<td>10 755</td>
<td>11 454</td>
</tr>
</tbody>
</table>

NOTE: The dotted lines delineate between groups of mussels fed for different lengths of time at the same level of zinc-loading.
levels, particularly zinc, as this metal is incorporated in the spawn (Simpson, 1979; La Touche & Mix, 1982).

Taylor & Simkiss (1984) used “the binding capacity of metal ions as equivalent divalent cations” to calculate metal/phosphorus ratios in metal-containing granules which could then be compared to the ratio of calcium to phosphorus in known calcium phosphate compounds (Table 3.3), resulting in an understanding of the form of phosphate in the granules. Such calculations were applied to the results in Table 3.2. However, the results were inconclusive with ratios ranging from 0.58 to 2.55 (mean 1.47 ± 0.25), and no inferences could be made regarding the likely form of phosphate within the kidney granules of *Mytilus galloprovincialis*.

Table 3.3: Ca:P ratios for various calcium phosphate compounds (after Taylor and Simkiss, 1984).

<table>
<thead>
<tr>
<th>Calcium phosphate compound</th>
<th>Common name</th>
<th>Ca:P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(H$_2$PO$_4$)$_2$</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>CaHPO$_4$</td>
<td>monetite</td>
<td>1.0</td>
</tr>
<tr>
<td>CaHPO$_4$ 2H$_2$O</td>
<td>brushite</td>
<td>1.0</td>
</tr>
<tr>
<td>Ca$_5$H$_7$(PO$_4$)$_5$ 5H$_2$O</td>
<td>octacalcium phosphate</td>
<td>1.33</td>
</tr>
<tr>
<td>β-Ca$_3$(PO$_4$)$_2$</td>
<td>whitlockite</td>
<td>1.5</td>
</tr>
<tr>
<td>Ca$_3$(PO$_4$)$_2$OH</td>
<td>hydroxyapatite</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>bone</td>
<td>1.76</td>
</tr>
<tr>
<td>Ca$_3$P$_2$O$_7$</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Ca$_3$P$_2$O$_7$ 2H$_2$O</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Ca$_3$P$_2$O$_7$ 4H$_2$O</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Ca$_4$(PO$_3$)$_n$</td>
<td>amorphous calcium phosphate (ACP)</td>
<td>1.45</td>
</tr>
<tr>
<td>Ca $(n + 3)$ (PO$_3$)$_n$ + PO$_4$</td>
<td>as $n \rightarrow \infty$ Polyphosphate</td>
<td>0.50</td>
</tr>
</tbody>
</table>
3.4 Discussion

The results clearly suggest that the kidney granules of *Mytilus galloprovincialis* sequester zinc when it is presented to the mussel in particulate form. Since particulate zinc is not available in a radioactively labelled form (\(^{65}\text{Zn}\)), it could not be determined whether the particulate metal is being directly sequestered by the granules or whether it undergoes dissolution prior to uptake. However, previous studies have shown that, in *Mytilus edulis*, a substantial portion of zinc adsorbed to particulates was desorbed and metabolised (Davies & Simkiss, 1996) and that zinc is released from dissolved complexes in the gut (Vercauteren & Blust, 1996). The increase in the amount of zinc in kidney granules from mussels exposed to particulate zinc-loading indicates that the granules are sequestering the excess zinc from the tissues. In addition, the increase in the yield of granules over longer periods of zinc exposure shows that granules are being produced in response to the excess levels of zinc present.

The concentration of zinc in granules extracted from the kidney tissue of mussels is related to both the weight of the granules extracted and the number of days the mussels were exposed to particulate zinc. The variation in weight of granules can be accounted for by using the amount of zinc, rather than the concentration, as an indicator of zinc accumulation. Thus, while the concentration of zinc remained virtually steady between the freshly harvested mussels and fed controls, the amount increased somewhat. In addition, the concentration of zinc decreased between mussels loaded at 0.5 µg.g\(^{-1}\) for 21 days and those loaded at the same level for 28 days, while the amount of zinc and the amount of granules harvested actually increased. Therefore, the amount of zinc presents a more realistic picture of the levels of zinc present in the kidney granules.

Interpretation of the results of this study suggests the following sequence of events occurs. When the mussels are exposed to a rapid increase in the level of particulate zinc in their environment they begin to accumulate the excess in the kidney (Burbidge *et al.*, 1994). The majority of this excess zinc is sequestered by the lysosomal granules within
the kidney cells. The rate of granule excretion increases and many of the granules are removed from the mussel as particulates in the urine. At the same time, the synthesis of granules is stimulated and thus, the rate of granule production is increased as new granules are formed to detoxify the excess zinc. At some stage, between 21 and 28 days of zinc-loading, the rate of granule production overtakes the rate of granule excretion, accounting for the increase in granule yield during this period. In fact, new granules are almost certainly produced before existing granules have accumulated the maximum amount of material. This results in a number of granules where the membrane-bound space appears to be only partially filled (see Chapter 4 for more details).

The half life of the granules in the kidney of *M. edulis* under control conditions has been estimated at more than 50 days (George & Pirie, 1980). The result obtained by these authors would suggest that the majority of the granules in the control mussels in this study would not be excreted within the experimental period. However, the decrease in the total weight of granules extracted between the control and zinc-loaded mussels, despite the increase in zinc concentration of the kidney tissue seen in this study, suggests that this is not the case in the zinc-loaded animals. A number of processes may be working in combination to produce this result. Granules that have a high concentration of zinc and cannot easily bind more metal ions, may be actively excreted by the mussel. This would promote the synthesis of new granules to continue the process of zinc accumulation. In addition, the stress that the mussels are under purely by being held in a laboratory environment could also increase the rate of excretion of granules. The granules may be excreted by either rupture of the cell membrane or via a “budding” process both of which are described by George and Pirie (1979). Finally, the tissue in the zinc-loaded mussels suffered a gradual decline in the integrity of the cell membranes causing an additional loss of granules into the kidney lumen, particularly at higher levels of zinc-loading (see Chapter 4). One group of mussels loaded at 1.0 µg.g⁻¹ yielded a very small amount of granules (8 mg). Due to high levels of mortality in this group, the mussels were only loaded for 21 days and only 35 animals were used for
granule extraction. The lower yield of granules may have been due, in part, to accelerated loss of granules via cell membrane breakdown.

A comparison between granules isolated from *M. galloprovincialis* in this study and those isolated from *M. edulis* (George *et al.*, 1982) reveals that the overall composition of the two sets of granules is different, although some similarities can be seen (Table 3.4). Granules analysed from control *M. edulis* contained lower quantities of phosphorus, sulfur, sodium and copper while calcium, zinc and iron levels were higher (George *et al.*, 1982) when compared to kidney granules from freshly harvested (control) *M. galloprovincialis* (this study). These variations are most likely to be due to differences between the environments that the mussels lived in, with *M. edulis* being collected from Scotland, while *M. galloprovincialis* were from south-west Western Australia. Granules from *M. edulis* loaded with 0.1 µg.g\(^{-1}\) cadmium (George & Pirie, 1979) had lower phosphorus, but higher sulfur levels, while the level of calcium was similar to that in *M. galloprovincialis* loaded with 2.5 µg.g\(^{-1}\) zinc. The cadmium-loaded *M. edulis* and the control mussels of both species had high levels of iron relative to the zinc-loaded *M. galloprovincialis* (Table 3.4).

Many of the previous studies into the composition of kidney granules in bivalves have been carried out on animals that have had no exposure to elevated concentrations of metals. These animals may be regarded as “day 0 controls”, making it possible to draw some comparisons between these animals and the freshly harvested mussels in this study.

The calcium content of kidney granules isolated from *Mytilus* appears to be much lower than that found in kidney granules from many other bivalve species. The calcium content of the kidney granules in this study ranged from 2.7% in freshly harvested animals to 2.3% in zinc-loaded (2.5 µg.g\(^{-1}\)) *M. galloprovincialis*, while granules from *M. edulis* contained approximately 6% calcium (George *et al.*, 1982). In contrast, kidney granules from *Mercenaria mercenaria* contained approximately 30% calcium.
Table 3.4: Percentage composition of elements in granules isolated from *M. edulis* and *M. galloprovincialis* under control and metal-loaded conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percentage composition of elements</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn</td>
<td>P</td>
</tr>
<tr>
<td>Control <em>M. galloprovincialis</em></td>
<td>10.7</td>
<td>17.4</td>
</tr>
<tr>
<td>Control <em>M. edulis</em></td>
<td>14.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Zinc-loaded <em>M. galloprovincialis</em></td>
<td>23.2</td>
<td>23.2</td>
</tr>
<tr>
<td>Cadmium-loaded <em>M. edulis</em> (0.1 µg.g⁻¹)</td>
<td>20.7</td>
<td>7.1</td>
</tr>
</tbody>
</table>
(Sullivan et al., 1988), Margarita margaritifera had 32% (Roinel et al., 1973), Argopecten irradians had 49% and A. gibbus had 67% (Carmichael et al., 1979). However, granules isolated from the kidney of the scallop, Pecten maximus contained only 10.7% calcium (George et al., 1980).

Phosphorus levels in the kidney granules did not change significantly between treatments in this study. Levels in granules from both freshly harvested (17.4%) and zinc-loaded mussels (23.2%), although they were much higher than those in granules isolated from M. edulis (1.7%), were lower than those from other bivalve species. For example, granules isolated from M. margaritifera contained 18% phosphorus (Roinel et al., 1973) while kidney granules from A. irradians contained 30% (Carmichael et al., 1979) and those from P. maximus had 47% phosphorus (George et al., 1980).

Levels of sulfur were also quite stable amongst granules isolated from all treatments in this study. The level of sulfur in the kidney granules from M. galloprovincialis was high in both the freshly harvested and zinc-loaded animals when compared to that in other species. The level of sulfur in the freshly harvested granules was 19.6%, compared to 0.4% in the clam M. margaritifera (Roinel et al., 1973), while no sulfur was detected in those from A. irradians and A. gibbus (Carmichael et al., 1979). Granules from M. galloprovincialis loaded with 2.5 µg.g⁻¹ particulate zinc contained 24.8% sulfur and X-ray analysis of granules from the kidney of the oyster Crassostrea gigas exposed to excess metals revealed a large sulfur peak (Thomson et al., 1985). Sulfur was also detected in kidney granules from the bivalve Donacilla cornea, both before and after exposure to sub-lethal concentrations of copper and cadmium (Regoli et al., 1992).

The vast differences seen in calcium, phosphorus and sulfur content in different species of bivalves are almost certainly due to the type of granule present. Taylor and Simkiss, (1989) classified granule types according the major anion they contained, for example
carbonate, phosphate, sulphate, or oxalate. Granules containing sulfur binding atoms were also defined. Systems with phosphate as the major anion are very complex with some having a simple calcium phosphate composition, or a pyrophosphate or polyphosphate, while others also contain a range of cations (Taylor & Simkiss, 1989). The granules isolated from *M. margaritifera* (Roinel *et al.*, 1973), *A. irradians* (Carmichael *et al.*, 1979) and *P. maximus* (George *et al.*, 1980) appear to be in this group. All these granules also contained a range of cations, such as manganese, magnesium, zinc, iron, cadmium and lead.

The results of this study and those of George *et al.* (1982) would indicate that the granules isolated from *M. galloprovincialis* and *M. edulis* are lysosomal and in the sulfur group. This is a broad group of granules which are electron dense, contain sulfur and generally class ‘b’ metals, and have a role in the regulation and detoxification of these metals (Taylor & Simkiss, 1989). The metals they most commonly contain are copper, zinc, cadmium and mercury (Taylor & Simkiss, 1989). Since these are also the metals most commonly bound to metallothionein, the presence of sulfur in these granules may be due to the degradation of this sulfur-rich protein within the lysosomes. Indeed, Taylor & Simkiss (1989) calculated that the granules from *M. edulis* (George & Pirie, 1979) could contain metallothionein. Chromatographic separation of the proteins from the granules could help to identify metallothionein, but it is important to note that the cross-linking within the lipofuscin matrix of the granule makes such separation problematic.

The high sodium content observed in the kidney granules isolated from both freshly harvested (22.0%) and zinc-loaded (21.5% after loading at 2.5 \( \mu \text{g.g}^{-1} \)) *M. galloprovincialis* appears to be unusual. For example, granules isolated from *M. edulis* contained only between 1.4% and 8.4% sodium (George *et al.*, 1982). Results from the analysis of kidney granules isolated from other bivalve species, such as *M. mercenaria* (Sullivan *et al.*, 1988), *M. margaritifera* (Roinel *et al.*, 1973), and *P. maximus* (George *et al.*, 1980) indicated that they contained no detectable sodium. External
contamination of the samples during granule extraction cannot be ruled out as the source of the very high levels of sodium observed in this study compared to the lower levels in *M. edulis*. However, it is not obvious what the source of such contamination could be.

The relatively high levels of iron and copper in the granules from freshly harvested mussels indicates that these elements are found naturally in the mussels' environment. However, the dramatic decrease in the percentage of these elements in the granules between freshly harvested mussels and the other treatments, and the concurrent increase in the percentage of zinc, suggests that zinc may be replacing the iron and copper within the granules. Thus, the iron and copper appear to be reversibly bound and presumably detoxified in some other manner and/or excreted once replaced. The involvement of both copper and iron ions in lipid peroxidation may enhance the production of granules in this case, allowing more efficient detoxification of the zinc and subsequent detoxification of the released iron and copper. In addition, zinc may be preferentially bound by the granules at the expense of iron and copper in order to maintain a zinc equilibrium within the cell. The relative binding affinities of zinc, iron and copper ions would depend on the type of binding sites available on the granule’s surface.

These results confirm the findings of George (1983) who suggested a model of the kidney granules of *M. edulis*, which contained numerous metal-binding sites. Initially, binding would be reversible allowing exchange of metal ions between the granule and the cytoplasm. However, as lipid peroxidation occurred, the metal ions would become trapped by intramolecular cross-linkages. Thus, zinc could replace iron and copper already bound in the granule, and then become trapped, resulting in a high concentration of zinc in the granule.

Lipofuscin, the end product of reactions between peroxidation proteins and lipids, almost certainly makes up the organic portion of the granules (George *et al*., 1982). If the sulfur was originally from an organic source, it may also form a part of this
component. Alternatively, sulfur, together with phosphorus and calcium, may form part of the inorganic matrix of the granule. In this latter scenario, these elements would be irreversibly bound within the matrix and thus, could not be replaced by ions of the excess metal, in this case zinc. This explanation would also account for the relatively consistent percentage composition of sulfur, sodium, phosphorus and calcium between treatments in this study.

The results of this study have shown that the kidney granules from *M. galloprovincialis* accumulate excess zinc when it is presented in a particulate form. The concentration of zinc increased as the level of loading increased. In addition, synthesis of new granules was initiated in response to the excess zinc. The composition of the kidney granules indicates that they belong to the sulfur group of granules as described by Taylor & Simkiss (1989). While similar sulfur-type granules have been found in the oyster *Ostrea edulis* (George *et al.*, 1978), the majority of other bivalves have granules with quite a different composition (see Table 1.1 for further information). However, it appears that these latter granules have phosphate as their major anion and, thus, are likely to be more involved in the regulation and storage of calcium than detoxification of metals (Taylor & Simkiss, 1989). The organic portion of the granules appears to have a fairly stable composition dominated by the presence of lipofuscin, whether the mussels are exposed to excess metal or not. However, much of the non-organic portion, which in these granules is made up of zinc, iron and copper, appears to be reversibly bound and, furthermore, the data indicate that zinc ions are able to replace iron and copper ions in the granule.

Thus, exposure of mussels to acute levels of particulate zinc results in changes to the composition of the granules. The next part of the study concentrates on internal changes in both the kidney cells and the granules themselves.
4.1 INTRODUCTION

The kidney of marine mussels often contains metal-containing granules which are involved in accumulation and detoxification of excess metals obtained from its environment. The kidney cells of *Mytilus edulis* have been shown to contain three types of granules based on microscopic and morphological features (Pirie & George, 1979 and Table 4.1). Type 1 have a regular shape, are relatively electron dense, and approximately 1 µm in diameter. Type 2 granules have an irregular shape and size and apparently have no internal structure. Finally, Type 3 granules are either electron dense, or contain many bodies of uneven electron density which has led to them being referred to as multivesicular bodies. Type 3 granules may be up to 5 µm in diameter.

In some regions of the kidney, particularly near the excretory duct, the lumen of the kidney is filled with granular debris. As such, it has been suggested that the granules are excreted from the kidney cells, either by rupture of the membrane of individual cells or by exocytosis of single granules (Pirie & George, 1979). The excreted granules then leave the body via the particulate urine.
Although the morphology of the kidney tissue of *Mytilus* sp. has been examined in detail under normal conditions, there are no reports of the changes that occur when the mussel is subjected to stress in the form of metal loading, as would occur if the animal was exposed to metal pollution. Thus, the objective of this section of the study was:

- to identify any differences in the structure or composition of both the kidney tissue, and any granules that the cells may contain, under different conditions of zinc-loading.

Both light and electron microscopy were used to observe the intact tissue and the isolated granules in *Mytilus galloprovincialis*. 
4.2 MATERIALS AND METHODS

4.2.1 Light and transmission electron microscopy of kidney tissue

Whole kidneys were fixed in situ from freshly harvested commercially grown mussels *Mytilus galloprovincialis*, laboratory control mussels and mussels loaded with particulate zinc at levels described previously (Chapter 3). The control mussels together with those loaded with 0.5 µg.g\(^{-1}\) and 2.5 µg.g\(^{-1}\) particulate zinc were maintained for 28 days prior to sampling. Mussels loaded with 1.0 µg.g\(^{-1}\) zinc were sampled after 10 and 28 days of zinc-loading. Some of these latter animals were then maintained for a further 14 days without zinc-loading prior to the tissue being collected.

Tissue removal and fixation was achieved by opening the shell, flooding the tissue with 2.5% glutaraldehyde in filtered seawater, removing the kidney and placing it into fresh 2.5% glutaraldehyde/seawater. As much contaminating tissue as possible was then removed. The kidney tissue was left in the glutaraldehyde/seawater mixture overnight at 4\(^\circ\)C to ensure complete fixation, then washed with fresh filtered seawater, dehydrated through a graded series of ethanol-water mixtures and, depending on whether the tissue was destined for light or electron microscopy, finally passed through either chloroform or propylene oxide, followed by infiltration with either paraffin wax or epoxy resin respectively. Paraffin sections (6 µm) were stained with either haematoxylin and eosin (Pearse, 1972) to demonstrate cell structure, or Nile Blue Sulphate A (Pearse, 1972) to indicate the presence of lipofuscin granules. Resin sections (1 µm) were cut for the light microscope and stained with 1% methylene blue/1% Azur II in 1% borax. Light microscope sections were viewed under a Zeiss Photomicroscope III. Silver/gold sections (approximately 90 nm) were also cut from the resin blocks and stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1964) before being viewed using a Phillips 301 transmission electron microscope (TEM) at 80 kV.
4.2.2 Scanning electron microscopy and energy dispersive spectroscopy of isolated granules

Kidney granules were isolated using the centrifugation technique described previously (Chapter 2). Isolated granules were attached to aluminium stubs using an adhesive (Microhesive - Probing and Structure, Aitkenvale, Queensland) and evaporatively coated with carbon. Imaging was carried out using a Philips XL20 scanning electron microscope (SEM) at 5 or 10 keV, with a working distance of 12 mm and a spot size of 2 or 3. A number of techniques were examined in an attempt to improve the quality of SEM images of isolated granules, including methods of placing the granules onto the stub, using different adhesives, and trialling carbon and gold coatings. However, the granules remained very difficult to view under SEM, with the resultant images being somewhat unclear.

Energy dispersive spectroscopy (EDS) was carried out using a LINK analytical system attached to a JEOL 6400 SEM with a beryllium window and a lithium-drifted silicon detector. This system provides for analysis of all elements with an atomic weight equal to, or greater than, that of sodium. Calibration with a copper standard allowed subsequent identification of other element peaks.

The EDS data obtained were corrected using the peak integration and background subtraction method of Reed & Ware (1973) and Ware (1981). The data for individual treatments were pooled from either 20 granules (control day 0), 15 granules (0.5 µg.g⁻¹ zinc-loading) or 10 granules (1.0 and 2.5 µg.g⁻¹ zinc-loading) to produce the final result. The variation in the number of granules analysed was related to the amount of granules extracted, which decreased with higher levels of zinc-loading (see Chapter 3), and the conglomeration of the sample on the SEM stub, which made visualisation and analysis of individual granules problematic. Full quantitative analysis was not possible due to the small size and uneven surface of the granules, resulting in possible underestimation of lighter elements in the granules.
4.2.3 Analysis of data

In order to determine whether different levels of zinc-loading affected the number of granules in kidney cells, electron micrographs of kidney tissue from mussels in each treatment were examined. The total number of granules in between 8 and 12 randomly selected cells was counted. The number of multivesicular bodies and granules with concentric rings were also counted within each cell. Granule size was determined by measuring, at random, the diameter of 50 granules. A mean and standard error (± SE) were calculated for each set of data.

To determine whether significant differences were present between granule diameters, total number of granules, the number of multivesicular bodies and the number of granules with concentric rings between each treatment, statistical analysis was undertaken as described in Chapter 2.
4.3 RESULTS

4.3.1 Light and transmission electron microscopy of kidney tissue

The kidney of *Mytilus galloprovincialis* is comprised of a convoluted tubule (Figure 4.2a) consisting of a single layer of columnar cells (Figure 4.2b). As such, the ultrastructure of the kidney of *M. galloprovincialis* is virtually identical to that of *M. edulis* as described previously by Pirie & George (1979) (see Chapter 2). The kidney cells of *M. galloprovincialis* are approximately 25 µm in length, with a basal nucleus and large numbers of mitochondria, the majority of which are in the basal portion of the cell (Figure 4.3a). The surface area of the cells is increased by microvilli on the upper, lumenal surface and infolding of the basement membrane.

All kidney cells from both control and zinc-loaded mussels contained numerous membrane-limited granules, which were typically found in the apical region of the cell. In freshly harvested *M. galloprovincialis*, two main forms of granules were present in the kidney cells (Figure 4.3a). The first form was generally spherical in shape, uniformly electron dense and, as such, corresponded to the Type 1 granule described by Pirie & George (1979) (Figure 4.4a, Table 4.1). The mean diameter of these granules was 0.6 µm, although one granule 1.25 µm in diameter was also present. The second form of granule was more irregular in shape, with the electron-dense portion not entirely filling the area enclosed by the membrane (Figure 4.3a). These latter granules had a mean diameter of approximately 1.0 µm with a maximum of 1.8 µm. Many cells contained a number of small electron dense bodies, and thus, could be described as Type 3 granules (Pirie & George, 1979) or multivesicular bodies (Figure 4.4b). A few Type 2 granules were present in the cells of *M. galloprovincialis* (Figure 4.3a), with the largest being oval in shape and measuring approximately 2.25 µm by 1.75 µm.
Figure 4.2: Light micrographs of kidney tissue from *Mytilus galloprovincialis*.  
(a) low magnification view, showing the convoluted nature of the kidney;  
(b) higher magnification view, showing columnar shape of individual cells.  Note also presence of granules in kidney cells.  
Scale bars = 25 µm.
Figure 4.3: TEM micrographs of kidney tissue from *Mytilus galloprovincialis*.

(a) freshly harvested mussel;
(b) mussel kept in the laboratory for 28 days;
(c) mussel loaded with 0.5 µg.g⁻¹ particulate zinc for 28 days;
(d) mussel loaded with 2.5 µg.g⁻¹ particulate zinc for 28 days.

Scale bars = 2 µm.
Figure 4.4: TEM micrographs of individual granules from freshly harvested *Mytilus galloprovincialis*.

(a) type 1 granule;

(b) type 3 granule.

Scale bars = 0.5 μm.
Table 4.1: Description of granule types found in the kidney cells of *Mytilus galloprovincialis*.

<table>
<thead>
<tr>
<th>Type of granule</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>spherical, electron dense, generally less than 1 µm in diameter</td>
</tr>
<tr>
<td>Type 2</td>
<td>irregular shape and size, generally devoid of contents</td>
</tr>
<tr>
<td>Type 3</td>
<td>of either uniform or uneven electron density, may be very large, up to 5 µm in diameter, and contain a number of smaller bodies, may also be referred to as multivesicular bodies.</td>
</tr>
<tr>
<td>Type 4</td>
<td>concentric rings of electron dense and translucent material, generally spherical, size approximately 1 µm in diameter.</td>
</tr>
</tbody>
</table>

A mean of 65.1 ± 5.8 (SE) granules per cell, with a range of 42-94 granules per cell, was calculated for kidney cells from freshly harvested mussels (Table 4.2). The mean granule diameter was 0.63 µm ± 0.02 µm (Table 4.3). Membranous whorls could be seen in some granules, possibly due to the presence of membranes taken up by the lysosome via autophagy (George *et al.*, 1982).

In animals that had been kept under control conditions in the laboratory for 28 days (that is, fed controls), the basic structure of the kidney cells remained the same, with numerous microvilli present at the apical end of the cell and the nucleus and mitochondria remaining at the basal end of the cell. However, some loss of membrane integrity was observed and the lumen of the kidney had shrunk somewhat compared to freshly harvested mussels (Figure 4.3b). There were slightly less granules in the kidney cells of the fed controls (mean of 59.1 ± 6.0 granules.cell$^{-1}$) than in the freshly harvested tissue (mean of 65.1 granules.cell$^{-1}$), although the difference was not significant (Table 4.2). A number of the granules in kidney cells from the fed controls had a concentric ring structure, (Figure 4.3b). Thus, in these cells, four types of granules were present, Type 1, Type 2, Type 3 and those with the ring structure, which have been nominally denoted as Type 4 granules (Table 4.1). The mean diameter of granules in these cells was 0.83 µm ± 0.02 µm (Table 4.3), a slight, but not significant, increase compared to the diameter of granules in mussels collected from the field, although this difference was not significant.
Table 4.2: Number of granules (mean ± standard error) present in kidney cells of *Mytilus galloprovincialis* exposed to different levels of zinc-loading.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells counted</th>
<th>Total number of granules.cell(^{-1}) (mean ± SE) (range)</th>
<th>Number of multivesicular bodies.cell(^{-1}) (mean ± SE) (range)</th>
<th>Number of granules with concentric rings.cell(^{-1}) (mean ± SE) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>freshly harvested</td>
<td>12</td>
<td>65.1 ± 5.8 (42-94)</td>
<td>3.8 ± 0.5 (2-7)</td>
<td>0.8 ± 0.3 (0-4)</td>
</tr>
<tr>
<td>fed controls</td>
<td>8</td>
<td>59.1 ± 6.0 (36-88)</td>
<td>3.5 ± 0.3 (2-5)</td>
<td>1.5 ± 0.7 (0-6)</td>
</tr>
<tr>
<td>0.5 µg.g(^{-1}) zinc day 28</td>
<td>8</td>
<td>56.4 ± 6.1 (28-71)</td>
<td>6.4 ± 0.7 (3-9)</td>
<td>1.1 ± 0.5 (0-3)</td>
</tr>
<tr>
<td>1.0 µg.g(^{-1}) zinc day 10</td>
<td>10</td>
<td>68.3 ± 5.2 (47-97)</td>
<td>2.6 ± 0.5 (1-6)</td>
<td>0.3 ± 0.2 (0-2)</td>
</tr>
<tr>
<td>1.0 µg.g(^{-1}) zinc day 28</td>
<td>10</td>
<td>53.4 ± 8.0 (23-93)</td>
<td>1.0 ± 0.3 (0-3)</td>
<td>0.8 ± 0.3 (0-2)</td>
</tr>
<tr>
<td>1.0 µg.g(^{-1}) zinc day 28 + 14</td>
<td>10</td>
<td>66.9 ± 6.8 (44-109)</td>
<td>4.8 ± 0.7 (2-8)</td>
<td>4.1 ± 0.8 (0-7)</td>
</tr>
<tr>
<td>2.5 µg.g(^{-1}) zinc day 28</td>
<td>8</td>
<td>54.0 ± 5.8 (40-92)</td>
<td>10.3 ± 1.5 (5-18)</td>
<td>1.2 ± 0.4 (0-4)</td>
</tr>
</tbody>
</table>

After 28 days of zinc-loading at 0.5 µg.g\(^{-1}\) zinc, the kidney cells were still intact and microvilli were still visible, although reduced in number when compared to cells from freshly harvested mussels (Figure 4.3c). The mean number of granules per cell was 56.4 ± 6.1 (Table 4.2), indicating a slight decline when compared to the number found
in kidney cells from freshly harvested mussels. The size of the granules (1.01 µm ± 0.09 µm) increased slightly compared to both control treatments (Table 4.3), although this difference was not significant. The majority of these granules were similar in appearance to those in the fed control sections and Type 1, Type 3 and Type 4 granules were visible (Figure 4.3c). A significantly higher (p < 0.05) number of multivesicular bodies (6.4 per cell) was recorded in these cells, compared to only 3.8 multivesicular bodies.cell⁻¹ in cells from freshly harvested mussels (Table 4.2).

Loading at 2.5 µg.g⁻¹ zinc for 28 days resulted in a loss of cell membrane integrity (Figure 4.3d) and a further decrease in the number of granules present, with only 54.0 ± 5.8 granules per cell recorded. There also appeared to be less microvilli present on these cells. Interestingly, many of the granules in these cells were Type 3, with 10.3 ± 1.5 being present, although in many cases the electron dense portion filled only part of the area enclosed by the membrane. Very few granules with concentric rings were present in this tissue (1.2 ± 0.4) (Figure 4.3d, Table 4.2). The mean diameter of granules was 1.08 µm ± 0.13 µm, the highest recorded for any treatment (Table 4.3) and significantly higher than the diameter of the granules from freshly harvested mussels (p < 0.05).

Kidney cells from mussels that had been loaded at 1.0 µg.g⁻¹ zinc for 10 days actually contained slightly more (although not significantly) granules per cell, at 68.3 ± 5.2, than the number recorded in cells taken from freshly harvested mussels. The membranes of individual cells were complete and there were numerous microvilli and mitochondria present (Figure 4.5a). The majority of granules were Type 1, although Types 2 and 3 were also present (Figure 4.5a). After 28 days of zinc-loading at this level, the cells were more squat in shape, that is they were shorter and fatter than the tall, narrow cells of the control tissue. Microvilli were no longer visible and the nucleus was more centrally located. The number of granules present had declined to 53.4 granules.cell⁻¹, with a few individual granules showing at least one ring as part of their internal structure (Type 4). A small number of granules of Types 1 and 3 were also present in
these cells (Figure 4.5b), with there being significantly less (p < 0.05) Type 3 granules in this tissue (1.0 ± 0.3) when compared to that from freshly harvested mussels (3.8 ± 0.5). The mean diameter of kidney granules decreased from 0.91 μm ± 0.05 μm in mussels loaded for 10 days to 0.88 μm ± 0.16 μm after 28 days of zinc-loading, although this difference was not significant.

When mussels which had been loaded at 1.0 μg.g⁻¹ zinc for 28 days were maintained for a further 14 days without zinc-loading, the cell membranes regained their integrity, numerous mitochondria were present and microvilli were again visible, although these appeared to be less in number than in sections from freshly harvested mussels (Figure 4.5c). A large number of granules was seen in these cells (mean 66.9 granules.cell⁻¹) with the highest number of granules with a concentric ring structure (Type 4) at 4.1 granules.cell⁻¹ (Table 4.2, Figure 4.6). In fact, there was a significantly higher number of granules with concentric rings in this tissue than in any other treatment (p < 0.05). The number of multivesicular bodies was also significantly higher (p < 0.05) (4.8 ± 0.7) than that in cells from mussels loaded at 1.0 μg.g⁻¹ for either 10 (2.6 ± 0.5) or 28 days (1.0 ± 0.3). Type 1 and Type 2 granules were also present in these cells. The mean diameter of granules in these mussels increased again, but not significantly, to 0.94 μm ± 0.08 μm.

EDS of individual granules within thin sections of kidney tissue revealed that the metal content of the granules was lost during processing of the kidney tissue. All granules analysed had an almost identical elemental composition, but contained no significant amounts of metals such as zinc, iron or copper. Vesk & Byrne (1999) indicated that aqueous chemical fixation and processing of samples results in dissolution of elements in calcium phosphate granules, so it is likely a similar process took place in these lysosomal granules.
Figure 4.5: TEM micrographs of kidney tissue from *Mytilus galloprovincialis* loaded with 1.0 µg.g\(^{-1}\) particulate zinc. (a) for 10 days; (b) for 28 days; (c) for 28 days and then transferred to clean seawater for a further 14 days. Scale bars = 2 µm.
Figure 4.6 a & b: TEM micrographs showing type 4 granules with concentric rings in kidney tissue from *Mytilus galloprovincialis* loaded with particulate zinc for 28 days and then transferred to clean seawater for a further 14 days.

Scale bars = 2 µm.
4.3.2 Scanning electron microscopy and energy dispersive spectroscopy of isolated granules

SEM revealed that the kidney granules from *Mytilus galloprovincialis* were spherical in shape with a smooth surface (Figure 4.7). SEM confirmed the size range of the granules to be approximately 1µm in diameter.

Analysis of isolated granules by EDS revealed a composition similar to that found previously, using inductively-coupled plasma spectroscopy, in mussel kidney granules (see Chapter 3). Thus, the major elements present were phosphorus, sulphur, calcium, and zinc with smaller amounts of sodium, magnesium, chlorine, iron and copper (Figure 4.8). The majority of the aluminium peak is likely to be due to contamination from the aluminium stub on which the granules were mounted.

While the same elements were present in individual granules throughout the range of zinc-loading levels used, variations in the amount of the various elements detected by EDS occurred between the different conditions. The levels of zinc and sodium in individual granules increased as the level of zinc-loading increased, with a substantial zinc peak being seen in the granules from mussels loaded at 2.5 µg.g$^{-1}$ (Figure 4.8d). The amount of iron and copper present, however, did not change markedly.

The number of granules containing higher levels of zinc appeared to increase in extractions from zinc-loaded tissue. This impression was gained during the EDS analysis when a greater proportion of granules in the sample looked brighter on the microscope screen indicating greater electron charging and higher metal levels. However, as the analysis was semi-quantitative, conclusive measurements could not be made.
Figure 4.7: SEM micrograph of isolated kidney granules from freshly harvested *Mytilus galloprovincialis*.

Scale bar = 1 µm.
Figure 4.8: EDS spectra of kidney granules isolated from *Mytilus galloprovincialis*.

(a) freshly harvested;
(b) loaded with 0.5 µg·g⁻¹ particulate zinc for 28 days;
(c) loaded with 1.0 µg·g⁻¹ particulate zinc for 28 days;
(d) loaded with 2.5 µg·g⁻¹ particulate zinc for 28 days.
It was very difficult to get a clear image of the kidney granules using SEM. The very small size of the granules resulted, initially, in them sinking in to the adhesive used to attach them to the stub. This was solved by using an adhesive specially designed for very small objects. Different coating techniques were also trialed, sputter coating versus evaporative coating and carbon coating compared to carbon followed by a coating of gold. Difficulties were also experienced with excess charging on samples. Despite trying many different techniques, very little improvement was seen in the clarity of SEM images.
4.4 DISCUSSION

A number of effects were seen following zinc-loading of *Mytilus galloprovincialis*. TEM revealed degeneration of the kidney tissue, the appearance of granules with concentric rings, an increase in size but a decrease in number of granules present in the cells, and an increase in the percentage of multivesicular bodies, while the EDS analysis confirmed that the major elements present in the granules were phosphorus, sulphur, calcium, zinc and sodium (as observed in ICP analysis of extracted granules in Chapter 3). While aluminium was also recorded from the granule samples, it is more likely that this peak resulted from the aluminium stub that the granules were mounted on. The uneven surface presented by the granules resulted in a number of backscattered electrons exciting the adjacent stub producing an aluminium peak in the spectrum. In addition, aluminium was not shown to be present by ICP analysis.

The EDS analysis was also able to distinguish between granules containing different levels of zinc. Granules with high levels of zinc had a much larger zinc peak and a much brighter appearance on the electron microscope screen, while granules with lower levels of zinc had a much smaller zinc peak and looked duller. This may be a result of higher zinc levels in the granules producing more back-scattered electrons and, thus, a brighter image. The fact that granules containing different levels of zinc are present and that the extractions from zinc-loaded mussels contained more “bright” granules, is further evidence that granules were accumulating the excess zinc.

Interestingly, the levels of both iron and copper did not appear to change markedly between granules extracted from control and zinc-loaded mussels. This is in direct contrast to the results obtained from ICP (Chapter 3) which showed a dramatic decrease in the concentration of these two metals after zinc-loading. This can be explained by the fact that ICP measures the elemental composition of the whole sample (digested granules) while SEM EDS only analyses the surface of the granules. Previous discussion of the ICP results (Chapter 3) indicated that zinc could replace iron and copper at metal binding sites in the granule if it was present in excess. However, iron
and copper bind more readily to the metal binding sites resulting in surface binding sites being taken up by iron and copper while binding sites within the granule have zinc ions bound to them. Thus, there is no apparent change in copper or iron levels at the surface of the granule.

The changes in kidney tissue and granule structure observed using TEM appear to be the result of a combination of two main stresses: the toxic effects of excess zinc and the fact that the mussels were taken from their natural environment and kept in the laboratory. One of the most visible effects with increased levels of zinc-loading is the degeneration of the kidney tissue. This was visible as a gradual decrease in the number of microvilli and mitochondria present and culminated in a loss of cell membrane integrity in kidney tissue from mussels loaded at 2.5 µg.g⁻¹. This degeneration is presumably mainly the result of zinc toxicity, as the most severe cases were seen in the mussels loaded at the highest level of zinc. However, a proportion of these changes may be due to the stress of being kept in the laboratory, since mussels that were maintained in the laboratory for 28 days without being exposed to excess zinc, also showed slight changes in the structure of the kidney tissue.

The decrease in the mean number of granules per kidney cell after 28 days of zinc-loading is at least partly a result of this tissue degeneration. Under normal conditions, the granules are excreted to the lumen of the kidney via exocytosis (Pirie & George, 1979). However, decreasing membrane integrity would facilitate the loss of granules to the lumen of the kidney. This observation is supported by the reduction in granule yield with increasing levels of zinc-loading (see Chapter 3) and a decrease in the total number of granules per cell after 28 days of zinc-loading at 0.5 µg.g⁻¹, 1.0 µg.g⁻¹ and 2.5 µg.g⁻¹ (Table 4.2). The result for mussels loaded with 1.0 µg.g⁻¹ zinc for 10 days appears to be slightly anomalous, in that the total number of granules per cell increased when compared to that in freshly harvested mussels. However, this may be explained by the relatively short period of time the mussels had been exposed to the excess zinc. There was no indication that cell integrity had been compromised in these animals and hence
no concomitant increase in granule loss. In fact, granule production is likely to have increased in these cells in order to detoxify the excess zinc, resulting in a higher number of granules per cell.

Granules with concentric rings have not been previously reported in kidney tissue of Mytilus. The appearance of concentric rings and changes in the proportion of multivesicular bodies may be linked to a change in the way the granules are formed. Under normal conditions, a lipofuscin matrix, made up of cross-linked proteins and lipids resulting from peroxidation, forms within the lysosome. Metal-binding sites are randomly distributed throughout the lipofuscin matrix of the granule and binding of the metal ions is governed by the size of the ion, the geometry of the binding site and the distance between the binding ligands (for further discussion see Chapter 1).

Metal uptake may occur via a combination of a number of processes, including fusion of phagocytotic vesicles with primary lysosomes (George et al., 1976), autophagy of degenerated organelles containing metals (George et al., 1982), the uptake of metallothioneins (George, 1983) and sequestration of metal ions by acidic molecules within the lysosomes (George, 1982). Metal accumulation is the result of irreversible binding of the metal ions as they are trapped in a lipofuscin matrix (George, 1983). Since lipofuscin is made up of lipid and protein, it contains ample metal binding sites so that any metal which is taken up into the lysosome is readily bound to the lipofuscin.

The way in which the internal structure of the granule is formed may change when metal is present in excess amounts, in combination with stress from being kept in the laboratory. Rather than forming a uniform matrix under these conditions, the lipofuscin may begin to form layers of material within the lysosome. Metal accumulation would still occur via the processes outlined above, with the only change being the location of the metal. That is, if lipofuscin is forming in layers, metal ions will be bound in these layers, resulting in a granule with concentric rings. The
formation of layers would mean that all but the metal ions bound to the outside of the outer layer would be trapped within the lysosome, as there would be no route for them to follow to the outside of the lysosome if released. This may provide a more efficient manner of detoxifying the excess metal.

The speed of granule formation and deposition of metal within the lipofuscin matrix may also influence the internal structure of the granule. This may account for the appearance of so many granules with concentric rings in the mussels loaded with 1.0 μg.g⁻¹ zinc for 28 days and then maintained for a further 14 days without zinc-loading. The removal of the additional stress of continued zinc-loading from these mussels allows them to produce granules as quickly as possible to remove the excess zinc from their tissues. The speed of granule production and utilisation may result in the formation of concentric rings.

Multivesicular bodies were seen in tissue from all mussels, indicating that they occur under all conditions of metal stress, including no stress. Interestingly, the number of multivesicular bodies.cell⁻¹ increased in mussels loaded with 1.0 μg.g⁻¹ for 10 days, decreased when mussels were loaded with 1.0 μg.g⁻¹ for 28 days and increased again, significantly, when these mussels were maintained for a further 14 days without zinc-loading. This apparent contradiction in response may be explained by changes in the process involved in production and excretion of granules.

It has been suggested that multivesicular bodies (Type 3 granules) are the result of an incomplete fusion of two or more smaller granules (George et al., 1982). It is possible that the excretion mechanism for kidney granules is also affected by metal stress and that the rate of granule excretion through normal processes, that is exocytosis from the cell, is reduced. An increased number of small granules within the cell could increase the chance of fusion creating multivesicular bodies. If this is so, the larger the number of small granules produced to cope with the excess zinc presented to the mussel, the larger the number which will fuse, resulting in an increased percentage of
multivesicular bodies. This hypothesis is supported by the fact that by far the highest number of multivesicular bodies.cell\(^{-1}\) was seen in the mussels loaded at 2.5 µg.g\(^{-1}\) zinc for 28 days.

Interestingly, the mean diameter of the granules actually increased after zinc-loading, with the largest mean diameter being seen in mussels loaded with 2.5 µg.g\(^{-1}\) zinc for 28 days. The mean diameter of the granules in mussels loaded with 1.0 µg.g\(^{-1}\) zinc was larger, compared to that in freshly harvested mussels, after both 10 and 28 days of loading. A positive relationship exists between the mean diameter of the granules and the number of multivesicular bodies in the cells. Since multivesicular bodies are the result of fusion of smaller granules, they tend to have a larger overall diameter. Hence, a larger number of multivesicular bodies in a cell would increase the mean diameter of the granules in that cell. This can be demonstrated by comparing the results for mussels loaded at 2.5 µg.g\(^{-1}\) where the mean diameter was 1.08 ± 0.13 µm and the mean number of multivesicular bodies.cell\(^{-1}\) was 10.3 ± 1.5, with those for freshly harvested mussels where the number of multivesicular bodies.cell\(^{-1}\) was only 3.8 ± 0.5 and the mean diameter of the granules was 0.63 ± 0.02 (Table 4.2).

Many of the granules seen in tissues examined in this study do not strictly fit the definitions given by George and Pirie (1979). These granules may have an even or uneven electron density and many could be described as multivesicular bodies or Type 3 granules. However, they all have one feature in common, namely the electron dense portion of the granule does not completely fill the space enclosed by the lysosomal membrane (see for example Figure 4.3a-d). While some of the shapes of the granules observed in these tissues may be the result of fixation and sectioning artefacts, this type of granule can also be seen in the photos of George and Pirie (1979, Plate IIIA) and can be considered to be a naturally occurring form. Thus, the definition of a Type 3 granule should be expanded to read “of either uniform or uneven electron density, may be very large, up to 5 µm in diameter, and contain a number of smaller bodies, may also be referred to as multivesicular bodies” (Table 4.1).
M. galloprovincialis appears to be able to quickly repair the damage to the kidney tissue caused by excess zinc when the pollution source is removed. The cells in tissue from mussels loaded with 1.0 µg.g\(^{-1}\) zinc for 28 days and then maintained for a further 14 days without zinc-loading, regained membrane integrity, and numerous microvilli and mitochondria were again present. Interestingly, the number of both granules with concentric rings and multivesicular bodies increased significantly in these cells, compared with tissue exposed to 1.0 µg.g\(^{-1}\) for 28 days. This may be partly due to the cells regaining membrane integrity and, therefore, retaining the granules within the cell rather than losing them to the lumen of the kidney.

This part of the study revealed a number of changes occurred in the internal structure of the kidney cells, and the granules themselves, after zinc-loading. These changes included the presence of a fourth type of granule with a concentric ring formation, not previously recorded in kidney cells of Mytilus. While the mussels in this part of the study were subject to loading with excess amounts of particulate zinc, those in the next part were subject to chronic long-term zinc-loading. Many of the changes described above may only be the result of this acute exposure to zinc and may not be present in the mussels exposed to chronic levels of excess zinc.
CHAPTER 5

INVESTIGATION OF THE KIDNEY GRANULES FROM *MYTILUS GALLPROVINCIALIS* FROM THE ESTUARY OF THE DERWENT RIVER

5.1 INTRODUCTION

The Derwent River and its associated estuary, situated at the southern end of Tasmania, is one of Australia’s most polluted river systems (Coughanowr, 1997). It has been severely affected by the input of contaminants from both point sources, including sewage treatment plants and industry, and more diffuse sources, such as urban runoff and leachate from contaminated sites (Director of Environmental Control 1972, Coughanowr, 1997). Although there are no known natural sources of lead, zinc, cadmium or mercury in the catchment area of the river (Bloom & Ayling, 1977), these metals are found to excess in the estuary waters, sediments and biota (Coughanowr, 1997).

A zinc refinery is the main source of metallurgical waste, which includes zinc, arsenic, copper, cadmium, lead and mercury (Cooper et al., 1982). From 1917 until 1981, liquid emissions from the refinery were discharged directly to the river without treatment (Coughanowr, 1997). Since this time, this effluent has been either recycled within the plant or treated and discharged, with a reduction in the levels of zinc and other metals released into the river. However, zinc, in particular, is still discharged from the refinery via stormwater runoff, flow of groundwater contaminated by leachate from stockpiles and dumps, and direct loss from ship loading wharves.

Commercial oyster (*Crassostrea gigas*) farming was begun in the estuary of the Derwent River in the late 1960s (Thrower & Eustace, 1973). The oysters were first marketed in 1970, but were almost immediately withdrawn from sale as they were suspected of causing nausea and vomiting. A survey carried out to determine the cause
of these symptoms found that the levels of zinc, cadmium and copper in the oysters were well in excess of those recommended for human consumption (Thrower & Eustace, 1973). At the time of the study, the recommended levels for human consumption were 40 µg.g⁻¹ wet weight for zinc, 5.5 µg.g⁻¹ for cadmium and 30 µg.g⁻¹ for copper. None of the oysters examined complied with these regulations, with individual animals containing concentrations of up to 21,000 µg.g⁻¹ zinc, 63 µg.g⁻¹ cadmium and 450 µg.g⁻¹ copper wet weight (Thrower & Eustace, 1973). The NH&MRC (National Food Standard Code) has since altered the recommended levels to 1,000 µg.g⁻¹ zinc, 2 µg.g⁻¹ cadmium and 70 µg.g⁻¹ copper in shellfish (Bloom & Ayling, 1977, Coughanowr, 1997).

Mussels (*Mytilus galloprovincialis*) are also common in the area and were often collected for human consumption. A survey in 1975 showed that, paralleling the situation in oysters, mussels in most parts of the Derwent estuary were unfit for human consumption. This was primarily due to levels of cadmium, mercury, zinc, lead and copper which were above those recommended by the NH&MRC (Bloom & Ayling, 1977). Metal levels in both oysters and mussels were assessed again in 1982 (Cooper et al., 1982) and in 1990 (Department of Environment and Land Management, 1995). Specimens of both species taken from contaminated sites were still unfit for human consumption, although the metal levels had decreased from the levels seen in the first survey and the metals levels were lower in mussels compared to oysters. However, it appears that the levels measured in 1990 were unusually low and higher levels have been recorded in subsequent years (R. Dineen, personal communication).

Mussels are sessile animals which means that they cannot directly avoid any stresses, including excess levels of metal pollutants, introduced into their environment. Therefore, it is reasonable to assume that they will have mechanisms other than movement to cope with stress. Mussels exposed to excess levels of many metals have been shown to produce a metallothionein-like protein which binds the metal ions, thereby playing a detoxification role (see for example Noel-Lambot, 1976; Frankenne et
al., 1980; Bebianno & Langston, 1991; Viarengo et al., 1985a). Another mechanism, which has been suggested to function in the detoxification of excess metals, is the production of lysosomal granules in the cells of the kidney of mussels (see Chapters 3 and 4). Although lysosomal granules are present in the kidneys of Mytilus from reference sites (Pirie & George, 1979 and Chapter 3), they are also produced at enhanced levels well above background, by mussels in response to excess metals in their environment (Chapter 3 and George & Pirie, 1979). Mussels living in parts of the Derwent River estuary are continually exposed to high levels of metals in their environment. Under these conditions of chronic exposure, the pattern of granule accumulation in the kidney cells of mussels from the Derwent provides a comparison with background/baseline levels in mussels from the control site at 7-mile Beach.

The aims of this section of the study were thus:

- to establish base-line data on kidney granules in M. galloprovincialis from a reference site outside the Derwent River Estury not impacted by metal pollution.
- to compare the composition and appearance of the kidney tissue and granules in control mussels to those in mussels collected from a polluted site in the estuary of the Derwent River.
- to compare the results from mussels from a zinc-polluted site in the estuary of the Derwent River and mussels exposed to high levels of zinc in the laboratory.

Both light and transmission electron microscopy studies were carried out on the kidney tissue and the composition of the granules was determined using inductively-coupled plasma spectroscopy and energy dispersive spectroscopy.
5.2 MATERIALS AND METHODS

5.2.1 Collection of mussels

Mussels (*Mytilus galloprovincialis*) were collected from Seven Mile Beach, a pristine site not impacted by metal pollution and which was used as a control site (Figure 5.1). *M. galloprovincialis* were also collected from Bellerive Bluff, in Tasmania, Australia. This site is approximately 1.5 km south of the zinc refinery, in the middle section of the estuary, and is considered to be seriously polluted (Coughanowr, 1997). At both sites, the mussels lived in an intertidal environment, with a tidal range of up to 1.3 m, and were exposed at low water. Consequently, they would experience periods when they were unable to feed. The water is relatively clear at both sites, with total suspended solids being slightly higher at Bellerive Bluff (R. Dineen, personal communication).

Mussels from both sites were packaged in ice as soon as possible after collection, and transported live to Perth by air. Upon arrival, each mussel was measured, shucked and the kidneys removed. Using techniques described previously, the kidneys were either used for the extraction of granules (Chapter 2) or fixed and processed for light or transmission electron microscopy (Chapter 4). Following purification, isolated granules were attached to aluminium stubs and processed as previously described (Chapter 4) for scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). Ten individual granules per mussel were analysed using EDS analysis and a mean determined. The remainder of the granules were acid digested and analysed using inductively-coupled plasma spectroscopy (ICP).

5.2.2 Analysis of data

To determine if there were differences between mussels from the control and the impacted sites, statistical analyses were undertaken as described in Chapter 2 on granule diameters, total number of granules, the number of multivesicular bodies and the number of granules with concentric rings in each treatment. Analysis was also
undertaken to determine if differences existed between these parameters in mussels obtained from Tasmania and Western Australia.
Figure 5.1: Map of Derwent Estuary region showing mussel collection sites.
5.3 RESULTS

5.3.1 Physical characteristics of mussels

The mean length of the control *Mytilus galloprovincialis* from Seven Mile Beach was 30.6 mm while that of the mussels from the polluted site, off Bellerive Bluff, was 51.5 mm (Table 5.1). The mean width:height ratio was 0.95 for the control animals from Seven Mile Beach and 0.72 for those from the polluted site at Bellerive Bluff (Table 5.1). The shells of the mussels from both sites had a rough surface. When viewed laterally, they were quite long and narrow and the hinge area was hooked, while a dorsal view showed a thickened, wide shell.

Table 5.1: Mean length (mm) and width:height ratios of Tasmanian mussels.

<table>
<thead>
<tr>
<th>Location</th>
<th>Type of site</th>
<th>Mean length (mm)</th>
<th>Width:height ratio</th>
<th>Number measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seven Mile Beach</td>
<td>Control</td>
<td>30.6</td>
<td>0.95</td>
<td>110</td>
</tr>
<tr>
<td>Bellerive Bluff</td>
<td>Polluted</td>
<td>51.5</td>
<td>0.72</td>
<td>119</td>
</tr>
</tbody>
</table>

5.3.2 Inductively-coupled spectroscopy analysis

A greater weight of granules was extracted from the zinc-contaminated mussels (61 mg) compared to the control animals (31 mg) (Table 5.2). ICP analysis indicated the presence of phosphorus, sulphur, calcium, zinc, sodium, iron and copper. Higher levels of zinc, copper and phosphorus, and reduced levels of iron and sodium were observed in the mussels from the polluted site at Bellerive Bluff compared to those from the control site (Table 5.2). Similar levels of sulfur and calcium were seen in mussels from both sites.
Table 5.2: Mean concentration (µg.g⁻¹) and amount (µg) of elements present and weight (mg) of granules isolated from *Mytilus galloprovincialis* from Tasmania.

<table>
<thead>
<tr>
<th>Collection Site</th>
<th>Weight (mg)</th>
<th>P</th>
<th>S</th>
<th>Ca</th>
<th>Zn</th>
<th>Na</th>
<th>Fe</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seven Mile Beach (control)</td>
<td>31</td>
<td>13277</td>
<td>7734</td>
<td>6181</td>
<td>1424</td>
<td>776</td>
<td>1660</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Conc. Amount</td>
<td>41</td>
<td>23</td>
<td>19</td>
<td>4.4</td>
<td>2.4</td>
<td>5.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Bellerive Bluff (polluted)</td>
<td>61</td>
<td>17746</td>
<td>7576</td>
<td>6873</td>
<td>13570</td>
<td>197</td>
<td>373</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Conc. Amount</td>
<td>108</td>
<td>46</td>
<td>42</td>
<td>83</td>
<td>1.2</td>
<td>2.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

EDS of individual granules revealed a picture that was very similar to that given by ICP analysis, although the presence of additional elements was observed. Granules isolated from the control mussels contained mainly phosphorus, sulphur and calcium, with smaller amounts of sodium, magnesium, silicon, chlorine, potassium, iron and copper (Figure 5.2a). The granules isolated from the zinc-contaminated mussels had a very similar composition except they contained much greater zinc and sodium (Figure 5.2b). The aluminium peak is likely to be from the stub the granules were mounted on (cf Chapter 4). While an increase was seen in spectral intensity in the zinc-contaminated granules that may indicate that more of all the elements were present in these granules when compared to the granules from mussels from the control site (Figure 5.2), the analysis was only semi-quantitative so this is not conclusive.

**5.3.3 Transmission electron microscopy of kidney tissue**

Electron microscopy of kidney tissue taken from mussels from the control and polluted sites also revealed some differences in the structure of the cells. The kidney cells from control mussels were elongate with basal nuclei, large numbers of well-developed microvilli, abundant membranous whorls and numerous electron-dense granules (Figure 5.3 a). The majority of the granules were electron dense Type 1 granules with a mean diameter of 0.78 µm ± 0.04 µm. Some of these, while retaining their regular
Figure 5.2: EDS spectra of kidney granules from *Mytilus galloprovincialis* from Tasmania. (a) Seven Mile Beach control site; (b) Bellerive Bluff contaminated site.
Figure 5.3: TEM micrographs of kidney tissue from *Mytilus galloprovincialis* from Tasmania.

(a) Seven Mile Beach control site;

(b) Bellerive Bluff zinc-contaminated site.

Scale bars = 5 µm.
shape, were less electron dense. There were a small number of Type 3 granules (2.9 ± 0.5 per cell) while granules with concentric rings (Type 4) were rare (0.3 ± 0.1 per cell) (Figure 5.3a and Table 5.3).

The kidney cells in *M. galloprovincialis* from the contaminated site were intact but were shorter and wider in shape compared to the control cells (Figure 5.3b). Microvilli were still abundant, the lumen of the kidney appeared somewhat shrunken, and numerous clear vacuoles, some of which were likely to have been Type 2 granules, were present. There was no significant difference between the total number of granules per cell in the control (55.5 ± 5.9) and zinc-contaminated mussels (57.4 ± 5.7). The majority of the granules present in the cells from zinc-contaminated mussels were Type 3 granules. In fact, there were significantly more (p < 0.05) Type 3 granules in this tissue (7.8 ± 0.8) compared to the number seen in cells from control mussels (2.9 ± 0.5). The electron dense portion of many of these granules did not entirely fill the area enclosed by the membrane. A small number of Type 1 granules were present while Type 4 granules were scarce (0.2 ± 0.1 per cell). The mean size of the granules was significantly smaller (p < 0.05) in the cells from zinc-contaminated tissue (0.64 μm ± 0.03 μm).

Table 5.3: Number and size of granules present in the kidney cells of *Mytilus galloprovincialis* from the Derwent Estuary region of Tasmania.

<table>
<thead>
<tr>
<th>Collection site</th>
<th>Cells counted</th>
<th>Total granules per cell (mean ± SE) (range)</th>
<th>Multivesicular bodies per cell (mean ± SE) (range)</th>
<th>Granules with concentric rings per cell (mean ± SE) (range)</th>
<th>Diameter of granules (μm) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seven Mile Beach (control)</td>
<td>12</td>
<td>55.5 ± 5.9 (30-92)</td>
<td>2.9 ± 0.5 (0-6)</td>
<td>0.3 ± 0.1 (0-1)</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td>Bellerive Bluff (polluted)</td>
<td>12</td>
<td>57.4 ± 5.7 (24-83)</td>
<td>7.8 ± 0.8 (4-13)</td>
<td>0.2 ± 0.1 (0-1)</td>
<td>0.64 ± 0.03</td>
</tr>
</tbody>
</table>
5.3.4 Comparisons with Western Australian Mussels

Chemical analysis revealed the same elements were present in kidney granules from mussels from both Tasmania (Derwent Estuary) and Western Australia (Garden Island), although the concentration of all elements varied between the two regions. Levels of phosphorus and calcium were higher and levels of sodium and sulphur lower in the Tasmanian mussels from both sites when compared to the Garden Island mussels, regardless of whether they were clean or zinc-loaded. However, iron, copper and zinc levels in the Tasmanian controls were much lower than those in the Garden Island controls (Table 5.2). The Tasmanian mussels also had detectable silicon in the granules (Table 5.2 and Figure 5.2), an element which was absent from the Garden Island mussels. The concentration of zinc in the Tasmanian controls was lower than that in the freshly harvested Garden Island mussels, while the animals from the polluted site at Bellerive Bluff had a much higher zinc concentration than that of any of the laboratory zinc-loaded mussels (compare Tables 3.1 and 5.2). The higher concentration of zinc in granules from the polluted site in Tasmania revealed by ICP analysis, accounts for the larger zinc peak seen in the EDS spectra from the Tasmanian mussels.

In contrast to the results of short term exposure to high levels of zinc (Chapter 4), electron microscopy of the Tasmanian mussels revealed that chronic exposure to excess levels of zinc produced only small changes in the ultrastructure of the kidney cells. Thus, the kidney cells from mussels collected from Bellerive Bluff maintained their integrity and columnar shape and still contained all organelles, although they were shorter and wider in shape (Figure 5.3b). The kidney cells from mussels exposed to 2.5 µg.g⁻¹ zinc in the laboratory had lost much of their integrity, had no microvilli and contained far less granules than cells from control tissue.

The results obtained for granule yield were also in complete contrast to the situation seen in the laboratory zinc-loaded mussels. The granule yield increased in the mussels from Bellerive Bluff (mean of 61 µg) compared to the Tasmanian controls (mean of 31
µg), while it decreased as the level of laboratory zinc-loading increased, from a mean of 90 µg in the controls to a mean of 35 µg in mussels loaded at 1.0 µg.g\(^{-1}\). The total number of granules per cell was lower in Tasmanian mussels from both the control (55.5 ± 5.9) and contaminated (57.4 ± 5.7) sites (Table 5.3) compared to the freshly harvested Garden Island mussels (65.1 ± 5.8) (Table 4.2).

Mean granule diameter was slightly lower in both the Tasmanian control (0.78 µm ± 0.04 µm) and polluted (0.64 µm ± 0.03 µm) mussels than any recorded for mussels either freshly harvested from Garden Island (0.63 µm ± 0.02 µm), or loaded with zinc in the laboratory (range: 0.88 µm ± 0.16 µm to 1.08 µm ± 0.13 µm). The diameter of granules in mussels from the polluted site at Bellerive Bluff was significantly lower (p < 0.05) than that from mussels loaded with either 0.5 µg.g\(^{-1}\) zinc or 2.5 µg.g\(^{-1}\) zinc in the laboratory.

The number of Type 3 granules, or multivesicular bodies, was significantly higher (p < 0.001) in the mussels from the polluted site (7.8 ± 0.8) than in freshly harvested Garden Island mussels, fed laboratory controls or mussels loaded at 1.0 µg.g\(^{-1}\) (range: 1.0 ± 0.3 to 4.8 ± 0.7). While the number of granules with concentric rings was lower in both the control and polluted Tasmanian mussels than that for all laboratory treatments, the only significant differences were seen between both the control and contaminated Tasmanian mussels and Garden Island mussels loaded at 1.0 µg.g\(^{-1}\) for 28 days and then transferred to clean seawater for a further 14 days.
5.4 DISCUSSION

The results of this study strongly suggest that *Mytilus galloprovincialis* from the polluted site at Bellerive Bluff are producing granules in response to the excess metal levels in their environment. Evidence for this includes the increase in the number of granules in the kidney cells and the increase in the concentration of zinc in granules from the mussels at this site. These results confirm that granule production is a “natural” process as these animals were exposed to excess metals in their natural environment rather than under experimental conditions in the laboratory.

The kidney granules extracted from the Tasmanian control mussels were very similar in structure and overall composition to those from mussels of the same species grown on a mussel farm at Garden Island, Western Australia (see Chapter 3). This was despite large differences in the level of zinc exposure and environmental conditions experienced by the mussels at the different sites. The Tasmanian mussels lived in an estuary subject to tidal fluctuations and were exposed to the air at low tide (see section 5.2). In contrast, the mussels farmed at Garden Island lived in an oceanic environment with clearer waters. They were not exposed at low tide and, because of farming practices, did not have to compete as intensely for either food or space.

Some of the variations seen in the composition of control mussels from Tasmania and Garden Island may be explained by differences in water quality at each site, although these data were not available. For example, the Tasmanian mussels had lower levels of iron and copper and the presence of silicon. However, uptake of other elements may be subject to physiological control. It should be noted that silicon has only ever been found as silica in biological systems. In the latter case, differences in the mussels’ environment may lead to differences in the physiology of the mussels. For example, the Tasmanian mussels experience periods of anoxia each day when the tide goes out and may also have to produce thicker shells to mitigate the effects of higher wave action. Thus, differences in levels of phosphorus, sulfur and calcium may be due to these physiological differences.
The differences observed in cell structure and granule yield between Tasmanian and Western Australian mussels are most likely to be due to a combination of the length of time over which the two groups of mussels were exposed to zinc and the level of zinc exposure. Thus, mussels from Bellerive Bluff, Tasmania were exposed to relatively low levels of zinc over a relatively long period of time (chronic exposure), while the cultivated mussels from Garden Island were exposed to high levels of zinc in the laboratory over a short period of time (acute exposure).

Under conditions of chronic exposure, granule production would be likely to be at a rate equal to or slightly greater than that the mussel needs to detoxify the excess metal. This would account for the increase in granules seen in mussels collected from the polluted Bellerive Bluff site compared with the Seven Mile Beach specimens. Similar results were obtained in *Donacilla cornea* exposed to sublethal concentrations of cadmium and copper with an increase in the number of granules in kidney cells (Regoli *et al.*, 1992).

In contrast, while the laboratory zinc-loaded mussels may maximise the production of granules in order to detoxify the vast amounts of zinc they were being exposed to, the rate of granule excretion in the laboratory-loaded mussels would also be much higher than that in the control mussels, reducing the total number of granules present. In addition, granules are lost through the disintegration of the cell and the subsequent loss of cell contents, further reducing the number of granules present in the cell and effectively reducing the mussel’s ability to detoxify the excess zinc.

The calcium content of the granules appears to be different depending upon whether exposure to excess zinc was chronic or acute. Viarengo (1989) presented arguments that an alteration of calcium metabolism is induced by oxidative stress, which is at least partly the result of exposure to excess metal. This author argued that the inactivation of the Ca-transporting ATPases by lipid peroxidation, resulting from the presence of excess metals, could lead to an alteration of the calcium homeostasis of the cell. Such an alteration could eventually lead to cell death and could explain the structural changes.
seen in the kidney cells in mussels from the polluted Bellerive Bluff site. The mussels collected from this site had a slightly higher calcium content than the Seven Mile Beach control animals (Table 5.2) Viarengo et al., (1988) has previously demonstrated that mussels collected from polluted field sites had a higher cellular calcium content when compared to mussels from uncontaminated environments.

Another major difference was noted using electron microscopy. While granules extracted from all mussels were spherical and electron-dense, the number of granules with the concentric ring formation was much lower in the Tasmanian mussels. This phenomenon may be due to the nature and time frame involved in the formation of the granules. While the actual amount of time taken to form a granule may not change when environmental conditions change, the time frame for deposition of the granule’s contents is likely to alter. Thus, the contents of granules in the laboratory fed mussels would have been deposited relatively quickly, over a period of only a few weeks, as the mussels had to cope with suddenly high concentrations of zinc in their environment. When granule contents are deposited relatively quickly, the deposition may occur in layers, giving rise to concentric rings (see Chapter 4). Conversely, the longer term exposure of the mussels in the Derwent Estuary to high metal concentrations could result in the contents of the granules being deposited over a longer period of time. This may allow for the end products of protein and lipid peroxidation (lipofuschin) and other waste products, including the excess metal, to be deposited into the granule in a more controlled fashion, resulting in the more uniform appearance of the granules.

The relatively large proportion of multivesicular bodies observed in the kidney cells of the mussels from Bellerive Bluff, compared to those from Seven Mile Beach, may also be a result of exposure to high levels of metal over extended periods of time. Multivesicular bodies are thought to be the result of fusion of a number of smaller, Type 1, granules. Such fusion may further reduce the availability of metals within the granules to the cell’s metabolism, enhancing cell survival. In addition, the simple fact
that more smaller granules are present increases the chance of fusion between them, which in turn increases the number of multivesicular bodies present.

Marked differences in shell morphology were seen between the mussels from Garden Island and those from the Derwent Estuary. Shell morphology in *Mytilus* is greatly influenced by both population density and individual growth rate (Seed, 1968). Mussels from areas of high density tend to have narrow, elongated shells while those from low density populations have shells with a broad, triangular shape (Seed, 1968). As age increases, growth in height decreases relative to increase in length and the shells become wider and dorsally rounded (Seed, 1968). The width:height ratio of mussels can be used as an indicator of both relative age and growth rate. A ratio of 0.6 - 0.8 indicates young, fast-growing mussels while animals with ratios of 1.0 - 1.1 are older, fatter and senescent (Lobel *et al.*, 1991).

The morphology of the shells of the mussels used in this study has been influenced by a combination of density and age, although age appears to have played the greater part. The Tasmanian mussels came from fairly densely populated areas while the Garden Island mussels were less densely populated due to ‘thinning’ techniques used in the farming process. This resulted in the Garden Island mussels being much larger and broader than those from Tasmania. However, the shells of the latter were wider than the animals from Garden Island, indicating greater age. The width:height ratio also indicated that the Tasmanian mussels were older than the Garden Island mussels.

The ratios of shell width to height obtained for mussels from Tasmania indicated that the Seven Mile Beach control animals were relatively young and growing relatively fast while those from the Bellerive Bluff site were older and growing at a much slower rate. This difference is likely due to a number of factors, such as, the physical environment, the presence of environmental metals and age. The increased energy expenditure involved in granule production for metal detoxification would reduce the energy available for growth. The greater age of the Bellerive Bluff mussels also allows the granules to be deposited over a longer period of time. As a result, the excess zinc that
these animals were exposed to was detoxified by the granules and did not cause the death of the mussels. In contrast, the mussels loaded with zinc in the laboratory were exposed to the excess metal over a very short period of time and detoxification mechanisms were unable to prevent their death in many cases.

In comparison to the Tasmanian mussels, the animals from Garden Island were very young, and had a clean and abundant food source, resulting in a faster growth rate. The wider shells of the Tasmanian mussels are more likely to be the result of many years of shell accretion rather than from living in low density populations as they were also thicker. In addition, the Garden Island mussels were living in quite deep water while the Tasmanian mussels were living on muddy shorelines with a tidal influence and quite high wave action (R. Dineen, personal communication), resulting in increased environmental stress and slower growth.

The level of zinc in individual mussels has been found to be affected by the size, and hence age and weight, of the individual, with smaller individuals usually having a higher concentration of zinc (Boalch et al., 1981; Boyden, 1974; Boyden, 1977; Jones & Walker, 1979). In order to eliminate this variation, Cossa (1989) advised using mussels of similar shell length, if possible within a size range of 50 - 100 mm with the centre of the range around 60 mm. The results of this study indicate that this advice does not hold true when different populations are compared, as levels of many metals were in fact lower in the smaller Tasmanian animals than the Garden Island mussels. This illustrates the importance of considering a number of factors when carrying out such comparisons between populations.

Overall, the results from this part of the study indicate that *M. galloprovincialis* produces kidney granules under natural conditions, and that they are involved in the detoxification of excess zinc. Furthermore, if mussels are exposed to metal pollutants in their natural environment, granule production is increased in order to detoxify the excess metal.
CHAPTER 6

STRUCTURE OF METAL-CONTAINING GRANULES AND MORPHOLOGY OF THE KIDNEY IN THE FRESHWATER MUSSEL, *VELESUNIO ANGASI*

6.1 INTRODUCTION

Freshwater mussels of the genus *Velesunio* (Unionacea: Eulamellibranchiata: Hyriidae) are relatively abundant in Australia, with *V. ambiguus* (Philippi) being among the most common and widespread of the Australian species (Jones & Walker, 1979). This species occurs throughout the Murray-Darling river system and in most other coastal rivers of eastern and southern Australia (Jones & Walker, 1979). The closely related *V. angasi* occurs abundantly in rivers, creeks and billabongs of the Alligator Rivers region of the Northern Territory (Jeffree, 1985).

Extracellular granules have been isolated from many freshwater mussels (Jeffree et al., 1993). In the genus *Velesunio*, the presence of extracellular granules has been reported in *V. ambiguus* by Ch'ng-Tan (1968) and in *V. angasi* by Jeffree & Simpson (1984). The granules occur in the intercellular spaces of the connective tissue of the gills, mantle, labial palps, the dorsal portion of the foot and the renal organs Ch'ng-Tan (1968). They may occur either individually, or in aggregations of varying size and colour, depending on their location in the body. The granules are primarily composed of calcium phosphate (50-75%) but may also contain iron, manganese, barium, magnesium, aluminium, zinc, silicon (as silica) and sulfur within an organic matrix (Jeffree et al., 1993). The structure of the granules is invariably amorphous.

The main element within the granules, phosphorous, is present both as orthophosphate (PO$_4$) and pyrophosphate (P$_2$O$_7$) in approximately equal amounts, although, on the basis of M$^{2+}$/P ratios, the latter is indistinguishable from HPO$_4$ and may have been incorrectly
identified (Jeffree et al., 1993). The best evidence for the form of phosphate comes from quantitative analyses of the granules and calculation of the $M^{2+}/P$ ratios (Taylor & Simkiss, 1984; Taylor & Simkiss 1989). Thus, isolated granules from the gills and visceral organs of *V. angasi* had an $M^{2+}/P$ ratio suggesting the presence of HPO$_4^{2-}$ and/or $P_2O_7^{4-}$ (Jeffree and Markich, unpublished results, cited in Jeffree et al., 1993). Jeffree et al. (1993) calculated a similar result for *V. ambiguus* from analysis of isolated granules carried out by Ch'ng-Tan (1968). In contrast, the former authors calculated that unionids from Europe and North America had $M^{2+}/P$ ratios indicative of amorphous calcium orthophosphate ($Ca_x(PO_4)_y$), whitlockite ($\beta$-$Ca_3(PO_4)_2$) and/or hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (see for example Roinel et al., 1973; Pynnonen et al., 1987; Silverman et al., 1989).

In most cases, elements that are accumulated by the extracellular granules in *Velesunio* will occur as insoluble phosphates (Jeffree, 1988). Accumulation of elements appears to be via adsorption or co-precipitation with calcium (Jeffree & Brown, 1992). The rate of loss of metal elements depends on the solubility of the hydrogen phosphate of that metal, with solubility being defined as the logarithm of the solubility product ($K_{sp}$). Thus, the lower the solubility, the slower the rate of loss of the metal from the granules will be (Jeffree, 1988). The granules play a major role in element dynamics in freshwater mussel tissues (Byrne and Vesk, 2000).

Although a considerable amount of study has been carried out on the extracellular granules of freshwater mussels in the genus *Velesunio*, no investigations have been undertaken into the possible presence of intracellular granules. As described previously in this thesis (Chapters 3, 4 and 5), many species of marine bivalves contain intracellular granules in their kidney. These granules may occur as insoluble deposits, or be lysosomal, and are often produced in response to excess metals in the animals’ environment. In Pine Creek and Copperfield Creek, in the Northern Territory, *V. angasi* are often exposed to a variety of metals, including iron, manganese, zinc, nickel, aluminium and copper (Milne et al., 1992). Many of these metals are found naturally in
the creek water as the area contains mineralised rocks. In addition, temporary increases in the levels of some metals may occur during the wet season when overflow of dams and ore stock piles at the nearby Pine Creek Gold Mine occurs (Milne et al., 1992). Many of these metals are present in particulate or colloidal forms.

In view of the large amounts of metals in the environment, it was thought possible that intracellular kidney granules may be involved in detoxification of metals in *V. angasi*, in a similar fashion to that described previously for marine mussels (see Chapters 3, 4 and 5). As such, the aims of this study were thus:

- to determine whether intracellular granules exist in the kidney of *V. angasi* collected from Copperfield Creek in the Northern Territory of Australia; and
- to determine elemental composition and structure by analysis of both the kidney tissue and the granules.

Both light and electron microscopy were used to analyse the structure of the kidney tissue and the extracellular granules, and to determine the presence of intracellular granules.
6.2 MATERIALS AND METHODS

6.2.1 Animals

Freshwater mussels (*Velesunio angasi*) were collected in July, 1993 from two sites in Copperfield Creek, downstream from the Pine Creek gold mine, in Northern Territory, Australia (Figure 6.1). Both sites were used by the Northern Territory Department of Mines and Energy (Milne *et al.*, 1992; Noller, 1999) when assessing the possible impacts of overflow from a process water dam at the Pine Creek gold mine. Site 1 was upstream of the point where the overflow entered the creek, while site 2 was downstream from this point.

The mussels live in the creek sediment, and so were collected from the banks of the creek. They were transported live to the laboratory by air as quickly as possible following collection. Upon arrival, the shell length and width of the mussels were measured, and the animals were weighed and shucked. For all animals, the distribution of granules was scored by examining the major organs following a slightly modified version of the procedure of Allison and Simpson (1989) (Figure 6.2). Briefly, this consisted of a visual assessment of where the granules occurred and the extent and density of coverage on each of the individual organs. If there were no granules present on an organ it was given a score of one, while a high coverage and high density of granules scored six.

6.2.2 Extraction and analysis of extracellular granules

The labial palps, mantle and the visceral mass were removed from five mussels from each site. Organs of the same type were pooled and placed in 3% NaOCl for 5 minutes before being rinsed in DDI H$_2$O with this procedure being repeated until only granules remained. A portion of the sample of granules was then removed and fixed in
Figure 6.1: Locations of mussel and water sample collection sites (after Noller, 1999), Copperfield Creek, Northern Territory.
Figure 6.2: Diagrams of opened mussels showing the extent of coverage by extracellular granules for scores of 1 to 6 (modified from Allison and Simpson, 123.
2.5% glutaraldehyde in 0.25M phosphate buffer overnight. These granules were then washed in phosphate buffer and processed for transmission electron microscopy (TEM) as previously described (Chapter 4). Sections of the resin in which the granules were embedded were cut and mounted on a copper 200-mesh grid coated with formvar. They were viewed using a Philips 301 TEM at 80kV. The remainder of the granules were dried under vacuum in a desiccator. A sample of these granules was acid-digested as previously described (Chapter 2) and analysed using inductively-coupled plasma spectroscopy (ICP) while another sample was mounted onto aluminium stubs using techniques described previously (Chapter 4). Samples were viewed and photographed using the scanning electron microscope (SEM) and energy dispersive spectroscopy (EDS) was carried out, all as described in Chapter 4. Granule diameters were also measured while the samples were in the SEM.

6.2.3 Morphology of kidney tissue

Whole kidneys were removed and fixed overnight at 4°C in 2.5% glutaraldehyde in 0.25M phosphate buffer. They were then washed in phosphate buffer, dehydrated through a graded series of ethanol, followed by chloroform and infiltration with paraffin wax for light microscopy, or dehydrated with ethanol and propylene oxide and infiltrated with Epon/Araldite epoxy resin for TEM. Tissue sections were prepared for either light or transmission electron microscopy as previously described (Chapter 4). Sections were viewed and photographed also as described in Chapter 4.

6.2.4 Extraction and analysis of intracellular granules

Kidney tissue was removed from mussels using the aspiration technique described previously (Chapter 2), then homogenised and the granules extracted, also as described in Chapter 2. Vacuum-dried granules were mounted on aluminium stubs, and evaporatively coated with carbon only or carbon and gold again as described previously (Chapter 4). Photography and EDS were carried out as described in Chapter 4. Granule
diameter was also measured whilst the sample was in the SEM. Unfortunately, not enough granule matter was extracted from the kidney in order to be able to carry out an acid digest and ICP analysis.
6.3 RESULTS

6.3.1 Extraction and analysis of extracellular granules

Extracellular granules were mainly found on the labial palps, mantle edge and at the junction of the foot and the remainder of the visceral mass. The granules were orange in colour and ranged in diameter from 0.2 µm to 1.6 µm (Figure 6.3). In mussels from both sites, granule scores were highest for the labial palps, followed by the mantle and visceral mass, and lowest for the adductor muscle which had a score of 1.00 ± 0 (Table 6.1). The foot of some animals from site 1 had granules on the upper margin which was not catered for in Allison and Simpson’s (1989) original procedure. As such, slight modifications to this procedure have been made to take this into account (Figure 6.2). The foot from animals from site 1 had a mean score of 2.00 ± 0.35 while the foot from site 2 animals had no granules resulting in a score of 1.00 ± 0.

Table 6.1: Granule scores for organs from *Velesunio angasi* collected from Copperfield Creek in the Northern Territory.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Granule score (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site 1 (n=20)</td>
</tr>
<tr>
<td>Labial palps</td>
<td>5.89 ± 0.07</td>
</tr>
<tr>
<td>Mantle</td>
<td>5.53 ± 0.12</td>
</tr>
<tr>
<td>Visceral mass</td>
<td>3.26 ± 0.02</td>
</tr>
<tr>
<td>Gills</td>
<td>2.89 ± 0.27</td>
</tr>
<tr>
<td>Foot</td>
<td>2.00 ± 0.35</td>
</tr>
<tr>
<td>Adductor muscle</td>
<td>1.00 ± 0</td>
</tr>
</tbody>
</table>
Figure 6.3: SEM micrograph of extracellular granules extracted from the mantle of *Velesunio angasi*.

Scale bar = 0.5 µm.
EDS analysis showed that the major elements present were phosphorus, calcium and iron with smaller amounts of sodium, sulphur, chlorine, potassium, barium and manganese (Figure 6.4). Aluminium was also detected but is likely to be a result of contamination from the stub the granules were mounted on. There were no obvious differences between the composition of granules isolated from mussels collected from the two sites in Copperfield Creek. All granules were bright when viewed using the backscattered electron detector, indicating the presence of elements with a relatively high atomic weight. When the granules were sectioned, less than 1% had concentric rings, with many appearing to have no internal structure at all (Figure 6.5), although often all forms of granules were not intact due to the problems of sectioning such hard material.

6.3.2 Morphology of kidney tissue

The kidney of *V. angasi* consists of a continuous tubule that projects off the pericardial wall. It loops back on itself and ends in a ureter, which opens into the left exhalent gill passage. A series of lobes exists on the posterior end of the kidney. The kidney cells (Figure 6.6) are long, narrow columnar epithelial cells approximately 35 µm in length. The nucleus is located basally and the basal membrane is finely infolded. The base of the cells adjoin a blood sinus, in which amoebocytic blood cells and clusters of dark inclusions can be seen. The apical membrane has a brush border with microvilli approximately 2 µm in length. Numerous mitochondria were visible throughout the cell and electron dense granules were clearly visible in the cell cytoplasm, thus achieving the first aim of this section of the thesis.
Figure 6.4: EDS spectra of extracellular granules from *Velesunio angasi*, from Copperfield Creek.
Figure 6.5: TEM micrograph of extracellular granules from *Velesunio angasi* showing lack of concentric rings.
Scale bar = 5 µm.
Figure 6.6: (a) TEM micrograph of kidney cells of *Velesunio angasi*. Scale bar = 2 µm. The small arrow indicates the electron dense, spherical type of granule, while the larger arrow indicates the less electron dense, irregularly shaped type of granule. (b) higher magnification showing intracellular kidney granules. Scale bar = 0.5 µm.
Figure 6.7: EDS spectra of intracellular granules extracted from the kidney of *Velesunio angasi*, from Copperfield Creek. (a) “bright” granule; (b) “dull” granule.
Figure 6.8: SEM micrograph of kidney granules extracted from *Velesunio angasi*, from Copperfield Creek. Scale bar = 2 µm.
6.3.3 Extraction and analysis of intracellular granules

The granule extraction technique described previously for *M. galloprovincialis* (Chapter 2) proved to be successful for *V. angasi* and allowed the extraction of 84 mg (site 1) and 46 mg (site 2) of intracellular granules. Analysis using the SEM showed that the intracellular granules were slightly smaller (range 0.2 µm to 1.2 µm) when compared to the extracellular ones (range 0.2 µm to 1.6 µm).

SEM imaging and EDS analysis, using a backscattered electron detector, of the intracellular kidney granules showed that two forms of granules were present - bright and dull. The bright granules were similar in composition to the extracellular granules and, as such, contained mainly phosphorus, calcium and iron, with smaller amounts of sulphur, chlorine, potassium, barium, manganese and copper (Figure 6.7a). The dull granules were composed mainly of phosphorus and sulphur with smaller amounts of sodium, aluminium, magnesium, silica, chlorine, calcium and iron (Figure 6.7b). There were no obvious differences between the composition of granules isolated from mussels collected from the two sites in Copperfield Creek.

SEM imaging revealed that the kidney granules were spherical in shape (Figure 6.8). TEM (Figure 6.6) revealed that the intracellular granules were round in cross-section and membrane-bound. There appeared to be at least two types of granules in the kidney cells: a) very electron dense, spherical particles and b) less electron dense, irregular particles (Figure 6.6). Both were membrane-bound, and neither had concentric rings.
6.4 DISCUSSION

This study details for the first time the presence of intracellular granules in the kidney of the freshwater mussel *Velesunio angasi*. Indeed, Pynnonen et al. (1987) indicated that there was no evidence for metal-containing granules in the kidney of freshwater mussels, although they do mention a personal communication (HJ Herwig) which suggests that metal-containing structures are present in the kidney of *Anodonta*. While intracellular granules have not been reported previously from the kidney of any freshwater bivalve, such granules have recently been identified in the mantle and gonad of *Hyridella depressa* (Adams et al., 1997; Byrne & Vesk, 2000). The only other record in the literature of intracellular granules in freshwater mussels was made by Silverman et al. (1989). These authors observed that extracellular granules in the connective tissue of the gills of *Anodonta* sp. were formed inside phagocytic type cells. However, once formed, the granules were released to lie between the cells of the connective tissue, so being defined as extracellular rather than true intracellular granules.

The results of this study suggest that three forms of metal-containing granules are present in the freshwater mussel, *V. angasi*: extracellular insoluble deposits, and two types of intracellular granules, one of which appears to be insoluble deposits very similar to the extracellular ones. It is possible that the insoluble deposits thought to be isolated from the kidney are actually extracellular in origin and through sheer numbers have contaminated the sample. It would be necessary to undertake TEM EDS to determine this. While this technique was trialled, it was discovered that the metal content of the granules was lost during processing of the kidney tissue (cf Chapter 4). Studies such as Vesk & Byrne (1999) indicate that aqueous chemical fixation and processing of samples results in dissolution of elements in calcium phosphate granules.

The extracellular granules found on the palps, gills and mantle edge have been shown to be insoluble precipitates of predominantly calcium phosphate/pyrophosphate (Jeffree & Simpson, 1984). The nuclei for these granules may be large particles that have been rejected by the food sorting system. Bivalve molluscs sort particulate material based
primarily on size, with the largest particles being discarded by the ctenidia. Particles are then passed from the ctenidia to the labial palps for further sorting, before those of an acceptable size are passed onto the mouth where they are ingested (Morton, 1973). This sorting system may also account for the abundance of granules on the palps.

If the granules are deposited around a nucleus and increase in size by depositing new layers, it would be expected that concentric rings would be present. However, the vast majority of the extracellular granules isolated and sectioned in this study (approximately 99%) exhibited no internal structure at all, which agrees with the findings of Ch'ng-Tan (1968). This incorporation of elements into the granule is more likely to occur via another method that maintains the spherical shape of the granules.

It has been suggested that the presence of large numbers of extracellular granules is related to the high demand for calcium shown by *V. angasi*, enabling the mussel to build a large shell in relation to its tissue mass (Jeffree & Simpson, 1984). *V. angasi* also lives in an environment which is poor in calcium, with the calcium concentration being two orders of magnitude lower than that found in many other systems around the world (Jeffree, 1988). As such, the ability of the calcium uptake mechanism in *V. angasi* to discriminate between calcium and other elements appears to be reduced, accounting for the ease in which other elements, besides calcium, are sequestered by the extracellular granules. Indeed, Markich & Jeffree (1994) argue that the discriminatory mechanism is so diminished that, in effect, any element is treated as an analogue of calcium and sequestered into the granules. The very low solubility of some elements in phosphate/pyrophosphate matrices, for example zinc, lead and cobalt, results in them remaining in the granules once sequestered and their concentration increasing with the age of the mussel (Jeffree, 1988).

The EDS spectra of isolated extracellular granules from the palps of *V. angasi* sampled at Copperfield Creek are very similar to those from animals from the Magela Creek area of the Northern Territory (Jeffree & Simpson, 1984). This may indicate that mussels
from both areas are exposed to similar environments and are using similar mechanisms to sequester metals present.

Metal content of the extracellular granules extracted from *V. angasi* in this study did not appear to have been affected by the overflow from the Pine Creek gold mine process water dam. However, the marked increase in metal levels, including zinc and lead, seen in the creek water is very short term (Milne et al., 1992). As overflows only occur in the wet season as a result of the dam’s capacity being exceeded, the overflow is very quickly diluted by large amounts of rainwater. The mussels used in this study were collected in the dry season (July) and any changes that may have occurred in the metal content of the granules during the wet season were no longer evident. Alternatively, the semi-quantitative nature of the EDS analysis used in this study may not have been sensitive enough to show changes. Mussels collected at the same time and analysed quantitatively using ICP analysis (Noller, 1999) showed very slightly elevated levels of cadmium, zinc and lead in mussels from site 2, compared to those from site 1. These elements were also elevated in the water analysed from just upstream of site 2, where the process water dam overflow enters Copperfield Creek (Noller, 1999). It is also possible that the excess metal may have been accumulated by the mussel and detoxified by another method, for example intracellular granules, metallothionein or another detoxification, storage or transport protein and then excreted, and therefore, were not present in the excess granules.

Of the two types of intracellular kidney granules, one form also appears to be an insoluble precipitate of predominantly calcium phosphate. Again, these granules lack concentric rings. Phosphate granules almost always have an organic component and are invariably amorphous (Taylor & Simkiss, 1989). The function of phosphate granules is the subject of some debate but many have suggested they are involved in the regulation of cations particularly calcium (Simkiss, 1977; Silverman et al., 1987; Jeffree & Brown, 1992). Phosphate granules also accumulate trace metals (Jeffree & Simpson, 1984; Taylor & Simkiss, 1989). A possible process for the formation of these granules may be
linked to the high levels of iron, and perhaps manganese, which could affect the calcium homeostasis of the mussel by inhibiting Ca\(^{2+}\)-ATPase activity and activating voltage-dependent calcium channels in the plasma membrane (Viarengo, 1989; Viarengo & Nott, 1993). This would lead to an excess of free Ca\(^{2+}\) ions in the cytosol of the kidney cells, causing precipitation of calcium phosphate/pyrophosphate in membrane-limited vesicles. Any other elements present, for example iron, manganese and barium, would be trapped in the granules via co-precipitation.

The second type of intracellular granule appears to be a sulfur granule and could be lysosomal. Sulfur granules have a heterogeneous appearance, no concentric rings and are membrane-bound and commonly contain copper, zinc, cadmium and mercury (Taylor & Simkiss, 1989). They appear to be involved in metal regulation and detoxification, a function that may be related to the binding of metals to metallothionein (Taylor & Simkiss, 1989). The high sulphur content may be derived from the breakdown of metallothionein within the lysosome. While metallothionein has not been isolated from Velesunio, it has been isolated from other freshwater bivalves (for example Anodonta (now Pygandon) grandis (Couillard et al., 1993), Dreissena polymorpha (High et al., 1997) and Corbicula fluminea (Baudrimont et al., 1999)) as well as a large number of marine bivalve species, and so it is quite likely to be present in Velesunio.

Lysosomal granules are common in the kidney of marine bivalves. The sulfur-type kidney granules, isolated from V. angasi, are composed mainly of sulphur and phosphorus, with smaller amounts of iron and calcium. Granules with the same composition have been isolated from various tissues, but not the kidney, of the freshwater mussel Hyridella depressa (Adams et al., 1997).

The relatively low concentrations of metals seen in the intracellular granules compared to those in the extracellular granules could be a function of the relative amounts of these two types of granules, and their locations in the mussel. The number of extracellular granules isolated from an individual mussel is far in excess of the number of
intracellular granules. As the extracellular granules are on the outside of tissues involved in filtering the water (gills) and collecting food (palps), they are directly exposed to much higher levels of any metal present in the environment. This provides an opportunity for metal sequestration by the extracellular granules. In addition, as much of the metal is accumulated by the extracellular granules, metal levels in food are reduced, leaving less metal to be detoxified by the intracellular granules. The presence of lower metal levels in internal organs compared to extracellular granules is confirmed by Jeffree & Simpson (1984), who found much lower levels of $^{226}$Ra in the kidney/heart than in the palps and visceral mass. There is also the strong possibility that some of the metal is accumulated by the shell. Indeed, the shell of freshwater mussels has been used as an environmental indicator of pollution events (Mutvei & Westermark, 2001).

There is no direct evidence to suggest that the granules formed in the kidney cells of *V. angasi* are excreted. However, lysosomal granules in the kidney cells of the marine mussel *M. edulis* (Pirie & George, 1979) and the clam *Mercenaria mercenaria* (Sullivan *et al.*, 1988) are excreted to the lumen of the kidney. It is, therefore, reasonable to suggest that the lysosomal granules in *V. angasi* may be excreted in a similar manner, that is via a budding process or breakdown of cell membranes into a particulate urine.

The intracellular granules formed from insoluble deposits of calcium phosphate/pyrophosphate may be either retained in the cell or excreted via the urine. It is unlikely that these granules can be utilised as a calcium store. Evidence presented by Mason & Nott (1981) indicates that calcium is unable to be mobilised from phosphate-rich granules found in the secretory cells of *Littorina littorea*. These same authors suggest that such granules are most likely to act as a detoxification mechanism for excess metals. However, further studies would be required to confirm or deny this hypothesis in the case of the insoluble kidney deposits found in *V. angasi*.

The lack of internal contents in the kidney cells of *V. angasi* when compared to a marine organism such as *M. galloprovincialis* is typical of a freshwater animal. This is due to
the need for freshwater animals to maintain a much lower osmotic pressure within their cells. The kidney cells of *V. angasi* are not much larger than those of *M. galloprovincialis*. While *V. angasi* has a greater need to adjust the osmolarity of its tissues and hence, has more active cells, compared to the osmoconformer *M. galloprovincialis*, this does not appear to lead to larger cells. For example, the freshwater mollusc *Anodonta grandis*, has the lowest recorded osmolarity of any animal, but not very large cells in comparison to other animals (Schmidt-Neilsen, 1990).

In conclusion, it appears that the freshwater mussel *Velesunio angasi* has at least two means of granule formation, one or both of which may be involved in the detoxification of excess metals in the mussels' environment. This is the first time that intracellular granules have been extracted from the kidney of *V. angasi*. Further studies on the exact nature of the intracellular granules need to be carried out in order to elucidate the ability of both the sulfur and the calcium phosphate granules to accumulate excess metal ions in either a soluble or particulate form. Studies to determine whether the intracellular lysosomal granules are involved in the detoxification of different metals to those accumulated by the extracellular granules would serve to clarify the role of the granules. The use of EDS analysis of thin tissue sections, and the application of metal to phosphate ratios to determine the form of calcium phosphate would assist with such studies.
CHAPTER 7

GENERAL DISCUSSION

The fundamental reason for carrying out these studies was to investigate the validity of using the metal-containing granules found in mussel kidney as a biomarker for zinc pollution. However, before this could be determined, the form, composition and function of these granules needed to be elucidated further. In this thesis, such a study was carried out on the metal-containing kidney granules in the marine mussel *Mytilus galloprovincialis* and the freshwater mussel, *Velesunio angasi*. The study had a number of different parts, each with more specific aims. Chemical and energy dispersive analysis measurements were carried out on isolated kidney granules, under a number of conditions and treatments, to determine their composition. Microscopic examination revealed changes in the kidney tissue and granular structure of *M. galloprovincialis* in the presence of excess amounts of zinc.

Metal-containing granules are present in all of the major invertebrate phyla, including both marine and freshwater bivalves. They are mainly present in organs with digestive, excretory and/or storage functions (Brown, 1982). Generally, granules appear to come in two forms: those which involve the lysosomal system in their formation, and those which are formed from insoluble metal precipitates (Viarengo & Nott, 1993). In marine bivalves, the majority of these granules are intracellular, with the occurrence of extracellular granules appearing to be scant. However, freshwater bivalves contain extensive deposits of extracellular granules, with the study of intracellular granules in this group being very limited.

The composition of the kidney granules of *Mytilus edulis* has been studied under natural and laboratory-induced pollution conditions (Pirie & George, 1979; George & Pirie, 1979; George *et al.*, 1982). However, only the impact of soluble metals on the composition of the granules was determined. This study used particulate zinc and
looked at the closely related species, *M. galloprovincialis*. Extracellular granules in the freshwater mussel *Velesunio angasi* have also been quite well studied. However, the presence of intracellular granules in the kidney had not, prior to this study, been reported.

The first step was to ascertain the involvement of the intracellular kidney granules of *M. galloprovincialis* in the accumulation of excess particulate zinc. Mussels were loaded with 0.5 µg.g⁻¹, 1.0 µg.g⁻¹ or 2.5 µg.g⁻¹ particulate zinc in the laboratory. Freshly harvested mussels were used as a control group. The results of this study have shown that the kidney granules accumulate excess zinc when it is presented in a particulate form in the laboratory. These granules are in the sulfur group described by Taylor & Simkiss (1989). The organic portion appears to have a fairly stable composition, made up mostly of the end result of the breakdown of cell components, lipofuscin (George et al., 1982). The metallic component, which in this case is predominantly zinc, copper and iron, appears to be reversibly bound, with the results indicating that zinc ions can replace copper and iron already bound to the granule.

The next part of the study aimed to identify any differences in the structure or composition of both the kidney tissue, and any granules that the cells may contain, under different conditions of zinc-loading. Different levels of zinc-loading resulted in differences in the numbers and types of granules present in the kidney cells of *M. galloprovincialis*. Degeneration of the kidney tissue was most obvious at higher levels of zinc-loading with cell membrane integrity being breached in kidney tissue from mussels loaded at 2.5 µg.g⁻¹. This degeneration is thought to be the result of zinc toxicity, as it is only observed at very high levels of zinc-loading. A decrease in the number of granules per cell and a reduction in granule yield was also seen and both are, at least partly, the result of this degeneration, as decreasing membrane integrity would facilitate loss of granules to the lumen of the kidney.
The type of granules present in the kidney cells changed with different levels of zinc-loading. Freshly harvested mussels contained type 1, 2 and 3 granules in their cells. The mean diameter of granules in kidney cells increased with increasing zinc-loading, and this can be attributed to an increase in the number of type 3 granules, as these tend to be larger than other types of granules. It was hypothesised that this increase in the number of multivesicular bodies in the kidney cells was due to the higher number of small granules as a result of increased granule production, to manage the excess zinc being presented to the mussel. As multivesicular bodies are thought to be the result of fusion of small granules (George et al., 1982), a higher number of small granules would increase the chance of fusion occurring resulting in more multivesicular bodies.

Granules with concentric rings (Type 4) appeared in control *M. galloprovincialis* tissue after the mussels were kept in the laboratory for 28 days with no zinc-loading. While these granules were present in tissue from mussels in all zinc treatments, kidney cells from mussels loaded at 1.0 μg.g⁻¹ zinc for 28 days, and then maintained for a further 14 days without zinc-loading, showed by the far the largest proportion of Type 4 granules. Thus, the formation of granules with concentric rings may be due to the presence of excess metal in combination with the stress of being kept in the laboratory. In addition, the speed of granule formation and utilisation may impact on the granule’s internal structure.

It is interesting to note that the stress of being kept in the laboratory appears to contribute to the type of granules observed in the different treatments. For example, mussels kept in the laboratory for 28 days with no zinc-loading showed different types and proportions of granules than freshly harvested mussels. To provide a better indication of the role that this added stress has in bringing about the changes to granule structure, further experiments would be required. These could include exposing the mussels to sublethal concentrations of particulate zinc over an extended period of time in the laboratory, rather than very large doses over a relatively short period of time as in
this study, and maintaining mussels with no zinc-loading for extended periods, and observing any subsequent changes in the granule structure.

The next section of the study was designed to determine if there were any differences in the appearance of the kidney tissue and granule structure in mussels exposed to particulate zinc in the laboratory and those exposed in a natural, but zinc-polluted, environment. Thus, the composition and appearance of the kidney tissue and granules in *M. galloprovincialis* collected from a polluted site in the estuary of the Derwent River, Tasmania, were compared to those at a control site outside the estuary. In addition, the results from mussels from this polluted site were compared to those from mussels collected in Western Australia and exposed to high levels of zinc in the laboratory, in an earlier part of the study.

It was shown that *M. galloprovincialis* accumulated zinc in metal-containing granules in the kidney when they were exposed to excess levels of zinc in their natural environment. A substantially higher granule yield in these zinc-contaminated mussels indicated that the mussels increased granule production to assist with zinc detoxification. Kidney cells from mussels collected from the polluted site showed some signs of degeneration, although they retained their membrane integrity. This is in direct contrast to the loss of membrane integrity observed in kidney cells from mussels loaded with zinc in the laboratory. Both this observation and the increased granule yield are likely to be the result of the Tasmanian mussels being exposed to excess levels of zinc over a longer period of time (chronic exposure) compared to the acute exposure of the laboratory-loaded mussels. This longer period of exposure would allow the mussel to more efficiently use its detoxification mechanisms, including the production of kidney granules, to absorb the excess zinc. In addition, the Tasmanian mussels were exposed to relatively lower levels of zinc in their natural environment, rather than being fed high levels of zinc in a laboratory environment, resulting in lower stress and a better ability to employ natural defence mechanisms.
Cells from *M. galloprovincialis* collected from the Seven Mile Beach control site in Tasmania contained mainly type 1 granules, while the cells from mussels from impacted sites contained a significantly higher number of type 3 granules (multivesicular bodies). Type 4 granules were scarce in both tissues. The mean number of granules.cell\(^{-1}\) was higher in mussels from Bellerive Bluff compared with those from Seven Mile Beach, but the mean diameter of granules was significantly lower. This is an intriguing anomaly since the results of the laboratory zinc-loading experiments indicated that a higher number of type 3 granules equated to a higher mean granule diameter, as the type 3 granules, which result from the fusion of a number of smaller granules, tend to be larger. It may be that the average size of type 1 granules in the zinc-contaminated Tasmanian mussels from Bellerive Bluff was much smaller, as the granule production system was producing granules at a much higher rate than in animals from Seven Mile Beach. Thus, fusions between much smaller granules would produce much smaller multivesicular bodies and a smaller overall mean granule diameter.

The lack of type 4 granules in Tasmanian mussels is also interesting and again may be related to the time over which the exposure to zinc occurred. The chronic exposure of the Tasmanian mussels provided a longer period of time in which to detoxify the excess zinc, compared to the acute exposure of the laboratory-loaded mussels, possibly resulting in the more uniform appearance of the granules. Conversely, during acute exposure, granule contents may be laid down layer by layer, producing the concentric rings seen in many granules.

Chemical analysis revealed the same elements were present in kidney granules from both Tasmanian and Western Australian sites, although the concentration of the various elements varied. In addition, silicon was detected in the Tasmanian mussels but not in those from Western Australia. These differences are likely to be a function of the mussels’ environment and water composition at each site. The concentration of zinc was higher in the mussels from the polluted site in Tasmania than even that in mussels loaded at the highest level of zinc (2.5 \(\mu\)g.g\(^{-1}\)) in the laboratory, again, perhaps a
reflection of the time over which the Tasmanian mussels were exposed to excess levels of zinc.

In the final part of the study, intracellular metal-containing granules were isolated for the first time from the kidney of the freshwater mussel, *Velesunio angasi*, collected from Copperfield Creek in the Northern Territory of Australia. Two types of granules were observed in the kidney tissue, both of which were membrane-bound. The first were very electron dense, spherical particles, while the second type were less electron dense with an irregular shape. Neither showed concentric rings.

Chemical analysis of granules isolated from the kidney also revealed two types of intracellular granule. The first of these was composed mainly of phosphorus, calcium and iron, with smaller amounts of sulphur, chlorine, potassium, barium, manganese and copper. The second form contained mainly sulphur and phosphorus with smaller amounts of sodium, aluminium, magnesium, silicon, chlorine, calcium and iron. While EDS analysis of thin sections of kidney tissue would have assisted in determining which physical type of granule corresponded to which chemical type, this was unable to be carried out. Thus, based on the chemical analysis, it is hypothesised that the first type of granule is a calcium phosphate deposit, while the second bears more resemblance to a lysosomal-type granule, similar to those present in the kidney of *M. galloprovincialis*. The high sulphur content in the latter form of granule may be the result of the breakdown of metallothionein in the lysosomal granule. While metallothionein has not been isolated from *V. angasi*, its presence in other freshwater mussels (Couillard *et al.*, 1993; Vesk & Byrne, 1999) and a large number of marine bivalves (Casterline & Yip, 1975; Olafson *et al.*, 1979; Bebianno & Langston, 1992; Bebianno *et al.*, 1992; Bordin *et al.*, 1994; Roesijadi, 1994) would strongly suggest that it is present in this species.

Extracellular granules were also isolated from *V. angasi*, from the labial palps, mantle and visceral mass, and they were clearly visible as orange deposits on the surface of many major organs. Chemical analysis revealed them to be calcium phosphate deposits,
which also contained iron with smaller amounts of sodium, sulphur, chlorine, potassium, barium and manganese. They appear to be able to accumulate excess metals from the environment (Jeffree & Simpson, 1984; Jeffree, 1988; Jeffree & Brown, 1992; Jeffree et al., 1993).

The number of intracellular granules isolated from *V. angasi* is far outweighed by the number of extracellular granules present. This may be a function of their location. The extracellular granules are mainly found on the outside of organs involved in sorting food (labial palps) and filtering water (gills), and so may absorb any excess metal during these stages. This would reduce the metal levels in the food, decreasing the need for intracellular granules to detoxify excess metals.

More frequent sampling throughout the year and laboratory testing would assist in determining whether freshwater mussels utilise the intracellular granules as a detoxification mechanism. This should include TEM EDS to identify which physical type of granule corresponds to which chemical type and function.

It would appear from the results of this study that the use of the lysosomal kidney granules from marine or freshwater molluscs as biomonitors of metal pollution is fraught with difficulty. Firstly, the extraction of granules is time-consuming and requires relatively specialised equipment. There is a need to extract granules from many animals in order to have enough sample to analyse for contaminant content; 50 animals were used for each sample in this study. EDS analysis could be carried out on thin sections, but this is also lengthy, requires very specialised techniques and equipment and can be expensive. In addition, while there is a converse relationship between the level of zinc in the environment and the concentration of zinc in the kidney granules in *M. galloprovincialis*, more studies would need to be undertaken in order to calculate the exact nature of this relationship. This relationship may not be the same for different pollutants or in different animals. However, this is a problem common to many biomarkers, and initial studies to determine the responses of animals in both the
presence and absence of the pollutant are required for any biomarker (Ringwood et al., 1999a).

The use of whole soft tissues for biomonitoring purposes can also be problematic. While bivalve molluscs can be easily collected and deployed at sites of interest, many current biomonitoring studies require extended periods of exposure and the use of a well-defined population of organisms of a particular size, age and condition (Steinert, 1999). The development of more versatile, simple, reliable and sensitive assessment methods is needed. The fact that effects such as growth, development and survival are initiated at a cellular level has led to the identification of cellular biomarkers, which have the advantages of responding to stress predictably and more rapidly (Steinert, 1999). One major advantage of using such biomarkers is that they can provide information on a time-integrated response, which alleviates the difficulties associated with frequent sampling (Wu & Lam, 1997).

It is becoming clear that a suite of biomarkers should be used to detect the impacts of pollutants on animal species (Ringwood et al., 1999a; Ringwood et al., 1999b; Porte et al., 2001b; Viarengo et al., 2001) as each biomarker responds differently to various pollutants and environmental parameters. It is also essential to improve the understanding of basic cellular and biochemical responses to pollutants in a range of animals, the variability of responses both in the presence and absence of the pollutant, and the integrated responses of the organism at a number of levels to the pollutant in the presence of natural stresses and cycles (Ringwood et al., 1999a). Cellular biomarkers are somewhat limited in determining impacts at a population level, and the development of an understanding of how changes in cellular biomarkers translate into implications for individuals and populations is also critical (Ringwood et al., 1999a).

A large number of cellular detoxification responses and stress proteins have been investigated as biomarkers of pollutants. These include metallothionein, the cytochrome P-450 monooxygenase system, multixenobiotic transporter proteins, anti-
oxidant enzymes, cholinesterases, ferrihaemoprotein reductase, catalase, diaphorase, gamma glutamyl traspeptidase, glutathione S-transferase, glutathione, heat shock proteins, lipid peroxidation, lysosomal destabilisation, lysosomal accumulation and DNA damage (Ringwood et al., 1999a; Ringwood et al., 1999b; Narbonne et al., 1999; Steinert, 1999; Viarengo et al., 1999a).

Metallothionein is a metal-binding protein that occurs in a wide variety of vertebrate and invertebrate animals, and can be considered as an important specific biomarker to detect the response of organisms to metal pollutants such as zinc, cadmium, mercury and copper (Viarengo et al., 1999a). The development of a simple and rapid spectrophotometric method of determining metallothionein content of tissues (Viarengo et al., 1997) enhances the potential use of this biomarker. Significant correlations were seen between metallothionein concentration and sediment levels of cadmium and copper in the oyster Crassostrea virginica (Ringwood et al., 1999b). However, Viarengo et al. (2001) indicated that exposure of M. galloprovincialis to copper only raised metallothionein levels in digestive gland at intermediate levels, while lower (0.078µM) and higher (1.258µM) copper levels were detected by using lysosomal membrane stability. Metallothionein is also not a good biomarker for organic pollutants, such as hydrocarbons (Viarengo et al., 2001). Lysosomal membrane stability is generally used to detect stress in organisms, but it is not a suitable biomarker for zinc exposure as zinc acts to stabilise the membranes (Viarengo et al., 2000; Viarengo et al., 2001).

Cytochrome P450 is an enzyme of the mixed function oxygenase system that catalyses the oxidation of a wide range of structurally diverse compounds (Peters & Livingstone, 2001). Cytochrome P450 dependent monoxygenation may be induced following exposure to specific contaminants, making the induction of this enzyme system a potential biomarker. Many different cytochrome P450 genes have been sequenced in both eukaryotes and prokaryotes. Molluscan cytochrome P450 has unique physical and catalytic properties and is apparently induced by changes in season and water
temperature, and the presence of polychlorinated biphenyls (PCBs), benzo(a)pyrene (BAP) and water soluble fractions of crude and fuel oils (Peters & Livingstone, 2001). However, before this biomarker can be used in the field, molecular probes must be developed and used to define the range of responses of cytochrome dependent oxidative pathways to specific contaminants (Peters & Livingstone, 2001).

Other enzymes being investigated as potential biomarkers are the cholinesterases and carboxylesterases, particularly for organophosphate pollution (Porte et al., 2001a; Dizer et al., 2001). Acetylcholinesterase hydrolyses acetylcholine and is inhibited by high levels of organophosphates, while butyrylcholinesterase hydrolyses butyrylcholine and does not show substrate inhibition. Carboxylesterases hydrolyse a wide range of esters and are rapidly inhibited by low concentrations of organophosphates. Using primary cell cultures of *M. galloprovincialis* digestive gland, Porte et al. (2001a) showed inhibition of carboxylesterases and acetylcholinesterase by an organophosphate, fenitrothion. The activity of acetylcholinesterase in the gills of *M. edulis* and *M. galloprovincialis* showed a rapid biochemical response that provided an indication of environmental quality (Dizer et al., 2001). These authors concluded that low acetylcholinesterase activity equated to higher levels of pollution, and indicated that a 20% decrease in enzyme activity indicated exposure to neurotoxic compounds, while a 20–50% decrease indicated a sub-lethal impact of organophosphates.

Changes in the structure of DNA such as adducts, single-strand breaks and chromosomal damage can be used as biomarkers of exposure to certain chemicals. Some 20 classes of carcinogens and mutagens cause DNA structural changes, involving the covalent attachment of a chemical to DNA, known as adducts (Venier, 2001). DNA adducts can be determined by processes such as chemical uptake, rate of activating or deactivating reactions, spontaneous or enzyme-mediated adduct loss and fate of cells with damaged DNA (Venier, 2001). This author used an assay involving $^{32}$P-postlabelling to show DNA adduct formation following exposure of *M. edulis* and *M. galloprovincialis* to the genotoxic compound benzo(a)pyrene both in the laboratory and
in the field, but noted that it was complex and expensive. A simpler and cheaper technique using an alkaline DNA-unwinding assay measured DNA adducts in the gill of the same two mussel species (Dizer et al., 2001). The presence of “indigenous” adducts that are most likely to be generated by normal nutrient metabolism should be noted. However, these “indigenous” adducts are different in different species and appear to be influenced by age and diet (Venier, 2001). Thus, careful study of the existence of DNA adducts in a species needs to be undertaken prior to their use in biomarker studies. Single-strand breaks in DNA reveal the recent pollution status of the environment, and can be measured hours or days after the exposure has occurred (Bolognesi & Degan, 2001). However, the measurement of strand breaks alone is not always sufficient to detect genotoxic effects in the marine environment and further information on total crosslinks in the DNA is required (Vukmirovic et al., 1994). On the other hand, chromosome damage provides an index of accumulated genetic damage during the lifespan of the cells (Bolognesi & Degan, 2001). Both types of biomarker are subject to seasonal variations.

The majority of biomarkers have been developed to indicate exposure to pollutants. The enzymes glucose-6-phosphate dehydrogenase and lactate dehydrogenase can be used as biomarkers of hypoxia in mussels, or low dissolved oxygen in coastal environments (Wu & Lam, 1997). Both enzymes are inducible by oxygen stress, and were found to have raised levels of activity in mussels (Perna viridis) from environments with low levels of dissolved oxygen (Wu & Lam, 1997).

One of the ways of reducing the bias introduced into biomonitoring data by variations in environmental parameters is to used caged mussels for short periods of time in sites where these parameters are comparable (Viarengo et al., 1999a). Caged mussels also reduce the effects of endemic tolerance and the variability associated with different ages and exposure history (Ringwood et al., 1999a).
In freshwater mussels, either the mantle or shell appear to provide suitable samples for biomonitoring. A non-destructive method of sampling the mantle of freshwater mussels has been assessed by Berg et al. (1995). This involved inserting a wooden wedge between the valves to hold the shell open and then removing a small piece of mantle tissue, under field conditions. These authors could find no evidence of increased mortality or incidence of disease in mussels to which this technique had been applied. The mantle is a site of metal accumulation in freshwater mussels, although this may be due to the presence of metal-containing granules in this tissue. Extracellular calcium phosphate granules have been isolated from the mantle of *Velesunio ambiguus* (Ch'ng-Tan, 1968), *V. angasi* (Jeffree & Simpson, 1984), *Margaritifera margaritifera* (Roinel et al., 1973), *Amblema plicata perplicata* (Davis et al., 1982), *Anodonta anatina*, *A. cygnaea*, *Unio pictorium* (Pynnonen et al., 1987), *A. grandis* (Silverman et al., 1989), *Ligumia subrostrata* (Silverman et al., 1987) and *Hyridella depressa* (Byrne & Vesk, 2000). It would appear that the granules are so numerous in the mantle that enough sample can be collected for analysis in only a small section of the tissue. Due to its simplicity and non-destructive nature, this mantle biopsy technique holds promise for future biomonitoring programmes.

The shell also appears to be a reliable biomarker. In freshwater mussels, it is the inner aragonite layers that are generally used. These layers are neither exposed to the environment nor the pallial fluid and so reflect the metal levels in the environment at the actual time of deposition (Dermott & Lum, 1986). This allows comparisons to be made between modern and historical times and the pollution history of a number of industries or natural events to be determined. Such studies have been carried out using the shells of *Margaritifera margaritifera*, *Unio pictorium*, *U. tumidus*, *U. crassus*, *Anodonta anatina*, *A. cygnea* and *Pseudanodonta complanata* (Carell et al. 1987; Mutvei & Westermark, 2001). Imlay (1982) lists a number of species of freshwater mussel that may be suitable for monitoring heavy metals on the basis of annual shell growth due to their widespread distribution, age, pollution tolerance and/or conchological reflection of stream location.
The shell has also been investigated as a biomarker in *M. galloprovincialis*, in which the nacreous layer was assessed, and was shown to be a good indicator of lead (Sturesson, 1976; Bourgoin, 1990; Puente *et al.*, 1996) and cadmium (Sturesson, 1978). However, it does not appear to be useful for determining levels of zinc relative to levels in the environment, as the majority of this metal is taken up by the soft tissues. Goldberg (1986) also noted that there was not a strong relationship between metal levels in shells and soft tissues in mussels analysed during the US Mussel Watch. Thus, during monitoring programs in less contaminated sites it would be more useful to use soft tissues (Bourgoin, 1990). In addition, levels of metals in shells may reflect availability and physiological exchange rates during periods of growth rather than actual levels in the environment (Dermott & Lum, 1986).

While there has been a significant amount of study on a range of biomarkers in mussels, some believe that using mussels alone is insufficient to provide a picture of chemical contamination, and that other species such as birds, cetaceans and even humans may also need to be sampled (Kistner, 1984; Tanabe, 2000). In addition, the analysis of air, water and sediments could also be incorporated into future monitoring programs to give a much broader picture of the contamination being monitored (Tanabe, 2000).

This study has provided valuable insights into the changes seen in kidney tissue and metal-containing kidney granules from the marine mussel *M. galloprovincialis* under a variety of zinc pollution conditions. In addition, kidney granules were isolated for the first time from the freshwater mussel *V. angasi*. Furthermore, the potential of these kidney granules as biomarkers of zinc pollution has been assessed.


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