The Effects of Trace Metals on the Australian Abalone, *Haliotis rubra*

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Declaration

I, Jacquelle Gorski, hereby declare that:

Except where due acknowledgement has been made, the work submitted is that of the candidate alone;
The work has not been submitted previously, in whole or in part, to qualify for any other academic award;
The content of the thesis is the result of work carried out since the official commencement date of the approved research program; and,
Material contained in the work previously published or written by another person is noted.

Signed

JACQUELLE TERESA GORSKI
My PhD experienced on a part-time basis has been shared with so many people who have contributed so much on an emotional, technical and supportive level. I have to express my gratitude for their perseverance through this arduous task so lovingly known as research.

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<th>Full Form</th>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>µg</td>
<td>Micro-gram</td>
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<tr>
<td>µM</td>
<td>Micro-mol</td>
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<tr>
<td>%</td>
<td>Percent</td>
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<tr>
<td>±</td>
<td>Plus or minus</td>
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<tr>
<td>AAS</td>
<td>Atomic Absorption Spectrometry</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ANZECC</td>
<td>Australian and New Zealand Environment Conservation Council</td>
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<td>ANZFA</td>
<td>Australian and New Zealand Food Administration</td>
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<tr>
<td>ARMCANZ</td>
<td>Agriculture and Resource Management Council of Australia and New Zealand</td>
</tr>
<tr>
<td>ASTM</td>
<td>American Society for Testing and Materials</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>ATSDR</td>
<td>Agency for Toxic Substances and Disease Registry</td>
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<td>av.</td>
<td>Average</td>
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<td>BCF</td>
<td>Bioconcentration factor</td>
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<td>Ca</td>
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<td>CCME</td>
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<td>Cd</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>Cl</td>
<td>Chloride</td>
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<td>cm</td>
<td>Centimetres</td>
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<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>DWAF</td>
<td>Department of Water and Forestry (South Africa)</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Concentration that effectively inhibits normal development in 50% of the exposed population</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamineetraacetic acid</td>
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<td>For example</td>
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<tr>
<td>EPA</td>
<td>Environment Protection Authority, Victoria</td>
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<td>H₂O</td>
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<td>LC₅₀</td>
<td>Concentration that is lethal to 50% of the exposed population.</td>
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<td>LOEC</td>
<td>Lowest observable effect concentration</td>
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<td>Milli-mol</td>
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<tr>
<td>MPC</td>
<td>Maximum permissible concentration</td>
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<tr>
<td>n</td>
<td>Number of samples</td>
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<tr>
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<td>Sodium</td>
</tr>
<tr>
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<td>No observable effect concentration</td>
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<tr>
<td>NRC</td>
<td>National Research Council</td>
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<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>Pb</td>
<td>Lead</td>
</tr>
<tr>
<td>PIRSA</td>
<td>Primary Industries and Resources, South Australia</td>
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<tr>
<td>SE</td>
<td>Standard error</td>
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<tr>
<td>s</td>
<td>Second</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>Sulfate</td>
</tr>
<tr>
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<td>United States of America</td>
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<td>USEPA</td>
<td>United States Environment Protection Agency</td>
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<tr>
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ABSTRACT

The purpose of this research was to determine the tolerance of a commercially and environmentally important species to trace metals. Blacklip abalone, *Haliotis rubra* are distributed along the southern Australian coastline. This species of abalone is extensively farmed and an important species for the wild stock fishery. The demise of worldwide abalone populations can be attributed to exploitation by fishing activities and decline in quality of their natural habitats. Limited emphasis has been placed on the impacts of water quality within coastal waters that abalone inhabit. The development of *Haliotis* species is complex, and successful transition through its various phases is integral to the species survival and distribution in the marine environment. This research provides an indication of the sensitivity of the blacklip abalone to trace metal exposure in the water column at various periods of development.

The importance of this research lies in the fact that abalone have not been extensively studied to determine their sensitivity to trace metal exposure. This thesis focussed on the effects of a range of both essential and non-essential trace metals on various stages of *H. rubra* development. The trace metals assessed in this thesis were the essential metals Cu, Zn and Fe; and, the non-essential metals Hg, Cd and Pb. Acute and chronic exposures to trace metals were investigated and the effects on survival, development, and ATPase enzyme activity are the key components of this research. Copper and Hg proved to be the two most toxic metals to each of the life stages of *H. rubra* studied.

In the first series of experiments, fertilised eggs of *Haliotis rubra* were exposed to a range of dissolved nominal concentrations of Cd, Cu, Fe, Pb, Hg, and Zn in individual solutions for 48h. After 48h of exposure, the test was completed by recording morphological abnormalities
of pelagic veliger larvae in each trace metal treatment. The mean 48h median effective concentrations affecting normal morphological development of veliger larvae determined in this test showed a decreasing order of toxicity of 7µg Cu/L, 20µg Hg/L, 42µg Zn/L, 4,102µg Fe/L, 4,515µg Cd/L, and 5,111µg Pb/L.

Settlement and metamorphosis are key characteristics to the successful recruitment of populations of *H. rubra*. In the next series of experiments, veliger larvae of *H. rubra* were exposed to dissolved concentrations of Cu, Zn, Hg and Cd for 48h. After this time, larvae aged 5 days displayed the characteristics of competent larvae with the ability to commence the benthic existence. Artificial nursery microcosms were developed containing microscope slides inoculated with the settlement inducing microalgae, *Ulvella lens*. Within 24h of introduction into the nursery microcosms, 82% of control *H. rubra* larvae were actively crawling on the settlement surface. Crawling success was impaired by 128µg/L Cu and Hg, and 1250µg Cd/L. After 48h in the nursery microcosm, 50% of control larvae displayed settlement characteristics. Settlement was inhibited by 128µg Cu/L, 32µg Hg/L, and 1250 Cd/L. Metamorphosis of larvae 96h after addition into the microcosms was inhibited by 32µg Cu/L, 512µg Zn/L, 32µg Hg/L and 625µg Cd/L compared to 90% of control larvae that had either settled or metamorphosed. The rate of larval metamorphosis was enhanced after exposure to Cu and Hg at 0.5µg/L and 64-256µg Zn/L. Exposure to Zn at concentrations 64, 128 and 256 µg Zn/L caused an increased rate of settlement and metamorphosis after 96h.

The concentrations of trace metals that resulted in mortality of *H. rubra* were investigated by exposing juveniles to acute concentrations of Cu, Zn, Hg and Cd for 96h. Copper produced the most toxic response and a 96h LC₅₀ of 87µg Cu/L. Hg resulted in more sudden mortality rate after 24h exposure compared to Cu yet produced a 96h LC₅₀ of 173µg Hg/L. Juvenile *H. rubra* were relatively insensitive to Zn and Cd with the 96h LC₅₀ established for these metals.
at 1730µg Zn/L and 3700µg Cd/L, respectively. During exposure, *H. rubra* displayed alterations in their behaviour including increased mucus production from the gills, decreased sensory capacity, and the inability to adhere using the foot muscle.

To determine the effects of chronic trace metal exposure and the ability of *H. rubra* to bioaccumulate metals, juveniles were exposed in individual exposure tests to three concentrations each of Cu, Zn, Hg and Cd for 28 days followed by 28 days depuration in clean seawater. The bioaccumulation of each individual metal was determined in the tissue; compartments; the mantle, viscera and edible foot muscle. Exposure to Cu, Zn and Cd produced significant accumulation in the viscera<mantle<edible foot muscle. Accumulation of Hg was greater in the mantle<viscera<edible foot muscle. Depuration for 28 days produced varying results for each metal and tissue compartment.

Changes in the ouabain sensitive sodium-potassium activated ATPase (Na$^+$.K$^+$-ATPase) activity were examined in gills of juvenile *H. rubra* to assess the sublethal effects of the selected trace metals, Cu, Zn, Hg and Cd on enzyme activity. *H. rubra* were exposed to individual trace metals in solution for 28 days followed by 28 days depuration in clean seawater. The dissolved trace metals significantly affected the Na+, K+-ATPase activity in gills of the abalone, with Hg producing the greatest effect. The decreasing order of effect on Na$^+$.K$^+$-ATPase activity was Hg>Cu>Cd>Zn. Depuration of *H. rubra* in clean seawater resulted in the recovery of Na$^+$.K$^+$-ATPase activity to varying degrees after exposure to each of the trace metals. The recovery of ATPase activity was more efficient following exposure to Cd>Zn>Cu>Hg. The abalone species, *H. rubra* appeared to have a higher ATPase activity than other marine invertebrate species, and this may be attributed to the isolation and measurement of other gill ATPases such as Ca$^{2+}$, Na$^+$, and Mg$^{2+}$-ATPase in the methodology employed.
The overall results of this thesis provide initial baseline information to evaluate the sensitivity of *H. rubra* to trace metal toxicants, and these results may be utilised by regulators for the setting of marine water quality guidelines to protect *H. rubra* and other abalone species in their natural habitats.
LIST OF COMMUNICATIONS

Papers Published in Peer Reviewed Journals


Electronic Format Accepted for Publication


Paper Accepted for Publication in Peer Reviewed Journals


Papers Submitted for Publication in Peer Reviewed Journals


5

**Conference Presentations**

**Oral presentations:**

Abalone Health Workshop, October 2004, Sydney New South Wales titled “The development of blacklip abalone larvae and the effects of heavy metals”.


8th Annual Abalone Aquaculture Workshop, July 2001, Fremantle Western Australia, titled “Morphological effects of abalone larvae (*Haliotis rubra*) following exposure to heavy metals”.

**Posters:**


Chapter 1

GENERAL INTRODUCTION

Over the years, the increasing prevalence of pollutants introduced by human activity has been the subject of much scrutiny. Anthropogenic pollutants resulting from industrialisation have entered our natural marine waterways, and for many years, the marine environment has been used as an endless sink for environmental contaminants (Batley, 1995). The legacy of industrialisation has enhanced the use of over 30,000 chemicals within Australia. A proportion of these are discharged either intentionally or unintentionally into marine environments, where some are highly toxic to marine organisms (Chapman, 1995a).

1.1 TRACE METALS

One particular group of environmental contaminants that have caused major concern in marine environments are trace metals. Trace metals are introduced into marine environments from non-point sources which include the natural weathering of rocks and soils, decomposition of detritus, precipitation and atmospheric deposition, surface runoff from industrial activities and urban stormwater, or from point sources which include domestic and industrial wastewater effluent (Lee, 2005).

Trace metals are natural components of marine ecosystems and many are integral constituents of cellular processes within an organism (Deb and Fukushima 1999). The trace metals, essential for biological function include copper (Cu), iron (Fe), and zinc (Zn). Other trace
metals that appear to have no direct biological function include cadmium (Cd), mercury (Hg) and lead (Pb). Essential trace metals can be regulated to some degree by organisms, whereas regulation of non-essential metals depends on their concentrations in the medium (Devineau and Amiard-Triquet, 1985). Once the regulatory mechanisms or the threshold of the organism is overloaded, either by the presence of essential metals in excess or unusually high levels of the non-essential metals, deleterious effects may occur (Langston, 1990).

1.1.1 Copper

Copper (Cu) is a common metallic element in the rocks and minerals of the earth’s crust, and is an essential micro-nutrient required for vital structural components of organisms (Brown and Depledge, 1998). Copper can be toxic when exposures exceed physiological needs (Moore, 1997). In most circumstances, a number of homeostatic mechanisms involving regulation of absorption, cellular uptake, intracellular transport, sequestration/storage, cellular efflux, and excretion from the body maintain a physiologically essential concentration of Cu (ATSDR, 2004).

Anthropogenic sources account for 33-60% of the total annual global input of Cu into the aquatic environment (DWAF, 1996). The main inputs of Cu into the marine environment include effluent from sewerage treatment plant; runoff from soil treated with Cu-containing fungicides and pesticides; corrosion of Cu-based pipes; atmospheric fallout from industrial sources such as mining and refining industries, coal burning and metal-producing industries; and antifoulant paints (Anon, 2001).

1.1.2 Zinc

Zinc (Zn) is one of the most common elements in the Earth's crust and is present in the air, soil, and water (ATSDR, 2005). Zinc is essential for normal biological function in all
organisms, and is necessary for the function of many metalloenzymes. In animals, Zn is an essential nutrient present in over 300 enzymes and plays a role in membrane stability, and in the metabolism of proteins and nucleic acids (WHO, 2001). Zinc is one of the most ubiquitous and mobile of the trace metals, and can be bioavailable in both the dissolved and particulate form.

In seawater, much of the Zn is found in dissolved form as inorganic and organic complexes. Zn does not volatilise from water but is deposited primarily in sediments through adsorption and precipitation, and severe Zn contamination tends to be confined to areas near emission sources (ATSDR, 2005). Zinc may enter the aquatic environment through natural processes such as weathering and erosion, or enter through anthropogenic input. Pollution of marine environments by Zn as a consequence of anthropogenic input is far greater than input from natural sources. Anthropogenic sources of Zn into the marine environment include sewage treatment plant effluents, runoff from fertilisers and insecticide soil applications, urban runoff, mine drainage, dye and pigment manufacturing, printing processes; and processes involving metal galvanising, battery and pharmaceuticals manufacturing (DWAF, 1996).

1.1.3 Iron

Iron (Fe) is an essential micronutrient for all organisms. It is the fourth most abundant metal in the earth’s crust and may be present in natural waters in varying quantities (DWAF, 1996). Sources of Fe within marine environments include natural processes such as weathering and erosion, or anthropogenic input from household chemicals, fungicides, chlor-alkali industry, and the petrochemical industry (DWAF, 1996). Iron is usually insoluble in water and occurs predominantly as particulate species in marine environments (Fabris et al. 1999), confined to sediments rendering it unavailable in the dissolved form.
1.1.4 Mercury

Mercury (Hg) is non-essential but highly toxic element for living organisms. Consequently, Hg and its compounds are one of 132 substances included in the “black list” of all international conventions (McLusky, *et al.* 1986). Mercury has been used as a catalyst in chlor-alkali plants, as a slimicide in pulp and paper mills, as a chemical reagent in the plastics industry; in pharmaceuticals and paints, in special heat engines of power plants, in metal-refinement operations by amalgamations, and in the manufacture of electric switches, batteries and lamps (Eisler, 1981). Due to the high toxicity of Hg in most of its forms, many applications and uses in industrial activities have been abolished because of attempts to reduce exposure to Hg (ATSDR, 1999).

Mercury has proven to be one of the most toxic metals to marine organisms when compared with other metals (MacInnes and Calabrese, 1979; Calabrese *et al.* 1977; Krishnaja, 1987). It is suspected that the Hg concentration alone is the dominant factor determining toxicity and is more commonly unrelated to temperature or salinity variations. Low concentrations of inorganic Hg can be transformed into organic methylmercury through biological processes, increasing the toxicity of this metal (Phillips, 1995). If absorbed into the bloodstream, inorganic Hg can readily combine with the plasma membrane, causing Hg to be efficiently distributed within an organism (Boening, 2000). Hg has an extremely low excretion rate once within an organism, and is readily biomagnified within the biological food chain (Quig, 1998).
1.1.5 Cadmium

The non-essential trace metal cadmium (Cd) is defined as “potentially hazardous” to most forms of life, and is considered to be toxic and relatively accessible to aquatic organisms (USEPA, 1986). Cadmium occurs naturally in association predominantly with Zn, and to a lesser extent lead and Cu ores, and can enter the environment through the weathering process of these ores. Cadmium is widely used in industry and is a common component of household cleaning products. Cadmium enters the marine environment from anthropogenic activities and naturally from land runoff. Anthropogenic sources such as sewerage treatment plants, mining and smelting activities, electroplating of steel, the manufacture and disposal of plastics, batteries and paints contribute significantly to cadmium pollution in the marine environment (DWAF, 1996).

Cadmium has no constructive purpose within an organism, and has proven to be an extremely toxic metal (Depledge and Rainbow, 1990). Having a similarity with Zn, cadmium may interfere with the action of Zn-containing enzymes. The toxicity of dissolved cadmium to a variety of marine organisms is related to salinity with decreased toxicity observed at high salinities (McLusky et al. 1986). The chloride content within water controls the free cadmium ion concentration, as chloride concentrations increase, the toxicity of cadmium decreases. It has been suggested that with a lower chloride concentration in the water, i.e. lower salinity, cadmium readily competes with calcium for uptake sites (George and Coombs, 1977; Phillips, 1980). In higher salinities, cadmium is complexed with chloride thus removing the toxic ion from competing with calcium in metabolic pathways. Temperature is also another factor controlling the fate of cadmium within an organism, and increasing temperature causes an increase in toxic effects.
1.1.6 Lead

Lead (Pb) is a non-essential metal that is potentially hazardous to most forms of life (USEPA, 1986). In marine environments, Pb is predominantly present as particulate metal species (Fabris et al. 1999). Major sources of Pb input into marine environments include atmospheric deposition, mining operations, lead smelters, inappropriate disposal of batteries, industrial and domestic wastewater discharges, and the combustion of fossil fuels (DWAF, 1996; ANZECC and ARMCANZ, 2000).

1.2 Metal Availability in Marine Environments

Metals in the marine environment are either available in the dissolved or particulate form. Dissolved concentrations of metals in the marine environment vary greatly over time, and can be influenced by input concentrations, tidal cycles, and freshwater run-off (Rainbow, 1995). The free metal ion is the most abundant and bioavailable form to marine organisms (Phillips, 1995). Metals that become bound to particulate material such as silt and sediments are removed from the water column and trapped, the sediments temporarily acting as a sink for the metals (Harris et al. 1996). Once the sediment is disturbed, the metals have the capacity to leach from the sediment back into the water column, rendering the metal bioavailable for absorption from the water column. Dredging of shipping channels has the capacity to physically disturb sediment-bound metals and reintroduce them into the water column (Harris et al. 1996). Therefore, coastal sediments may act as a secondary source of trace metal contamination regardless of the cessation of direct discharge (Riba et al. 2002).

1.2.1 Metal Availability to Marine Organisms

Metal pollution within marine environments has been widely reported to impact marine organisms including finfish, crustaceans and molluscs (Nugegoda and Rainbow, 1995; Sri
Lakshmi et al. 2002; Zauke et al. 2003; Yilmaz et al. 2004; Wang et al. 2005). The effect of elevated environmental contaminants on the physiology, behaviour and cellular responses of any given species within the marine environment is varied (Rainbow, 1990). Each species exhibits its own tolerance, avoidance and excretory mechanisms that determine the trace metal impacts. The uptake of metals into an organism can occur from water by passive absorption, absorption across the body surface or a combination of both (Fowler, 1982). The bioavailability of the metal in the marine environment determines the dose, and the effective dose is the concentration of a pollutant in an organism’s tissues; hence the starting point for adverse effects (Luoma, 1996).

1.2.2 Toxicity Assays

Research on trace metals and aquatic biota assays fall into two general categories: the toxicity test, where there are various procedures employing lethal and nonlethal responses, and the bioconcentration test, where the accumulation of a chemical is assessed (Chapman, 1995b). All toxicity testing measures a particular biological response or end-point for an organism. Over the years, the development of a whole suite of assays has allowed for the assessment of the toxicological threshold of a large range of marine species. Traditionally, lethal effects i.e. LC50 assays have been widely used to determine the mortality rate of an organism exposed to a given contaminant (Ahsanullah, 1976; Nelson et al. 1976; Martin et al. 1977; Gentile et al. 1982; Devi, 1987; Govindarajan et al. 1993). Chronic toxicity tests have been favoured more recently than short-term, high exposure assays to encapsulate an organism’s response to prolonged exposure over a significant portion of the organism’s life span.
1.2.3 Choice of Bioindicators

Ideal bioindicator and biomonitor species should be sedentary, easy to identify, abundant, long lived, available for sampling throughout the year, large enough to provide sufficient tissue for (individual) analysis, and resistant to handling stress caused by laboratory studies of metal kinetics and/or field transplantations (Rainbow 1995). They should also be tolerant to exposure to environmental variations in physico-chemical parameters such as salinity, and net accumulators of the metal in question with a simple correlation between concentration in tissues (body) and average ambient bioavailable metal concentration over a recent time period (Rainbow, 1995). Species of mussels are routinely used worldwide as the bioindicator species of choice (D’Silva and Kureishy, 1978; Chan, 1988; Nicholson et al. 1992; Phillips et al. 1992; Blackmore, 1998; Blackmore and Wang, 2002; Romeo et al. 2003; Wang et al. 2005). Anderson et al. (1990) have developed toxicity assays utilising the early life stage of *Haliotis rufescens* as a bioindicator species for testing toxicity of discharges to marine waters. These developed methods have been adapted and utilised by other authors to assess toxicity to other species of abalone (Shackleton et al. 2000; Gorski and Nugegoda, 2006a). Abalone are high prized for their commercial value both within Australia and worldwide and as a consequence there has been limited emphasis on assessing the local species, *Haliotis rubra* as a biomonitor for metal toxicity.

1.3 WATER QUALITY GUIDELINES

The United States, South Africa, Canada and Australia all have water quality guidelines set by the regulatory agencies to form the basis for protecting the survival of aquatic organisms (Table 1.1). Water quality criteria are set using ecological risk assessment, frameworks which consider exposure and effects and indicate the effects of a contaminant on a defined recipient under specific environmental conditions (USEPA, 1999; ANZECC and
Criteria specific to a region or country are established by compiling a range of toxicity data produced by both acute and chronic exposure to a variety of life stages of endemic species (ANZECC and ARMCANZ, 2000). Laboratory ecotoxicity tests can help establish suitable guidelines for minimum acceptable concentrations of a metal (Chapman 1995a). Effects of exposure to contaminants are generally estimated by reviewing published literature reporting toxicity experimental results, and risk to a species is determined by comparing the estimated exposure to the estimated effects (USEPA, 1999). To account for uncertainty in the assessment, water quality criteria are usually kept to at least an order of magnitude below the level at which significant risk is thought to occur (Chapman 1995a).

### 1.3.1 Australian Water Quality Guidelines

Australian water quality guidelines are comparable to existing regulatory water quality criteria established for marine waters of the world (DWAF, 1996; CCME, 1999; ANZECC and ARMCANZ, 2000; USEPA, 2000). Australian guidelines have been designed to protect all forms of aquatic life and all aspects of the aquatic life cycle during indefinite exposure to water (ANZECC and ARMCANZ, 2000). This is a difficult task, for in order to accomplish this goal, every species within an ecosystem would ultimately need to be tested to determine both acute and chronic toxicity to any given contaminant. Australian guidelines for protection of marine environment have been developed from both acute toxicity data and chronic NOEC data for at least five test species incorporating three fish species, two invertebrate species, and a marine plant (ANZECC and ARMCANZ, 2000). Similar to the Canadian water quality guidelines, Australian water quality criteria provide guideline metal concentrations for levels of protection ranging from 80% to 99% species protection (CCME, 1999; ANZECC and ARMCANZ, 2000). Alternatively, the USA and South African approach is to protect 95% of species using two figures: an acute figure to protect against
short-term exposure; and a chronic figure to protect against longer-term exposure (DWAF, 1996; USEPA, 2000).

Australian water quality guidelines define concentrations for protection of species, and are applied by regulatory agencies in each state. The southern mainland state of Victoria is one state of Australia that has applied the water quality guidelines established by ANZECC and ARMCANZ (2000) as state-wide policy for the protection of marine organisms from trace metal contamination. Victorian legislation utilises the water quality criteria specified by ANZECC and ARMCANZ (2000) to develop specific water quality guidelines for Victorian coastal and estuarine environments.
Table 1.1: Summary of background trace metal concentrations in Australian and international marine waters and water quality guidelines for the protection of marine species (µg/L). nd=no data available.

<table>
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<tr>
<th></th>
<th>Copper</th>
<th>Zinc</th>
<th>Mercury</th>
<th>Cadmium</th>
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<td>0.25-9.7&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.0008-0.005&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.015-0.07&lt;sup&gt;ac&lt;/sup&gt;</td>
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<td>0.02-15&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>0.1</td>
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<td>5000</td>
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</tbody>
</table>

<sup>a</sup>Hatje <i>et al.</i> 2003; <sup>b</sup>Munksgaard and Parry, 2001; <sup>c</sup>Fabris and Monahan, 1992, <sup>d</sup>Stauber <i>et al.</i> 2005; <sup>e</sup>Apte <i>et al.</i> 1998).

1. Australian guidelines are based on trigger values for the protection of 99% of marine species (ANZECC and ARMCANZ, 2000).
2. South African guidelines indicate a target water quality range as the management objective for protection of water quality (DWAF, 1996).
3. USA guidelines are an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect (USEPA, 2000).
4. Canadian guidelines are intended to protect and maintain all forms of aquatic life and aquatic life stages (CCME, 1999).
5. Indian guidelines specify concentrations permissible in marine environments (Kumari and Nair, 1992).
1.4 PORT PHILLIP BAY

Port Phillip Bay is a large salt water embayment located in south-central Victoria, Australia. This semi-landlocked marine bay encompasses an area of 1,950 km$^2$ and a coastline length of 264 km (DSE, 2006). The state’s capital Melbourne is located at the north of Port Phillip Bay (Figure 1.1). Melbourne’s suburbs extend around much of the northern and eastern shoreline, and Geelong, Victoria’s second largest city is located on Corio Bay, a subsidiary bay in the southwest. For its size, Port Phillip Bay is extremely shallow, the deepest portion is only 24m and half the volume is in waters shallower than 8m (Harris et al. 1996). Port Phillip Bay is connected to the ocean of Bass Strait via a narrow entrance known as “the heads”. The narrow entrance isolates the Bay and restricts exchange of water with the open ocean. Dissolved or suspended material within the waters of Port Phillip Bay has an average residence time of one year (Murray et al. 2001).

Port Phillip Bay supports a diverse abundance of marine life (Harris et al. 1996). A large proportion of the seafloor is comprised of sand and silt, and rocky reefs can also be found on some margins of the Bay, which are often dominated by hundreds of different seaweeds (DSE, 2006). The Bay also provides a wide range of recreational and commercial activities of various popular finfish and mollusc species including snapper, whiting, flathead, mussels, scallops and abalone spp.

1.4.1 Metal Input into Port Phillip Bay

The structure of marine communities in Port Phillip Bay has changed in the last 30 years (Harris et al. 1996). Habitats have been altered, exotic marine species have been introduced and water quality has changed. During the years of industrialisation, trace metals have long been deposited into Port Phillip Bay. Major industrial facilities are located on the shores of
Port Phillip Bay, including shipping ports, an oil refinery, and a sewage treatment facility discharging treated wastewater directly into the Bay. Along with industrial discharge from both point and non-point sources, urban runoff is also a significant source of metals like Zn and Pb and also organic contaminants such as petroleum oils and industrial chemicals (Harris et al. 1996). Twenty-one natural drainage basins discharge into Port Phillip Bay, and contribute to metal pollution in the Bay (Harris et al. 1996). Areas of concern have been identified around the sites of immediate discharge, and consequently there are many ‘hotspots’. These ‘hotspots’ include sections of Corio Bay; areas around Werribee and Mordialloc; and Hobson’s Bay, which is the body of the Yarra River feeds into and also home to the Port of Melbourne (Quinn and Keough, 1993). The Yarra River is responsible for 60% of metal input into the Bay, equivalent to all other rivers, creeks and drains (Harris et al. 1996).

Over the last 30 years, tighter government regulations have been implemented and the rate of metals discharged into waterways feeding Port Phillip Bay has decreased significantly. Yet despite these tighter regulations, metals continue to be discharged and can still be found within the Bay (Fabris et al. 1999). A review of trace metals, organochlorines and hydrocarbons within Port Phillip Bay determined that the pollution in particular areas of the Bay was of a similar magnitude to urbanised bays and estuaries in Europe and the USA (Phillips et al. 1992; Fabris et al. 1999).
Figure 1.1  Map of Port Phillip Bay
Members of the Haliotidae family are commonly known as abalone, and are distributed throughout temperate and tropical marine waters worldwide. These gastropod molluscs inhabit every continent of the world. There are in excess of 100 species of abalone distributed worldwide inhabiting coastal environments ranging in depth from the intertidal to greater than 100m (Hahn, 1989).

### 1.5.1 *Haliotis rubra*

*Haliotis rubra* are more commonly known as blacklip abalone. This species inhabits Port Phillip Bay and the Victorian coast, and also Australian temperate waters of New South Wales, Tasmania and South Australia (Fleming and Hone, 1997). *H. rubra* can reach an age of 15 years and a maximum length of 20cm (Hahn, 1989). *H. rubra* congregate on rock crevices in bays and oceans in shallow waters to a maximum depth of 40m (Shepherd, 1973). This species is usually localised in their distribution, and populations of *H. rubra* usually remain within a short distance from the family line. *H. rubra* are relatively inactive during the day and become active at night, feeding on drift weed or microalgae growing on the rock surface.

Abalone have one large shell encapsulating a foot that they use for attachment to their chosen substrate (Figure 1.2). The foot muscle is commonly eaten, and is regarded as a delicacy. Epipodial tentacles are present on the fringe of the foot, protruding outside the shell and are used for sensory perception. The shell is secreted from the mantle shell gland, depositing nacre at the leading edge of the shell, allowing for growth of the shell as the abalone matures (Fretter and Pilkington, 1971). The shell is comprised of respiratory pores that draw water into the gills for respiration and also allow for the elimination of the abalone’s waste. The
mantle also acts as a protective barrier for the internal organs of the abalone. The radula, a tongue-like organ with rows of teeth is used to rasp the rocks, or break the macroalgae they feed on into digestible pieces. The internal organs are atrophied during the early stages of abalone development and are present on the right hand side of the shell (Fretter, 1969).

Figure 1.2: Photograph of wild *Haliotis rubra* (mag x0.75)
1.5.2 Commercial Importance of *Haliotis rubra*

As the wild populations of abalone decline, the interest in abalone aquaculture has increased substantially (Daume and Ryan, 2004). Much of the abalone fisheries shortfalls continue to be replaced through abalone farming (Gordon and Cook, 2004). Australia has one of the world’s largest abalone fisheries and its abalone aquaculture industry is expanding (Fleming, 2000). Farming of abalone was first developed in China and Japan as a means of re-seeding the wild-fishery, and later techniques were applied to the USA and South America (Aquaculture SA, 2003). *H. rubra* are a delicacy in Asia, and the majority of *H. rubra* from wild catch and farmed stock are imported to Hong Kong and China. The interest in culturing *H. rubra* has intensified in recent years and this species is farmed extensively in Victoria, South Australia and Tasmania.

The impact of over-fishing, disease, disturbed and lost habitat, and the failure of government to manage the illegal abalone catch has contributed to the decline in abalone populations over the past 30 years; and a subsequent loss of 30% of the world’s wild fishery in 10 years (Gordon and Cook, 2004). Wild populations of all five major abalone species inhabiting the central and southern Pacific coast of California are now completely depleted (Stevens, 2003). In 2002, the United States and South Africa either closed their abalone fisheries entirely or threatened closure (Gordon and Cook, 2004).

Pollution of waterways is also a factor that can be attributed to the decline in populations, but the true nature of metal toxicity to abalone has not been fully assessed. Abalone meet all the prerequisites of a bioindicator yet abalone are far too valuable as a commercial species to be routinely used as a bioindicator or as a species in toxicity assays. The establishment of baseline metal concentrations that impact abalone at each stage of development will assist regulators and ecologists to protect wild abalone populations.
1.6 PROJECT AIMS

The availability of research investigating the impacts of metals on abalone, specifically *H. rubra* is limited. Therefore, this current project was aimed at exploring the effects a range of essential and non-essential trace metals have on the complex early life stages of *H. rubra* development. The first phase of the research investigates the impact of laboratory-based exposure of trace metals on larval development and metamorphosis. This research phase also allowed for the development of healthy *H. rubra* larvae to be documented and illustrated. The second phase focuses on juvenile *H. rubra* and the effects of trace metals following acute and chronic laboratory exposure.

The results gathered in this research will provide an indication of the sensitivity of abalone to trace metal exposure in the marine environment compared with other standard bioindicator species. This research will also provide additional valuable information on effects of trace metals on an economically important species that may assist regulators in the assessment and development of water quality guidelines for the protection of marine organisms.
Chapter 2

LARVAL DEVELOPMENT OF HALIOTIS RUBRA

This chapter has been accepted as:


2.1 INTRODUCTION

Haliotis rubra reach sexual maturity when they are between two and three years old, depending on their environment. Most temperate species of abalone have an annual reproductive cycle, and the periodicity and duration of spawning varies both intra- and interspecifically (McShane, 1992). All species of abalone are broadcast spawners, releasing their eggs and sperm from the gonad into the surrounding water column. The exact time of spawning varies by location and environmental cues such as increased water temperature (Aquaculture SA, 2003). Young females can release 10,000 eggs, while an older females can release 1,000,000 million eggs into the surrounding waters. The eggs are fertilised by the sperm in the water column and the embryo develops into free-swimming, motile veliger larvae (Fretter and Pilkington, 1971).

The pelagic veliger larvae remains in the water column until a cue for settlement induces the larvae to begin its benthic existence (Kang et al. 2004). Settlement is a critical phase when
the larva begins crawling over surfaces to select an appropriate habitat. Cues that promote the settlement of larvae include substrates supporting appropriate microalgal growth and the presence of adult *H. rubra* in the settlement location. The natural mortality of larvae is thought to be quite high, with mortality rates ranging from 35% to 90% (McShane, 1992; Tetschulte, 1976). Abalone have been shown to be extremely sensitive to stress and it has been suggested that larval viability, predation, and export to unsuitable environments by ocean currents may cause high mortality of wild abalone larvae (Stevens, 2003).

During progression from fertilised eggs to the fully developed veliger, *H. rubra* pass through six distinct stages of development. This chapter provides a comprehensive description of each stage accompanied by photographs depicting the early development of *H. rubra*.

### 2.2 MATERIALS AND METHODS

Sexually mature *H. rubra* broodstock were induced to spawn under controlled conditions at Ocean Wave Seafoods located in Lara, Victoria, Australia. The female and male abalone produce eggs and sperm that leave the gonads and escape into the branchial chamber. The gametes were released through the respiratory pores into the surrounding seawater. Eggs were gently siphoned from the spawning tanks, collected and fertilised with fresh sperm within 1h of spawning at a proportion of 15 sperm for a single egg. Immediately following fertilisation, eggs were washed to remove excess sperm from the egg sac to avoid polyspermy, and washed again 15min later. Embryo and larval samples were gently pipetted from the larval housing tanks and photographed with a digital camera (Canon G2 Powershot) mounted on a compound light microscope at regular intervals throughout the development of the larvae.
2.3 OBSERVATIONS AND NOTES ON DEVELOPMENT OF \textit{H. rubra} LARVAE

2.3.1 Gamete Fertilisation

Within the water column, the motile sperm penetrated the egg membrane to fertilise the single-celled zygote (Figure 2.1). The abalone sperm achieved successful fertilisation by binding at its anterior tip to the egg vitelline layer, dissolving a hole in the vitelline layer, or yolk sac and passing through this hole to fuse with the plasma membrane (Hahn, 1989).

The first sign of fertilisation was the appearance of the polar body 10-15 minutes post-fertilisation (Figure 2.2). The egg then underwent mitotic cleavage and the egg was divided into two cells (Figure 2.3). Further mitotic cleavage led to second division of the egg (Figure 2.4) occurring at a right angle to the first division, giving rise to four large cells (Bevelander, 1988). Consecutive cell division of the egg produced a multi-celled morula, approximately 4h post-fertilisation (Figure 2.5). The yolk supplied nourishment to the egg, and diffused oxygen was received from the surrounding water column through the egg membrane.
Figure 2.1: Photograph of *Haliotis rubra* egg within 0.5h of gamete release surrounded by motile sperm (mag x100).

Figure 2.2: Photograph of fertilised *Haliotis rubra* egg within 1.5h of gamete release with an obvious polar body, indicating successful fertilisation (mag x100).
Figure 2.3: Photograph of a fertilised *Haliotis rubra* egg after mitotic cleavage to produce two cells 1.5h post-fertilisation (mag x100).
2.3.2 Blastula Development Stage

The blastula stage of egg development was characterised by the close compaction of the embryonic cells surrounding a fluid-filled cavity called the blastocoel (Crofts, 1937). The gastrula stage followed shortly after, and during this phase the germ layers of the embryo were formed and the body plan of *H. rubra* was established. Once gastrulation was complete, all germ layers were in the correct location to begin organogenesis. The thee germ layers, the endoderm, mesoderm and ectoderm, begin development of the internal organs of *H. rubra* (Crofts, 1937). The endoderm developed into the organs, endocrine glands, respiratory system and gastrointestinal tracts, including the gastrocoel, which is the foundation for the gut. The mesoderm developed into the muscle, circulatory system, reproductive system, urinary and excretory systems, and the connective tissue of the gastrointestinal tract and integments. The ectoderm developed into the outer layer tissue of *H. rubra*, and included the nervous system and outer integumentary system, which incorporates the epithelium, exocrine glands, and the epipodial, respiratory and cephalic tentacles.
Figure 2.4: Photograph of a fertilised *Haliotis rubra* egg after second division, giving rise to four cells 3h post-fertilisation (mag x100).

Figure 2.5: Photograph of a fertilised *Haliotis rubra* egg 4h post-fertilisation after consecutive cellular division to produce the multi-celled morula (mag x100).
2.3.3 Trochophore larvae

Further development of the egg gave rise to the embryonic form known as the early trochophore (Crofts, 1937; Hahn, 1989). This occurred approximately 16-18h post-fertilisation (water temperature dependent) (Figure 2.6). The early trochophore within the egg membrane developed the prototrochal girdle. The prototroch, formed from the trochoblasts, gave the embryo locomotory ability by producing a circular band of cilia. The apical tuft, which is a pre-oral ciliated ring formed along the top of the embryo provided the larvae’s sensory ability, and the ocellus (eye spot) was also developed. Formation of the stomodeum began, which is the initial stage of the oral cavity.

The prototroch enabled the embryo to begin rotating within the fluid albumin in the egg capsule (Crofts, 1937). The increase in frequency and intensity of the trochophore larvae rotating within the egg caused the gradual thinning of the membrane (Figure 2.7). At approximately 24h, the egg membrane was destroyed and the larvae hatched through the membrane and entered the water column as free-swimming lecithotrophic larvae (Figure 2.8). The hatched larvae were negatively buoyant, and immediately swam towards the surface of the water with the aid of the powerful velar musculature and beating cilia (Figure 2.9). The velar musculature or velum is the organ responsible for locomotion in *H. rubra* larvae (Crofts, 1937). The larvae must continue swimming to stay within the water column.

The pelagic trochophore began the secretion of the shell (Figure 2.10). The shell glandular organ, located on the posterior end of the larvae is a derivative of the mantle (Crofts, 1937). The secretion of shell from the mantle edge continued to cover the body of the larvae from dorsal to ventral as the visceral mass expands (Figure 2.11 & 2.12). The growth of the
viscera and shell, the development of the foot and the enhanced ciliated swimming lobes on the velum transformed the trophophore to a veliger larvae (Fallu, 1991).

### 2.3.4 Veliger larvae

Attachment of the larval body to the shell occurred via the initial formation of the left retractor muscle, followed by the right integumental attachment (Figure 2.13). The retractor muscle passed from its posterior attachment on the shell to the velum, the stomodeum and the foot, and by active contraction it was responsible for the $90^\circ$ rotation of the cephalo-pedal mass and mantle membrane (Fretter, 1969). The cephalo-pedal mass was further rotated $180^\circ$ to position the larval foot to protrude from the top of the shell (Figure 2.14).

At 48h of age, the larvae could withdraw the cephalo-pedal mass into the shell. The operculum developed and served as a lid, closing the opening of the shell when the animal was retracted (Figure 2.15). The heart was further developed to provide circulation to the velar lobes. With the use of cilia on the velum, a continuous flow of water is maintained through the mantle cavity. This was evident not only when the larvae were free-swimming but also when they were partially withdrawn into the shell (Figure 2.15). The current of water entering the mantle cavity stops suddenly if adverse conditions cause a rapid withdrawal of velum and foot. When the current starts again and the larva was about to emerge, the current was regularly intermittent as though each sample of water is cautiously tested (Fretter, 1969).
Figure 2.6: Photograph of the embryonic form of *Haliotis rubra* 16-18h post-fertilisation called the early trochophore (mag x100).

Figure 2.7: Photograph of *Haliotis rubra* trochophore larvae 22h post-fertilisation rotating within the egg’s fluid albumin, facilitated by the maturing prototrochal girdle and beating cilia (mag x100).
Figure 2.8: Photograph of *Haliotis rubra* trophophore larvae 24h post-fertilisation hatching through the egg capsule and entering the water column as a free-swimming lecithotrophic larva (mag x100).

Figure 2.9: Photograph of free-swimming *Haliotis rubra* trophophore larvae 26h post-fertilisation (mag x100).
Figure 2.10: Photograph of *Haliotis rubra* trochophore larvae 29h post-fertilisation with the early stages of shell secretion from the shell glandular organ located in the mantle epithelium (mag x100).

Figure 2.11: Photograph of *Haliotis rubra* trochophore larvae 31h post-fertilisation with the developing shell and internal organs becoming more organised (mag x100).
Figure 2.12: Photograph of *Haliotis rubra* early veliger larvae 38h post-fertilisation with the developing shell and visceral mass, and enhanced ciliated swimming lobes on the velum (mag x100).

Figure 2.13: Photograph of *Haliotis rubra* veliger larvae 42h post-fertilisation following the 90° rotation of the cephalopedal mass and mantle membrane (mag x100).
Figure 2.14: Photograph of *Haliotis rubra* veliger larvae 48h post-fertilisation after 180° rotation of the foot mass to protrude from the shell, and completed development of the larval shell (mag x100).

Figure 2.15: Photograph of *Haliotis rubra* veliger larvae 48h post-fertilisation withdrawn into the shell (mag x100).


2.3.5 Pre-Competent Development Stage

After 72h of development, the veliger continued to mature and the eye spot became more prominent on the velum (Figure 2.16). The anterior portion of the foot, known as the propodium became pronounced on which cilia began growing and commenced beating (Hahn, 1989). A cephalic tentacle developed on the velum. As the larvae matured beyond 96h, cilia were formed on the mantle cavity and began beating. The propodium became more prominent with the appearance of an outgrowth, termed apophysis (Hahn, 1989). The cephalic tentacle became further pronounced, and a pair of epipodial tentacles was formed on the foot (Figure 2.17).

At this stage of development approximately 120h post fertilisation, larvae could use the foot to actively crawl. The larval retractor muscle attached to the larval shell draws the enlarged mantle cavity towards the back of the shell (Hahn, 1989). Short spines and the otolith formed on the cephalic tentacles, enabling balance and sensory positioning in the larvae. This was followed by the protrusion of the snout, which is the anterior facial part of the head from the velum (Figure 2.18). The development of four tubules on the cephalic tentacles indicated that the larvae were capable of crawling and actively exploring the surface for settlement (Seki and Kan-no, 1977).
Figure 2.16: Photograph of *Haliotis rubra* veliger larvae 72h post-fertilisation with a prominent eye spot, mantle cavity and foot muscle. The stomodeum and velum is becoming more mature (mag x100).

Figure 2.17 Photograph of *Haliotis rubra* veliger larvae 120h post-fertilisation with the capacity to crawl using the enlarged propodium (mag x100).
Figure 2.18: Photograph of *Haliotis rubra* veliger larvae 120h post-fertilisation with the larval retractor muscle pulling the enlarged mantle cavity towards the back of the shell (mag x100).

Figure 2.19: Photograph of settled *Haliotis rubra*, having laid down the shell and exploring the surface with the enlarged foot muscle (mag x100).
2.3.6 Competent Development Stage

Newly settled larvae still maintained the capability to swim with the use of the cilia on the velum (Figure 2.19). The post-larvae actively crawled over surfaces with the use of their developing foot. Once a suitable surface for settlement was found, the velum was lost, marking the end of the pelagic life phase (Figure 2.20).

A second phase of torsion occurred following settlement on a suitable surface and the settled larvae metamorphosed from the planktonic to the benthic form. The foot expanded and the right side of the mantle grew to shield the visceral mass from the growing larval shell. The development of the shell muscle and the pallial organs on the right side of the newly settled larvae were retarded. It is generally accepted that the asymmetrical coiling of the shell produces a pressure on the right side of the mantle cavity, resulting in the atrophy of organs on the right side (Fretter, 1969).

The shell gland continued to lay down calcium concretions from the mantle skirt, producing calcium carbonate, thus building the shell. The radula became functional and the metamorphosed larvae commenced its characteristic feeding by scraping the surface on which it had settled (Figure 2.21). The larval phase of *H. rubra* has been completed.
Figure 2.20: Photograph of *Haliotis rubra* crawling on the surface, with pronounced cephalic tentacles and having lost the velum (mag x100).

Figure 2.21: Photograph of metamorphosed *Haliotis rubra* with the appearance of the adult form (mag x100).
Chapter 3

SUBLETHAL TOXICITY OF TRACE METALS TO LARVAE OF THE BLACKLIP ABALONE, *HALIOTIS RUBRA*

This chapter has been published as:

3.1 INTRODUCTION

Abalone can only survive in ecosystems where environmental factors support their growth and reproduction. With approximately 115 species, abalone are an important marine gastropod both ecologically and commercially. Several factors appear to have combined in complex ways to weaken abalone, accelerate mortality and cause population declines (Davis *et al.* 1992). This decline can be attributed to both natural and anthropogenic factors including exploitation of fishing activities; decline in natural habitats and food availability; and possibly, the introduction of pollutants into marine environments (Schiel, 1993)

Trace metal pollution is widely accepted as a major problem within marine environments. The toxicity and bioavailability of trace metals to marine organisms is greatly influenced by the physicochemical condition in which the trace metal is present. The concentration of trace metals in marine environments is likely to be restricted to areas immediately surrounding the site of release, such a locations of industrial discharge, sewage outfalls and urban run-off. From a biological point of view, any toxic effect is significant if it influences, or is likely to
influence the physiology or behaviour of the organism in such a way as to alter its growth, reproduction, morphology, pattern of dispersal or cause mortality.

Aquaculture and harvesting of wild aquatic food species has been recognised as carrying a distinct environmental value within Australia (ANZECC and ARMCANZ, 2000). The success of marine aquaculture and harvesting of wild marine food species depends on the water quality of coastal areas. Each species exhibits a varied tolerance to water quality. To enhance protection of stocks, water quality guidelines for trace metals have been established to protect populations of seafood harvested for human consumption from Australian waters. Water quality guidelines propose acceptable concentrations of trace metals that should not cause direst toxic effects to exposed organisms in the marine environment. The survival or demise of these species under test conditions allows for the prediction of safe, acceptable levels of pollution within marine environments.

The availability of literature on sublethal and chronic toxicity of trace metals to early life stages of marine species is restricted to a few species of invertebrate molluscs, predominantly oysters and mussels (Wisely and Blick, 1967; Calabrese et al. 1979; MacInnes and Calabrese, 1977; Martin et al. 1981; Johnson, 1998). The early life stages of these species are routinely used as biological indicators of pollution levels. The data available on invertebrate toxicity to various metals are varied. An effect on embryonic and larval development probably has the greatest ecological significance in terms of preserving the health of marine communities (Langston, 1990). In the longer term, it is the effect of a pollutant at the most sensitive stage of the life cycle that provides the key to the species’ biological success, or failure in polluted environments (Beaumont et al. 1987).
Limited information has been published concerning trace metal toxicity, and the relevance of accepted water quality guidelines to maturation of abalone larvae. This study uses a standard toxicity test protocol to determine concentrations of the trace metals Cu, Hg, Pb, Cd, Zn and Fe that may affect the development of *Haliotis rubra* larvae. Each of these trace metals under investigation has a sufficient toxicological database, which has allowed water quality guideline concentrations to be determined and will provide a comparison with results presented in this study. The choice of metals in this study includes both non-essential and essential metals.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Animals

Toxicity tests using *H. rubra* larvae were based on methodology that used *Haliotis rufescens* as the test species (Hunt and Anderson, 1990). Sexually mature *H. rubra* broodstock were induced to spawn under controlled conditions at Ocean Wave Seafoods located in Lara, Victoria, Australia. Eggs were fertilised with fresh sperm within 1h of spawning. Immediately following fertilisation, eggs were washed to remove excess sperm from the egg sac to avoid polyspermy, and washed again 15min later. Eggs were obtained for experiments 1h after fertilisation, before the first cellular division occurred. Embryo density was determined to achieve a final test volume of 20 eggs/mL.

#### 3.2.2 Experimental Set-up

Range-finding tests were initially completed for each trace metal using a log scale to establish definitive concentrations. Stock solutions were added to 350 mL of filtered (pore size, 3µm) seawater and then treated with ultraviolet radiation to produce nominal trace metal concentrations. Fifty millilitres of fertilized eggs were added to each acid-washed sample jar
to reach a final test volume of 400 mL. Sample jars used in this larval test had a 500-mL capacity (height, 50 cm; diameter, 17 cm). These sample vessels were regarded as ideal, because their height allowed the vertical movement of larvae once hatching occurred.

Six trace metals were individually tested, and each concentration was replicated by four. A seawater control was used for each test. *H. rubra* embryos were exposed to dissolved salts of the following trace metals in solution: 1, 4, 16, 32, 64 μg Cu/L (CuSO₄·5H₂O); 8, 16, 32, 64, 128 μg Zn/L (ZnCl₂); 80, 320, 1,280, 5,120, 20,480μg Fe/L (FeCl₄); 8, 16, 32, 64, 128 μg Hg/L (HgCl₂); 80, 320, 1,280, 5,120, 20,480μg Cd/L (CdCl₂·2H₂O); or 80, 320, 1,280, 5,120, 20,480μg Pb/L (PbNO₃)₂.

Sample jars were covered and fertilised eggs were allowed to develop for 48h through the trochophore stage to the veliger larvae. The test was conducted in ambient conditions following conditions determined as optimal at Ocean Wave Seafood for the husbandry of *H. rubra* larvae. Dissolved oxygen (87±3%), pH (7.3±1) and temperature (20±2°C) were monitored in exposure jars. The test was concluded at 48h, at which time the no-observable-effect concentration (NOEC), 48h 10% effective concentration (EC₁₀), and 48h 50% effective concentration (EC₅₀) values were determined. Swimming behaviour of larvae and position within the water column was observed in each of the sample jars. Healthy larvae are positively buoyant in their swimming actions and can be observed swimming strongly in a vortex fashion. Larvae were collected on a mesh sieve (pore size, 37μm) and gently pipetted into 10mL polypropylene sample vials. A 1mL sample from each test solution was placed onto a Sedgewick-Rafter counting cell (Phyco-Tech, St. Joseph, MI, USA) under a compound light microscope, and the first 100 larvae sighted were counted at x100 magnification using a hand counter. Percentage normal development and survivorship of larvae were calculated in each test sample, and morphological abnormalities were recorded.
3.2.3 Larval Characteristics

Normally developed *H. rubra* larvae (refer to Chapter 2) exhibit smoothly curved shells that are striated with calcareous deposits and appear somewhat opaque (Hunt and Anderson, 1990). Larvae counted as abnormal exhibited at least one of the following deformities: indentation of the shell and an obvious lack of calcification in at least one part of the shell; a broken shell; a shell that was separated from the rest of the animal; arrested development (from one cell through to immature veliger stage); larvae found remaining in the egg membrane; abnormal cilia and velum; detached adductor muscle from the shell; and, decreased size (Hunt and Anderson, 1990).

3.2.4 Statistical Analysis

Results from the thee separate tests (n=3) for each individual trace metal were pooled, and the percentage normal larval development, expressed as a percentage of the average number surviving in the controls, was arcsine-transformed and subjected to single-factor analysis of variance. For all trace metals tested, NOECs were established via pairwise comparison using Dunnett’s test. Median effect concentrations (48h EC_{10} and 48h EC_{50}) were calculated using Toxcalc© statistical software (Tidepool Scientific, McKinleyville, CA, USA).

3.3 RESULTS

3.3.1 Controls

Survival in controls was 87% or greater for all tests conducted. This was deemed to be a successful survival rate based on U.S. Environmental Protection Agency criteria for *H. rufescens* assays that specify mean larval normality must be at least 80% in controls (USEPA, 1995). Physicochemical parameters remained constant throughout all tests performed (20 ±
H. rubra larvae within control sample jars were vigorously swimming in circles at the top of the water column. After 48h, larvae within controls had developed from embryo to larva with a well-defined foot, operculum and visceral mass, well-developed shell, defined muscle attachment to shell, and velum with pronounced, beating cilia (Figure 2.14).

### 3.3.2 Metal Toxicity

All six trace metals tested inhibited normal visceral and shell formation with increasing nominal concentrations. The calculated 48hEC$_{50}$ for each trace metal suggested a decreasing order of toxicity of Cu>Hg>Zn>Pb>Cd>Fe (Figure 3.1; Table 3.1). The most obvious deformity observed in H. rubra larval maturation was in shell development. Photographs demonstrating the increasing degree of morphological abnormalities in the veliger larvae exposed to each nominal metal concentration are depicted in Figures 3.2 to 3.7.
Figure 3.1: Mean percentage normal morphological development of *Haliotis rubra* veliger larvae after 48h relative to (A) Cu exposure; (B) Hg and Zn exposure; (C) Cd, Pb and Fe exposure (mean ± SE, n=100)
3.3.2.1 Copper

Cu was the most toxic trace metal, with a 48hEC$_{50}$ of 7.1µg Cu/L (95% CI, 6.69-7.5µg Cu/L). Obvious morphological deformities became apparent in larvae exposed to concentrations greater than the 48hEC$_{10}$ of 3.72µg Cu/L (95% CI, 3.83-3.50µg Cu/L), with obvious signs of immaturity in foot development and muscle attachment to the shell (Figure 3.2b). Larvae exposed to16µg Cu/L appeared abnormal, with obvious irregularities in velum, viscera and shell development (Figure 3.2c). The shells of these larvae exposed to 16µg Cu/L displayed lack of calcification and shell shape was deformed. These larvae were also smaller in size than the control larvae. There was an absence of calcification and shell development in larvae exposed to 32µg Cu/L and 64µg Cu/L (Figure 3.2d&e). The velum was abnormally developed, and the arrangement of cilia (though still beating) was malformed in these higher concentrations. Various sizes of irregular larvae were present at 32µg Cu/L and 64µg Cu/L when compared to control larvae. Larvae exposed to these higher concentrations survived and were predominantly at the top of the water column; though swimming behaviour was erratic and disorganised.

3.3.2.2 Mercury

Inorganic Hg was less toxic than Cu but more toxic than the other metals tested. The 48hEC$_{10}$ and 48hEC$_{50}$ were 11.9µg Hg/L (95% CI, 4.66-16.09µg Hg/L) and 19.84µg/L (95% CI, 14.02-27.97µg Hg/L), respectively. At 16µg Hg/L, larvae showed signs of immaturity (Figure 3.3b). The foot had not developed and irregularities in the velum, viscera and muscle attachment to the shell were evident. Larvae exposed to 16µg Hg/L exhibited abnormal shell and velum development. Larvae were unevenly distributed throughout the water column and displayed disorganised swimming behaviour compared to controls. Larvae exposed to 32µg
Hg/L were deformed and extremely small in size compared to controls, and distributed at the bottom of sample jars (Figure 3.3c). The larval shell was absent following exposure to 32µg Hg/L. There were signs of attempted shell development but the shell appeared detached from the visceral mass and muscle. The velum and viscera also appeared abnormally developed. Concentrations of 64µg Hg/L and 128µg Hg/L produced 100% mortality and embryo development had ceased in the gastrula stage. (Figure 3.3d&e).

3.3.2.3 Zinc

Larvae exposed to Zn displayed a 48hEC<sub>10</sub> and 48hEC<sub>50</sub> of 20.40 µg Zn/L (95% CI, 18.51-21.92µg Zn/L) and 42.25µg Zn/L (95% CI, 39.51-46.23 µg Zn/L), respectively. At Zn concentrations above the 48hEC<sub>10</sub>, larvae displayed abnormalities in foot development, and also muscle formation and attachment to the shell (Figure 3.4c). The shell and velum were also deformed with obvious lack of calcification at the leading edge of the shell where the mantle edge deposits the shell matrix. Larvae exposed to 64 and 128 µg Zn/L were alive, yet smaller in size than the controls and lacked calcification integral for shell development (Figure 3.4d&e). Swimming behaviour of larvae was erratic in these higher concentrations compared to control larvae, and they were distributed throughout the water column in a disorganised manner.
Figure 3.2: Development of *Haliotis rubra* veliger larvae after 48h exposure to (a) 1µg Cu/L, (b) 4µg Cu/L, (c) 16µg Cu/L, (d) 32µg Cu/L, and (e) 64µg Cu/L (Mag x400).

Figure 3.3: Development of *Haliotis rubra* veliger larvae after 48h exposure to (a) 8µg Hg/L, (b) 16µg Hg/L, (c) 32µg Hg/L, (d) 64µg Hg/L, and (e) 128µg Hg/L (Mag x400).

Figure 3.4: Development of *Haliotis rubra* veliger larvae after 48h exposure to (a) 8µg Zn/L, (b) 16 µg Zn/L, (c) 32 µg Zn/L, (d) 64µg Zn/L, and (e) 128µg Zn/L (Mag x400).
3.3.2.4 Lead and Cadmium

The trace metals Pb and Cd and produced effects on larval development at much higher concentrations than Cu, Hg and Zn. The NOEC for both Pb and Cd was 320µg/L. The 48hEC$_{50}$ for Pb and Cd were 4,102µg Pb/L (95% CI, 3,891-4,398µg Pb/L) and 4,515µ Cd/L (95% CI, 4,316-4,821µg Cd/L), respectively. In one test performed with Pb, there was 5% normal larval development at the highest exposure concentration of 20,480µg Pb/L. Larvae exposed to Pb and Cd at the higher concentrations exhibited abnormalities such as irregular velum formation and broken shells at 5,120µg Cd/L (Fig 3.5d & 3.6d). The highest concentration of Pb and Cd (20,480µg/L) inhibited normal velum and the larvae lacked the ability to develop a shell. Swimming behaviour of larvae exposed to Pb and Cd was affected at concentrations beyond 5,120µg/L, exhibiting erratic and disorganised behaviour. In comparison to controls, larvae exposed to the higher concentrations of Cd and Pb were confined to the bottom of sample jars.

3.3.2.5 Iron

Iron produced the least toxic effect, producing a 48hEC$_{10}$ and 48hEC$_{50}$ of 4,364µg Fe/L (95% CI, 4,058-4,578µg Fe/L) and 5,111µg Fe/L (95% CI, 4,860-5,375µg Fe/L), respectively. The calculated NOEC was 1,280µg Fe/L. Larvae exposed to Fe at 5,120µg Fe/L were swimming in the top half of sample jars in a disorganised manner, and were small and immature displaying an elongated body with obvious lack of calcification (Figure 3.7d). A high proportion of larvae exposed to Fe at 20,480µg Fe/L had not hatched from the egg membrane and displayed arrested development, yet these larvae remained alive and rotated within the egg, aided by deformed cilia (Figure 3.7e).
Figure 3.5: Development of *Haliotis rubra* veliger larvae after 48h exposure to (a) 80µg Pb/L, (b) 320µg Pb/L, (c) 1,280µg Pb/L, (d) 5,120µg Pb/L, and (e) 20,480µg Pb/L (Mag x400).

Figure 3.6: Development of *Haliotis rubra* veliger larvae after 48h exposure to (a) 80µg Cd/L, (b) 320µg Cd/L, (c) 1,280µg Cd/L, (d) 5,120µg Cd/L, and (e) 20,480µg Cd/L (Mag x400).

Figure 3.7: Development of *Haliotis rubra* veliger larvae after 48h exposure to (a) 80µg Fe/L, (b) 320µg Fe/L, (c) 1,280µg Fe/L, (d) 5,120µg Fe/L, and (e) 20,480µg Fe/L (Mag x400).
Table 3.1: Summary of mean no-observed-effect concentration (NOEC), 48h EC$_{10}$, and 48h EC$_{50}$ (μg/L) calculated using Dunnett’s test and Spearman-Karber analyses for each trace metal tested with Haliotis rubra larvae as the test species.

<table>
<thead>
<tr>
<th></th>
<th>NOEC</th>
<th>48hEC$_{10}$</th>
<th>48hEC$_{50}$</th>
<th>Port Phillip Bay$^a$</th>
<th>Nearshore and Estuarine$^a$</th>
<th>Water Quality Spp. Protection Levels$^b$ 95%</th>
<th>Water Quality Spp. Protection Levels$^b$ 99%</th>
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<tr>
<td>Copper</td>
<td>1</td>
<td>3.72</td>
<td>7.10</td>
<td>0.47 (0.40-0.63)</td>
<td>0.06-1.3</td>
<td>&lt;1.3</td>
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<td></td>
<td>(95% C.I)</td>
<td>(3.83-3.5)</td>
<td>(6.69-7.50)</td>
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<tr>
<td>Mercury</td>
<td>8</td>
<td>11.90</td>
<td>19.84</td>
<td>0.0017 (&lt;0.001-0.005)</td>
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<td>&lt;1</td>
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<td>(95% C.I)</td>
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<td>Zinc</td>
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<td>42.25</td>
<td>0.47 (0.25-1.05)</td>
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<tr>
<td>Lead</td>
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<td>3,718</td>
<td>4,102</td>
<td>0.06 (0.02-0.13)</td>
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<td>(95% C.I)</td>
<td>(3,650-4,159)</td>
<td>(3,891-4,398)</td>
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<td>(4,860-5,375)</td>
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</tbody>
</table>

Concentrations are reported as μg/L.

$^a$Port Phillip Bay mean concentrations, and nearshore and estuary data ranges have been derived from water samples taken at 25 sites within the Port Phillip Bay (Fabris & Monahan, 1992).

$^b$Water quality guideline concentrations for protection of either 95% or 99% of species within marine environments. Guidelines for 95% species protection have been derived using data from single-species toxicity tests (usually tests <96h duration) on a range of test species. Water quality concentrations for 99% species protection have been calculated from chronic NOEC, multiple-species data. ID = insufficient data to derive a reliable trigger value (ANZECC and ARMCANZ, 2000).
3.4 DISCUSSION

The results of the present study with *H. rubra*, using Cu, Cd, Hg, lead, iron and Zn provide baseline concentrations that inhibit normal development of *H. rubra* larvae. Concentrations higher than the calculated 48hEC\(_{10}\) for each trace metal tested produced morphological abnormalities in the typical development of the viscera, velum and shell of *H. rubra* larval. The rapid cellular reproduction in trochophore larvae from the visceral mass preceding shell formation appeared to be susceptible to trace metal toxicity. This resulted in abnormal development of the viscera, velum and shell. The thinning of the larval shell and subsequent disruption of shell growth may be caused by inhibition of the normal structural organisation of the shell by trace metal ions competing with calcium receptor sites as the shell is secreted (Hunt and Anderson, 1989). This interference by trace metals with cellular reproduction involved in shell formation may have resulted in the abnormal or limited deposition of the shell from the mantle gland in deformed *H. rubra* larvae.

### 3.4.1 Copper, Zinc and Mercury

Copper, Zn and Hg had deleterious effects on shell development at significantly lower concentrations than Cd, Pb and Fe. Copper was the most toxic trace metal tested with a 48hEC\(_{50}\) of 7\(\mu\)g Cu/L. *H. rubra* larval deformities became noticeable at 4\(\mu\)g Cu/L. This effective concentration that impaired larval development is an order of magnitude greater than the accepted water quality guideline concentration established for protection of 99% of marine species (ANZECC and ARMCANZ, 2000). South African, Canadian and USA water quality guidelines set at 1.4\(\mu\)g Cu/L, 2\(\mu\)g Cu/L and 3.1\(\mu\)g Cu/L, respectively are similar to those established for Australian waters (DWAF, 1996; CCME, 1999; USEPA, 2000). Maximum concentrations of 3000\(\mu\)g Cu/L have been reported acceptable in marine
environments as by the Indian Standard Institution (ISI) (Kumari and Nair, 1992). This concentration undoubtedly would be detrimental to abalone larvae if such exposure occurred.

Previous studies performed on larvae of molluscs suggest a sensitivity to Cu similar to that observed in this study for *H. rubra*. Larvae of the doughboy scallop (*Mimachlamys asperrimus*) and the Pacific oyster (*Crassostrea gigas*) are reported to be among the most sensitive Australian species, with development inhibited by Cu concentrations as low as 3μg Cu/L (ANZECC and ARMCANZ, 2000). This present study suggests that *H. rubra* larvae display a sensitivity to the larval stage similar to that of other molluscan species. The larvae of the oysters, *C. gigas* and *Crassostrea virginica* have been reported with a 48hEC\(_{50}\) of 5μg Cu/L and 15μg Cu/L, respectively (MacInnes and Calabrese, 1977; Martin et al. 1981). Larvae of the mussel (*Mytilus edulis*) have been reported with a 48hEC\(_{50}\) of 6μg Cu/L (CCME, 1999). Prolonged exposure of *C. virginica* and the crab, *Mercenaria merceneria* larvae for 8 to 12 days to Cu produced 50% lethal concentrations (LC\(_{50}\)) values of 33μg Cu/L and 16μg Cu/L, respectively (Calabrese et al. 1979). In retrospect, exposure of the oyster, *Crassostrea commercialis* larvae to Cu for 2h resulted in a LC\(_{50}\) of 22,300μg Cu/L (Wisely and Blick, 1967).

The limited published reports concerning the effects of trace metals to abalone larvae are confined to research completed with the North American species, *H. rufescens* using Cu and Zn. Exposure of *H. rufescens* embryos to Cu inhibited normal larval development at 9μg Cu/L (48hEC\(_{50}\)) (Hunt and Anderson, 1993). As a comparison Cu also has been reported to produce an EC\(_{50}\) of >80μg Cu/L, and an LC\(_{50}\) at 114μg Cu/L (Martin et al. 1977). *H. rufescens* larvae had already developed for 48h in that study (Martin et al. 1977), and this may have been the basis for the decreased toxicity observed. Tests completed with *H.
*rufescens* larvae exposed to Zn produced 48hEC$_{50}$ of 40µg Cu/L and 68µg Cu/L (Hunt and Anderson, 1989; Conroy *et al.* 1996).

In the present study performed with *H. rubra*, Zn began to cause morphological abnormalities in *H. rubra* larvae at 20µg Zn/L, and the 48hEC$_{50}$ was calculated at 42µg Zn/L. The calculated trigger level for the protection of 99% of marine species for Zn is 7µg Zn/L. This figure is slightly higher than the water quality guideline concentration of <5µg Zn/L specified for the protection of aquatic species in aquaculture systems. The U.S. Environment Protection Agency has suggested a water quality guideline concentration for Zn as high as 90µg Zn/L for short-term exposure without causing detrimental effects (USEPA, 2000). South African guidelines specify 2µg Zn/L as the target water quality range (DWAF, 1996) and Canadian authorities set 30µg Zn/L as the maximum permissible level for Zn (CCME, 1999). Reported permissible concentrations of Zn in Indian waters as stated by Indian Standards Institute report 5000µg Zn/L to be acceptable (Kumari and Nair, 1992). Our study demonstrates that marine waters concentrations this high would severely impair development of *H. rubra* larvae.

The concentrations of Zn we demonstrated to impair development of *H. rubra* larvae are lower than those reported to be deleterious for larvae of other molluscan species. The 48hEC$_{50}$ for Zn have been reported at 209µg Zn/L and 199µg Zn/L for larvae of *C. virginica* and *C. gigas* (MacInnes and Calabrese, 1977; Martin *et al.* 1981) and 175µg Zn/L for *M. edulis* larvae (Martin *et al.* 1981). Following exposure of *M. mercenaria* larvae for 8 to 10 days, Zn produced an LC$_{50}$ of 195µg Zn/L (Calabrese *et al.* 1979).

Copper and Zn are the most widely tested trace metals in published literature due to their acute toxicity and bioavailability within marine systems. Copper and Zn are readily absorbed
by organic material in larvae (ANZECC and ARMCANZ, 2000), and have been shown to inhibit normal osmoregulation and cause degeneration in cells and tissue (Hubschmann, 1967). Copper and Zn may be toxic to developing trochophore larvae after hatch-out from the egg membrane when osmoregulatory activity commences with the larvae entering the water column and beginning to swim. The mean concentration of both Cu and Zn within Port Phillip Bay is 0.47μg/L (Fabris and Monahan, 1992). This is below the effective concentrations determined in this study to be detrimental to *Haliotis rubra* larvae following acute exposure. The maximum concentration of Cu and Zn in Port Phillip Bay are 0.63μg Cu/L and 1.05μg Zn/L, respectively (Fabris and Monahan, 1992). Concentrations of Cu and Zn have been reported as high as 3μg Cu/L and 15μg Zn/L respectively, within marine waters in the United States (Protho, 1993). It may be assumed that concentrations of this magnitude in natural marine waters may affect the recruitment of abalone larvae and limit the success of the species’ subsequent development.

The toxicity of inorganic Hg and effects on *H. rubra* larvae were unlike Cu and Zn. Mercury is easily absorbed by aquatic organisms (DWAF, 1996), and appears to be more bioavailable to *H. rubra* larvae compared with Cu and Zn. The 48hEC₅₀ calculated in this test was 20μg Hg/L. The lethal concentration of Hg to *H. rubra* larvae was 64μg Hg/L, and development in test solutions caused arrested development of the embryo in the gastrula stage of development. This is different to the toxicity of Cu and Zn; the majority of larvae were alive and swimming within the highest test solution. It has previously been reported that Hg is more toxic than Cu and Zn to aquatic organisms (Wisely and Blick, 1967; Calabrese *et al.* 1979; Bryan, 1971; Connor, 1972). In this present study, however, Cu is more toxic than Hg, which in turn is more toxic than Zn. This may be a result of inorganic Hg used in this study when methyl Hg, the more bioavailable and toxic form may have been used in previous molluscan work.
*Mytilus edulis*, *C. gigas* and *C. virginica* larvae have been reported to produce a 48hEC$_{50}$ when exposed to Hg at 6µg Hg/L, 7µg Hg/L, and 11µg Hg/L (MacInnes *et al.* 1977; Martin *et al.* 1981). His *et al.* (1999) reported a 48hEC$_{50}$ of 7.8µg Hg/L for the sea urchin, *Paracentrotus lividus*. Exposure of *C. virginica* and *M. mercenaria* larvae to Hg for 8 to 12d produced a LC$_{50}$ at 12µg Hg/L and 15µg Hg/L, respectively (Calabrese *et al.* 1979). Exposure of *M. edulis* and *C. commercialis* to Hg for 2h produced a LC$_{50}$ of 13,000µg Hg/L and 180,500µg Hg/L, respectively (Wisely and Blick, 1967). The water quality guideline for protection of 99% of marine species is 0.1µg Hg/L (ANZECC and ARMCANZ, 2000). South African, Canadian and United States water quality guidelines have specified 0.04µg Hg/L, 0.1µg Hg/L and 0.94µg Hg/L as maximum acceptable concentrations within the marine environment for protection of species (DWAF, 1996; CCME, 1999; USEPA, 2000). The mean concentration of Hg within Port Phillip Bay was 0.0017µg Hg/L (Fabris and Monahan, 1992). The risk of Hg inhibiting larvae normal development of *H. rubra* within Port Phillip Bay is minimal, as maximum concentration of Hg recorded in the Bay was 0.005µg Hg/L.

### 3.4.2 Cadmium, Lead and Iron

Cadmium, Pb and Fe did not affect normal development of *H. rubra* larvae below 320µg/L. Concentrations of these thee metals affecting normal morphological development of *H. rubra* larvae were orders of magnitudes greater than the accepted water quality guideline concentrations and trigger levels. The mean Port Phillip Bay background concentration of Cd, Pb and Fe pose no threat to *H. rubra* larvae, as concentrations of less than 1µg/L for all thee trace metals have been reported in Port Phillip Bay (Fabris and Monahan, 1992). The toxic effects of Cd, Pb and Fe are reported to decrease with increasing salinity and water hardness (ANZECC and ARMCANZ, 2000). The toxicity of Cd to *H. rubra* larvae is well below that
documented for other aquatic species. Cd has been demonstrated to produce an EC\textsubscript{50} of 1,200 and 611\textmu g Cd/L for \textit{Mytilus edulis} and \textit{Crassostrea gigas} larvae, respectively (Martin \textit{et al.} 1981). A 96hLC\textsubscript{50} of 110\textmu g Cd/L has been reported for the mysid shrimps, \textit{Mysidopsis bahia} and \textit{Mysidopsis bigelowi} (Gentile \textit{et al.} 1982). This suggests that \textit{H. rubra} is not as sensitive to Cd as other marine invertebrate larvae. It has also been suggested that long-term exposure to Cd at lower concentrations is more detrimental than short-term tests at higher concentrations (Gentile \textit{et al.} 1982), and this remains to be investigated for \textit{H. rubra}.

Lead and Fe form complexations with organic matter and commonly become partitioned and accumulated in marine sediments (O'Donnell \textit{et al.} 1985). Both Pb and Fe remained relatively unavailable to larvae within the test solutions. These trace metals were not readily dissolved in the seawater in the highest concentration tested and flocculated out of solution, becoming heavily concentrated at the bottom of test containers despite soluble inorganic compounds being used in testing. Lead and Fe have the affinity to become tightly bound to particulates within marine environments, causing them to become unavailable in the dissolved phase. Therefore, it is difficult to accurately compare the toxicity of these metals to the water quality guidelines because of their behaviour in the tests performed here. On observation of \textit{H. rubra} larvae exposed to 20,480\textmu g Fe/L, larvae had developed enclosed within the egg membrane, and were rotating within the egg. Data regarding Pb toxicity to larval stages of marine species are not available. Fresh water aquatic species have displayed LC\textsubscript{50} for Fe of 1,500\textmu g Fe/L and higher (ANZECC and ARMCANZ, 2000). The \textit{H. rubra} larvae were not as sensitive to Pb as \textit{M. edulis} and \textit{C. gigas}. Reported morphological EC\textsubscript{50} for these two species when exposed to Pb were 758\textmu g Pb/L and 476\textmu g Pb/L for \textit{M. edulis} and \textit{C. gigas}, respectively (Martin \textit{et al.} 1981). His \textit{et al.} (1999) determined a 48hEC\textsubscript{50} for the sea urchin of 482\textmu g Pb/L. Those authors were unable to calculate a 24hEC\textsubscript{50} for \textit{C. gigas} because 31% of
larvae survived in the highest exposure concentration of 1,200μg Pb/L. Acute Pb toxicity has been documented to be as high as 27,500μg Pb/L (ANZECC and ARMCANZ, 2000).

This 48h acute toxicity test can offer an indication of long-term, chronic effects to larvae after a short exposure time. The deformations in the larvae caused by trace metals at this early stage would have adverse effects on larvae in later development, reducing the chance of progression beyond the planktonic stage, and settling to metamorphose into juvenile abalone. This is supported by previous research in which *H. rufescens* was exposed to nominal concentrations of Zn for 48h and allowed to recover for 8 days (Conroy *et al*. 1996). It was determined that deleterious effects were significant in larvae after initial 48h exposure, and larvae were unable to recover from short-term exposure. Consequently, metamorphosis and settlement were severely inhibited even after extended recovery periods. The present results also suggest that damage incurred at early life stages of *H. rubra* larval development as a result to trace metal contamination in the marine environment may not be reversed because of severe morphological damage sustained by larvae.
Chapter 4

THE EFFECTS OF TRACE METALS ON SETTLEMENT AND METAMORPHOSIS OF BLACKLIP ABALONE LARVAE

(HALIOTIS RUBRA)

This chapter has been submitted for publication and is currently being reviewed as:


4.1 INTRODUCTION

Haliotis species (Gastropoda: Haliotidae) are distributed throughout temperate and tropical marine waters worldwide and are important both environmentally and economically. Worldwide, abalone are one of the most prized seafood delicacies (Stevens, 2003). The culture of abalone in land based aquaculture facilities has increased throughout the world due to the high market demand for this edible species and the decreased availability of wild populations. Populations have decreased considerably in recent years. This decline can be attributed to many factors including exploitation of fishing activities, loss of habitat and increased input of anthropogenic pollutants into the marine environment. The larval stage of abalone is the most sensitive life stage (Gorski and Nugegoda, 2006a; Martin et al. 1977). The success of the larval development will ultimately determine the overall success of the individual.

Similar to many other benthic marine invertebrates, abalone begin their life as lecithotrophic larvae. The fertilised egg of Haliotis rubra develops for 24h through a blastula to
trochophore stage, and subsequently matures into a veliger larva that will eventually settle to begin the benthic existence. The planktonic phase can be divided two stages. The pre-competent stage lasts for 5-7 days when the larva is developmentally incapable of settling. This is followed by the competent stage when the larvae is capable of settling in response to an inducer (Raimondi and Schmitt, 1992).

Once competent and initiated by an appropriate inducer, settlement and metamorphosis of *H. rubra* larvae occurs typically within one to thee days (Anon, 2005). Settlement and metamorphosis are critical events in the life of *H. rubra*. Major physiological and morphological changes occur in the larvae at this time. The transition from the pre-competent to competent larval stage begins with the bouts of swimming becoming shorter and rarer, as the larva is looking for a morphogenetically suitable settlement substrate. The larva begins by crawling on the surface, while still maintaining the ability to swim. Once settled on a suitable substrate, metamorphosis of the abalone to the adult body form is typically completed within 24h (Morse, 1990). During metamorphosis, larval abalone shed the velum, develops enlarged gills and foot muscle, and start peristomal shell formation (Takami *et al.* 2002). Induction of metamorphosis depends on both the developmental stage of the larva and the chance contact with a morphogenetically-inducing substance (Degnan and Morse, 1995). Sublethal stresses experienced by larvae have the potential to dramatically reduce post-metamorphic performance (Pechenik *et al.* 2001).

Previous work has investigated the effects of metals on pre-competent stages of the abalone larvae (Gorski and Nugegoda, 2006a), yet the ability for the larvae of *H. rubra* to survive exposure to pollutants and continue to develop to the competent stage has not been investigated. This paper investigates the effects a range of sub lethal trace metals in solution have on the ability of the larvae to settle and begin metamorphosis once in clean seawater. *H.*
*Haliotis rubra* may be exposed to metals during their pelagic existence and this study provides information on the mechanisms of larval survival following short-term metal exposure.

### 4.2 MATERIALS AND METHODS

#### 4.2.1 Animals

*Haliotis rubra* larvae were reared at Ocean Wave Seafoods, Victoria, Australia following the methods of Gorski and Nugegoda (2006a). Larvae from the same spawning batches were used for each experiment. Three days (72h) after fertilisation, competent veliger larvae actively swimming in the top of the water column were very carefully siphoned from larval-rearing tanks and their density was determined. The suspension was diluted to achieve a final density of 15 larvae per mL. A 10mL volume of larval suspension was added to 30mL fresh, UV-filtered seawater (20°C, >90% DO, pH 8.02) test beakers containing the metal solution at the appropriate concentration. Larvae were exposed to the metals in solution for 48h. In culture systems, *H. rubra* are routinely settled at 5 days (120h) of age (20°C) as they display a well-developed foot, the third tubule of the cephalic tentacle has formed and larvae begin exploring the surface (Hahn, 1989).

#### 4.2.2 Experimental Set-up

Four concentrations of Cu, Zn, Cd and Hg were used, and each test container was replicated four times. Controls were replicated 8 times. Each metal was tested three times (n=3). Stock solutions were prepared by dissolving reagent grade metal salts in distilled water. Each individual stock solution was diluted to the appropriate concentration in the test container. The final nominal exposure concentrations 0.5, 4, 32 and 128µg/L for Hg and Cu; 64, 128, 256 and 512µg Zn/L; and 625, 1,250, 2,500 and 5,000µg Cd/L. The choice of exposure concentrations was based on a previous study investigating the sensitivity of fertilized *H.*
rubra embryos to these metals in solution (Gorski and Nugegoda, 2006a). Analyses of metal concentration in each test chamber performed by atomic absorption spectrometry (AAS) at 0h, 24h and 48h indicated that there was less than 12% variation of nominal and measured metal concentration.

4.2.3 Settlement Microcosms

Microcosm abalone nurseries were prepared in which the veliger larvae, in the pre-settlement stage of development (120h), would willingly settle. Previous research has highlighted the use of the microalgae, Ulvella lens as an ideal substrate on which H. rubra will readily settle (Daume et al. 2000). To mimic settlement plates utilised within the nursery stage of abalone culture, microscope slides (76.2mm x 25.4mm) were vertically placed within an U. lens culture tank. This culture tank contained a rich growth of U. lens and once placed in sunlight, the U. lens readily released spores, which settled on the microscope slides. The microscope slides were kept within the U. lens culture tank for 7 days. After this time, the spores had settled on the microscope slides and colonised the glass microscope slides and produced a film of U. lens growth. Non-specific diatoms that are also an added inducer of larval settlement and shown to be a food source for settled larvae (Daume, 2003) had also colonised the glass slides (Figure 4.1).
Figure 4.1: Photograph of *Ulvella lens* microalgae and unidentified diatom growth on microscope slides used as a settlement surface for *Haliotis rubra* in the nursery microcosm (mag x40).

### 4.2.4 Larval Endpoints

After 48h exposure to each metal, 120h old larvae (5 days) from each of the four replicate exposure beakers for each metal concentration were carefully sieved and transferred using a wide mouthed plastic pipette into individual glass nursery microcosms, each containing 400mL filtered seawater and a single settlement microscope slide with *U. lens* growth. The *U. lens* growth slides were maintained vertically to mimic settlement plates used in nursery culture systems. The stage of larval development in each nursery microcosm was quantified 24h, 48h, and 96h after exposure to the individual metals. This was achieved by gently placing each microcosm onto the stage of a dissecting microscope and 100 larvae were
counted in each to determine the stage of development. The number of larvae: 1) swimming; 2) crawling (actively crawling on substratum with shell vertical); 3) settled (crawling on substratum with shell horizontal); 4) metamorphosed (velum shed, and fourth cephalic tentacle formed); or 5) dead (no visual movement, obvious tissue necrosis) was counted. Physicochemical parameters remained constant throughout all tests performed (20 ± 1°C; 90 ± 2% O₂; pH 8.12 ± 0.03).

4.2.5 Statistical Analysis

Statistical analyses were performed using the statistical software package, GraphPad InStat V.3. Results from each replicate treatment counted after 24h, 48h and 96h were statistically analysed and as there was no significant difference between replicates, data for replicate containers were pooled for comparison of means. Assumption of normality and homogeneity of variance was checked graphically for each data set. One-way ANOVAs with Tukey HSD tests were used for all “no-choice” experiments. Groups were considered significantly different from each other if p<0.05.

4.3 RESULTS

4.3.1 Control Larvae

After five days, control H. rubra larvae had developed through the veliger stage and displayed characteristics including an enlarged foot, the first epipodial tentacle and an enhanced cephalic tentacle, and a pronounced mantle cavity with formed cilia. These characteristics are all indicative of the larvae approaching settlement competence (Figure 2.17). After 24h in the nursery microcosm, 82% of larvae were crawling on the microalgae slides, 13% had settled, and 3% were still swimming (Figure 2.20). The proportion of control larvae crawling significantly decreased to 43% after 48h, and the rate of settled larvae increased to 50%.
There were 3% larvae swimming, 1% appeared abnormal and 4% were dead after 48h. After
96h, 53% of control larvae were settled and 37% had metamorphosed. Metamorphosed larvae
were observed as having developed the juvenile shell and had lost the remnants of the
prototroch and apical tuft (Figure 2.21). The death of control larvae increased insignificantly
from 2% at 24h to 6% at 96h, as did the rate of abnormal development (0% at 24h; 1% at
96h), indicating a strong survivorship of control larvae throughout the test.

4.3.2 Copper

In the initial 24h after exposure to 0.5, 4 and 32µg Cu/L, there was no significant difference in
larval development in the microcosm nursery compared to controls (Figure 4.2a). Exposure
to 128µg Cu/L produced significant difference to control larvae with 2% crawling and 3% settled,
and 69% mortality of larvae and 26% appeared abnormal (p<0.05). After 48h, 100% of
larvae exposed to 128µg Cu/L had died. There appeared to be marginally more larval
settlement 48h after exposure to 0.5, 4 and 32µg Cu/L compared with controls. A significant
increase in metamorphosed larvae was evident 96h after exposure 0.5µg Cu/L, with 65% of
metamorphosed larvae compared with 37% in controls. Exposure to 4, 32 and 128µg Cu/L
caused a significant increase in mortality of larvae after 96h, with 20%, 23% and 100% of
larvae dead, respectively. A higher proportion of larvae remained actively crawling on the
settlement substrate after exposure to 4 and 32µg Cu/L compared to controls (Figure 4.2a).

4.3.3 Zinc

Twenty four hours after exposure to Zn, there was no significant difference in crawling
activity between the control and exposed larvae (Figure 4.2b). There were 82% of larvae
crawling in the control compared to 80% following exposure to 64 and 128µg Zn/L, and 73%
and 69% following exposure to 256µg Zn/L and 512µg Zn/L, respectively. After 24h, there
was an increase in the rate of settlement associated with the increasing concentrations. Larvae
exposed to 512µg Zn/L displayed a 28% settlement rate, significantly difference to 13% in
controls. After 48h, the proportion of crawling larvae decreased, and settlement success of larvae appeared to be insignificantly greater than controls following exposure to 64, 128 and 256µg Zn/L. After 48h exposure to 512µg Zn/L, there was significantly more larvae either abnormal (7%) or dead (17%) compared with the abnormality (1%) and mortality (4%) in controls. Larvae in the controls had not commenced metamorphosis after 48h, yet exposure to Zn at all concentrations caused 3 to 5% of larvae to begin metamorphosis. Also after 48h, there was no significant difference in the number of settled larvae following exposure to 64, 128 and 512µg Zn/L. Larvae exposed to 256µg Zn/L displayed significantly greater settlement success compared to control larvae. The ability for larvae to metamorphose 96h after exposure to 64, 128 and 256µg Zn/L was significantly increased compared to controls. Thirty seven percent of control larvae metamorphosed compared to 58%, 50% and 56% metamorphosis following exposure to 64, 128 and 256µg Zn/L, respectively. Only 19% of larvae had metamorphosed following exposure to 512µg Zn/L. After 96h, mortality of larvae increased significantly from 6% in controls to 19% and 42% following exposure to 256µg Zn/L and 512µg Zn/L, respectively.

4.3.4 Mercury

After 24h, 82% and 84% of larvae were crawling in the controls and following exposure to 0.5µg Hg/L, respectively (Figure 4.2c). Exposure to 4, 16 and 32µg Hg/L resulted in a significant decrease of 74, 69 and 27% of larvae crawling respectively, compared to controls. Larvae exposed to 4µg Hg/L after 24h experienced a significant increase in settlement with 24% compared to 13% in the controls. After 24h, 2% of larvae were dead in the controls compared to a significant increase of 15% and 69% mortality following exposure to 32 and 128µg Hg/L. Mortality of larvae after exposure to 32µg Hg/L and 128µg Hg/L significantly increased to 36% and 100% after 48h. Compared with the metamorphic rate of 37% for controls, 80% of larvae had significantly begun metamorphosis 96h after exposure to 0.5µg Hg/L. The rate of larval metamorphosis 96h after exposure to 4µg Hg/L was 35%, similar to control larvae. After exposure to 4µg Hg/L, mortality had significantly increased to 26% compared to 6% in controls. Larvae were significantly affected 96h after exposure to 32µg
Hg/L, with mortality of 28%, and significantly decreased metamorphosis success compared to controls.

4.3.5 Cadmium

Cadmium was the least toxic metal to *H. rubra* larvae, and the concentrations employed, based on previous range finding tests, indicated insensitivity to <650µg Cd/L (Figure 4.2d). *H. rubra* larvae were not significantly affected 24h after exposure to 625 and 1,250µg Cd/L compared to controls with 85 and 81% of larvae crawling, respectively. Twenty four hours after exposure to 2,500µg Cd/L and 5,000µg Cd/L, 51% and 58% of larvae were still actively swimming and 28% and 29% of larvae were crawling respectively, compared with 3% swimming and 82% crawling in controls. Settlement of larvae was significantly reduced to 37% and 32% 48h after exposure to 625 and 1,250µg Cd/L respectively, compared with 51% settlement in controls. After exposure to 2500µg Cd/L only 4% of larvae had settlement, 30% had died, and 31% appeared abnormal. Exposure to 5000µg Cd/L produced 32% mortality and 43% abnormality of larvae after 48h, significantly higher than the control mortality and abnormality of 4% and 1%, respectively. Larvae were either crawling or swimming after exposure to 5000µg Cd/L, and 0% had settled. After 96h, the mortality of larvae exposed to 625, 1,250, and 2,500µg Cd/L was comparable to control mortality of 6%. A higher proportion of larvae were still swimming 96h after exposure to 625, 1,250, and 2,500µg Cd/L. Metamorphosis was significantly reduced to 21% and 24% after exposure to 625 and 1,250µg Cd/L respectively, compared to control metamorphosis of 37%. Larvae exposed to 2,500 and 5,000µg Cd/L did not achieve metamorphosis. Exposure to 2,500µg Cd/L resulted in larvae either crawling (36%) or settled (42%), and 75% and 12% of larvae exposed to 5,000µg Cd/L had either died or were abnormal after 96h compared to 6% mortality and 1% abnormality in controls.
Figure 4.2: Development (%) of *Haliotis rubra* larvae for 96h following exposure for 48h to A) Cu, B) Zn, C) Hg and D) Cd from the age of 72h to 120h (n=3). Stages of development were assessed 24h, 48h, and 96h after exposure to four concentrations of each metal and a control (mean ± SE, n=100). Common letters shared represent no significant difference between concentrations for each stage of development (p<0.05).
4.4 DISCUSSION

4.4.1 Control Development

Most larvae of invertebrate organisms pass through the pelagic stages of swimming and crawling, to settle and explore the substratum before the metamorphic stage commencing the benthic existence (Kang et al. 2004). *Haliotis rubra* larvae utilised in this study displayed competent behaviour 120h (5 days) after fertilisation. Within 24h of introduction into the microcosm nurseries, the majority of control larvae began crawling, actively creeping on the substrate, assessing their environment. This involved the larvae still having the capacity to swim within the water column with the presence of cilia of the velum, but intermittently descending onto the prepared surface in search of a place to settle and commence metamorphosis into the adult form. Larvae will detach from the substratum and recommence swimming if they are not attracted to the attachment site (Bryan and Qian, 1998). The presence of thee pairs of long spines at the posterior end of the metapodium after the 180°C torsion of the foot, and the formation of the first epipodial tentacle is indicative of the veliger displaying crawling behaviour (Seki and Kan-no, 1977). In the 24h following exposure, crawling activity of *H. rubra* larvae was significantly affected by 128µg/L Hg and Cu, 512µg Zn/L, and 1,250µg Cd/L.

It is not until the fourth tubules were formed on the cephalic tentacles that *H. rubra* larvae showed the crawling, exploratory movements characteristic of settling larvae (Seki and Kan-Ho, 1977). This was obvious in *H. rubra* 48h after larvae were introduced to the nursery microcosms, and a large proportion of control larvae were either crawling or had settled onto the substrate. Normal settlement at 48h was significantly affected after exposure to 128µg Cu/L, 512µg Zn/L, >32µg Hg/L, and 1,250µg Cd/L. It is possible that the reduced settlement success observed for *H. rubra* exposed to >32µg Hg/L and 128µg Cu/L, 512µg Zn/L and
>1,250µg Cd/L may have been due to slowing of development, thereby increasing the time needed to become competent to settle and metamorphose.

After 96h, control larvae were either settled or had begun metamorphosis. Concentrations of 0.5µg/L Cu and Hg, 64, 128 and 256µg Zn/L and 625µg Cd/L caused a greater rate of metamorphosis 96h after exposure compared with controls. However, concentrations of Hg and Cu ≥4µg/L produced a significantly higher mortality rate in larvae. Exposure to concentrations of ≥32µg/L Cu and Hg, ≥625µg Cd/L, and 512µg Zn/L has the capacity to limit with the transition from the larval to the adult form of *H. rubra*. None of the successfully metamorphosed juveniles sampled from each of the metal exposures in this present study had deformities in their larval shells. This trend has also been observed in *Haliotis rufescens* (Hunt and Anderson, 1989), indicating that shell deformity precludes survival at the planktonic stage. Metamorphosed shells from both control containers and test containers in this present study showed the smooth shell surface with a pattern of flecks on the protoconch region, with the newly secreted juvenile shell flaring outward (Conroy *et al.* 1996), which is indicative of normally developed abalone post larvae (Figure 2.21). It is evident that pre-exposure to metals does not affect the structure of the shell once abalone were in the transition to final metamorphosis.

### 4.4.2 Copper

Copper is a successful inhibitor of invertebrate settlement, and is used as a major constituent of antifoulant paints used on the hulls of marine vessels. Copper toxicity is dependent of the concentration of the free hydrated ion (Wong *et al.* 1992). Our results indicate that pre-exposure of *H. rubra* larvae to 128µg Cu/L has the capacity to inhibit normal settlement 48h after exposure and metamorphosis was inhibited at ≥32µg Cu/L after 96h. Kobayashi (1980) exposed larvae of the sand dollar *P. japonica* to Cu for 4 days, and 60% and 100% retardation
occurred following exposure to 50µg and 100µg Cu/L respectively. Exposure of the sea urchin, *Heliocidaris erythrogramma* larvae for 2.5 days resulted in 60% abnormal metamorphosis following exposure to 20µg Cu/L, and 100% retardation at 50µg Cu/L. Lang *et al.* (1981) exposed larvae of the barnacle, *Balanus improvisus* to Cu for 96h and 64% of larvae died in 160µg Cu/L. Those larvae that survived, exhibited morphological abnormalities (Lang *et al.* 1981). Reichelt-Brushett and Harrison (2000) performed a similar exposure of Cu to the coral species, *Acropora tenuis*, and settlement was inhibited after exposure to 50µg Cu/L.

### 4.4.3 Zinc

Research investigating the toxicity of Zn to *Haliotis spp.* larvae has been conducted by Hunt and Anderson (1989). These authors exposed fertilized embryos of *H. rufescens* to Zn for 6 days, and determined the metamorphic success after placing the competent larvae in clean seawater for a further 3 days. Exposure to >19µg Zn/L produced significantly fewer metamorphosed larvae, and 50µg Zn/L produced 50% mortality of larvae. This increased sensitivity of *H. rufescens* to Zn exposure compared with *H. rubra* may be attributed to the extended exposure period of 6 days by Hunt and Anderson (1989) compared to pulse dose of Zn for 2 days in this present study. It may be suggested that either prolonged exposure of larvae or exposure of the younger larvae to Zn is more critical to survival then shorter exposure at an older stage of larval development. This is supported by Kobayashi (1980) who exposed the sand dollar to Zn for 4 days until the larvae were capable of metamorphosis and determined that 30µg Zn/L produced 85% abnormal larvae. As indicted in this present study, Zn appeared to be more stimulatory than toxic at all concentrations <512µg Zn/L.
4.4.4 Mercury

Mercury appeared to be the most toxic of the metals tested in this assay, yet only marginally more toxic than Cu. Hg and Cu have been reported to be neurotoxicants (Zhou and Weis, 1998) and both have been reported as the most toxic metal to larvae of other invertebrate species (Wisely and Blick, 1967; Calabrese et al. 1977; Kobayashi, 1977; Kobayashi, 1984; His et al. 1999; Reichelt-Brushett and Harrsion, 2000). Abalone embryos have been reported with high lipid content, which is utilised as a major energy source during development from egg to a stage competent of metamorphosis (Moran and Manahan, 2003). The affiliation of Hg for lipid soluble membranes may have enhanced the toxicity and mortality of H. rubra larvae before settlement and metamorphosis could occur. Mercury has shown to be extremely toxic to larvae of other invertebrate species (Calabrese et al. 1979; Glickstein et al. 1978; Kobayashi, 1980; His et al. 1999; Sánchez et al. 2005) at concentrations comparable to those used in this study.

4.4.5 Cadmium

Abalone larvae are negatively buoyant and must actively swim to remain in the water column. The consequence of a planktonic larva that prematurely sinks to the benthos is unknown, but it greatly reduces the probability of survival (Raimondi and Schmidt, 1992). Cadmium appeared to have limited toxicity on H. rubra survival, but appeared to affect the overall success of settlement and metamorphosis. Certain types of metals inhibit ciliary beating by altering normal activity of microtubules by competing with calcium (Tamm and Tamm, 1989). Cadmium generally acts as a broad-spectrum calcium channel blocker (Pechenik et al. 2001). It is possible that Cd competed with Ca and affected the ciliary beating in H. rubra. Forty-eight hours after exposure to Cd, a greater proportion of larvae appeared abnormal compared with 96h. At 96h, larvae that were abnormal appeared to have settled. This may have been a result of larvae recovering from exposure to Cd, yet appearing immature
compared to controls. Immaturity because of Cd exposure has also been observed by Lang et al. (1981) following exposure of the barnacle, *Balanus improvisus* larvae to Cd for 96h. Exposure to 200µg Cd/L caused mortality to 18% of larvae, and those larvae that survived appeared to have halted development (Lang et al. 1981). Cadmium was shown to retard metamorphosis in 90% of exposed sand dollars after 4 days exposure to 1200µg/L, which is comparable to concentrations utilised in this study.

### 4.4.6 Effects on Environmental Cues for Metamorphosis

Metals have the potential to retard growth, yet temporary stimulatory effects may also occur at low concentrations (Lang et al. 1981). Evidence from our study suggests that 0.5µg/L Cu and Hg, and Zn concentrations between 64 and 256µg Zn/L appeared to stimulate metamorphosis of larvae. These trace metal concentrations may have interfered in the specific receptors and signal transducers that control settlement and metamorphosis in *H. rubra* (Morse, 1990). Perception by larvae of the stimulus appropriate for settlement and metamorphosis involves the nervous system (Yool et al. 1986), and is controlled by larval sensory recognition and responsiveness to chemicals and environmental stimuli (Morse, 1990). The presence of the trace metals in the water column may imitate natural neurotransmitters released by the nervous system when the larvae detect settlement cues.

Calcium and potassium have shown to have significant effects on abalone settlement and metamorphosis (Baloun and Morse, 1984; Yool et al. 1986). Hunt and Anderson (1989) proposed that calcium ion-regulated channels affect the movement of potassium ions across the membranes of sensory cells, affecting the ability of sensory cells to respond to external stimuli that trigger metamorphosis. Potassium and calcium act by depolarising excitable cells involved in the perception of inductive stimuli or directly activating the nervous system (Zhou et al. 2003). Trace metals could interfere with such mechanisms and delay metamorphosis.
Enzyme kinetic studies have shown that Hg and Cd competitively, and Cu noncompetitively, can inhibit normal enzyme activity (Kramer et al. 1986).

Sublethal effects, such as delayed development, could indirectly contribute to significant reductions in the number of larvae available to recruit into benthic populations (Krause et al. 1992). Our results highlighted that some larvae were still actively swimming 96h after exposure to some metals when 90% of control larvae had either settled or begun metamorphosis. This indicates that exposure of larvae to metals may have adverse consequences on abalone survival at a later life stage. Larvae that delay metamorphosis may experience nutritional stress, as the amount of yolk reserves available for postlarvae declines as their larval period becomes longer (Takami et al. 2002). It has been hypothesized that extreme delays in metamorphosis may affect postlarval fitness by compromising the larva’s preparations for postlarval feeding and growth (Roberts and Lapworth, 2001).

Gorski and Nugegoda (2006a) determined the 48hEC$_{50}$ for Haliotis rubra development from the fertilised egg to veliger stage, and focused on determining the concentrations of trace metals that caused morphological abnormalities in the larvae following fertilisation. This current study has investigated the effects of metals on competent larval development preceding settlement. Mercury and Cu were again the most toxic metals tested and 48EC$_{50}$ results were 7µg Cu/L, 20µg Hg/L, 42µg Zn/L and 4515µg Cd/L. When comparing the results of both these studies, it appears that trace metals may impair development to an equal or greater extent than exposure when at an earlier larval phase. This current study demonstrates the importance of investigating effects of toxicants at different life stages since effects may differ.
Abalone larvae are locally dispersed as passively transported particles and settlement in natural environments (Takami et al. 2002). Reductions in densities of benthic populations caused by diminished recruitment are most likely to be detected for species with limited dispersal abilities and which are exposed to waters with low metal concentrations. Water quality effects abalone settlement and our study is supported by other authors. In an experiment by Raimondi and Schmitt (1992), *H. rufescens* larvae were exposed to produced water from a power plant for four days. It was determined after exposure that pre-competent larvae had greater difficulty settling when induced, compared with competent larvae, and effects on swimming behaviour persisted long after larvae were placed back into clean seawater. It was suggested by these authors, that exposure of *H. rufescens* to a plume of produced water reduces the probability that a larva would successfully complete the transition to the adult body form. This study demonstrates that short-term pre-exposure to selected trace metals are detrimental to the settlement and metamorphosis of *H. rubra* larvae. If ocean outfalls with high trace metal concentrations are located close to abalone settlement sites, or in the path of abalone dispersion, local coastal abalone populations may be severely depleted.
Chapter 5

TOXICITY OF TRACE METALS TO JUVENILE ABALONE, HALIOTIS RUBRA FOLLOWING SHORT TERM EXPOSURE

This chapter has been accepted and published as:


5.1 INTRODUCTION

The value of abalone both commercially and ecologically has increased due to a worldwide population decline. This decline has been attributed to many factors including exploitation by fishing activities, the deterioration of natural habitats and food availability, and possibly pollution of marine environments (Schiel, 1993). One particular group of pollutants, the trace metals, are toxic to marine species and can have severe effects on the health of an organism. Each species exhibits unique responses to trace metal exposure. Trace metals are predominantly concentrated in areas surrounding the site of release, such as industrial effluent pipes, sewage outfalls, dumpsites and urban run-off. For the majority of coastal and offshore environments, concentrations of trace metals are commonly below “effect levels” observed in field and laboratory tests (Langston, 1990).

Trace metals include both essential and non-essential metals. Examples of essential metals are Cu and Zn, these metals are vital components of enzymes, respiratory proteins and
certain structural elements of organisms (Depledge and Rainbow, 1990). Copper and Zn are the most widely tested trace metals due to their acute toxicity and bioavailability within marine environments. The mean concentration of Cu and Zn within Port Phillip Bay is 0.47µg Cu/L, with maximum concentrations reported at 0.63µg Cu/L and 1.05µg Zn/L, respectively (Fabris et al. 1999). The concentration of Cu and Zn in coastal waters in which Haliotis spp. inhabit worldwide range from 0.47-76µg Cu/L, and 0.47-3000µg Zn/L (Ferguson, 1983; Tarazona et al. 1991; Fabris et al. 1999; Lee et al, 1996; Apte et al. 1998; Stauber et al. 2005). Concentrations of Zn in Taiwanese waters, in which abalone are farmed have ranged from 60-300µg Zn/L (Lee et al. 1996; Lin and Liao, 1999).

Non-essential metals that have no known biological function include Cd and Hg. Non-essential metals can become incorporated within cellular processes and cause detriment to the cells. Mercury and Cd are listed as extremely toxic metals on the ‘blacklist’ (McLusky et al. 1986), and natural concentrations within coastal waters are maintained at exceptionally low concentrations. The concentration of Hg has been reported at 0.0002µg Hg/L in coastal waters (Fabris et al. 1999). The mean concentration of Hg within Port Phillip Bay was 0.0017µg Hg/L, with the maximum concentration of Hg recorded at 0.005µg Hg/L (Fabris et al. 1999). The concentration of Cd ranges from 0.03-72µg Cd/L in coastal waters (Tarazona et al, 1991; Abdullah and Mustafa, 2002; Fabris et al. 1999; Apte et al. 1998). Port Phillip Bay has a mean concentration of 0.026µg Cd/L, with a maximum measured Cd concentration of 0.07µg Cd/L (Fabris et al. 1999).

The effects of acute trace metal exposure to abalone in marine environments have not been extensively investigated. In this study, two essential and two non-essential metals of significance in the marine environment were tested to determine their toxicity to juvenile Haliotis rubra. A common endpoint employed in toxicity assays is the 96hLC_{50}, which is
the survival rate of 50% of a population following trace metal exposure for 96h. The objective of this study was to determine the 96hLC$_{50}$ for the trace metals Cd, Cu, Hg and Zn that cause mortality to *H. rubra*. Behaviour of abalone was observed to determine sublethal responses to metal concentrations that did not cause mortality.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Juvenile *Haliotis rubra* of approximately 1.5yrs of age (shell length 25-35mm) were obtained from Ocean Wave Seafoods (Lara, Australia). These juvenile abalone were stored in an insulated cooling container and transported to the laboratories of the Victorian Marine Science Consortium (Queenscliff, Australia) and acclimated in aerated 100L aquaria (17°C) for 48h prior to commencement of tests.

5.2.2 Experimental Set-up

Static 96h assays were conducted (ASTM, 1996) to determine 96hLC$_{50}$ for each trace metal tested in aerated glass aquaria containing 10L, 1µm filtered seawater with dissolved trace metals (n=3). Definitive concentrations for each trace metal were initially determined by performing range finding tests based on a log scale to produce nominal trace metal concentrations. Each concentration and seawater control was tested in triplicate tanks containing ten abalone in each. The trace metals tested were Cd, Cu, Hg and Zn as either chloride or sulphate salts. The concentrations used for each metal were 1000, 2000, 4000 and 8000µg Cd/L; 50, 150, 450, 1350µg Cu/L; 40, 120, 360, 1080µg Hg/L; and 750, 1500, 3000 and 6000µg Zn/L. Test solutions were completely renewed at 48h. The trace metal concentration within each test tank was tested by atomic absorption spectrometry.
(SpectrAA 220 Varian, Australia) at 0h and 48h. Abalone were not fed during the exposure period. Temperature, dissolved oxygen and pH were measured every 24h.

At 24h intervals following the start of exposure, abalone behaviour was observed and mortality was recorded. The behavioural characteristics observed were position of tentacles and their sensitivity to stimuli, surface adhesion by the foot, and the presence of mucus around the gills and in the water column. Results from the 3 separate tests for each trace metal were pooled and percent survival was arcsine-transformed and analysed by single-factor analysis of variance (ANOVA). Median effect (i.e. LC$_{50}$) concentrations were calculated using Spearman Karber analysis using the Toxcalc v.5.0© statistical software package (Tidepool®).

5.3 RESULTS

There was 100% survival of abalone in control tanks for all trace metals tested. Water parameters remained constant throughout all tests performed (18 ± 1°C; 90 ± 2% O$_2$; pH 8.06 ± 0.02). There was minimal measured loss of metals from test solution for each concentration over the 48h (Cd = 7%; Cu = 8%; Zn = 5%; Hg = 6%). Within the 96h exposure regime, significant differences in mortality were observed for each trace metal tested (p<0.05). Abalone mortality at 24h, 48h, 72h and 96h increased with exposure time and this was evident for all trace metals tested (Figure 5.1 A - D).

In the initial stages of exposure, Hg appeared to be the most toxic to _H. rubra_ with a calculated 24hLC$_{50}$ of 335µg Hg/L (95% CI, 270-413µg Hg/L). This is compared to Cu, which was the second most toxic metal after 24h, with a calculated 24hLC$_{50}$ of 691µg Cu/L (95% CI, 546-875µg Cu/L). Following the 96h exposure, Cu was overall the most toxic to juvenile _H. rubra_, with a calculated 96hLC$_{50}$ of 87µg Cu/L (95% CI, 76-98µg Cu/L). The
calculated 96hLC$_{50}$ for Hg was 173µg Hg/L (95% CI, 149-201µg Hg/L). Zn produced a 24hLC$_{50}$ of 4900µg Zn/L (95% CI, 4,305-5,563µg Zn/L). The mortality rate of the abalone increased with time to produce a 96hLC$_{50}$ of 1730µg Zn/L (95% CI, 1,524-1,971µg Zn/L). Abalone exposed to Cd for the initial 48h were not significantly affected at concentrations 1000µg Cd/L, 2000µg Cd/L and 4000µg Cd/L with 100% survival in these concentrations. The 24hLC$_{50}$ and 96hLC$_{50}$ were calculated to be 6200µg Cd/L (95% CI, 5,700-6,677µg Cd/L) and 3,700µg/L (95% CI, 3,209-4,188µg Cd/L), respectively.
Figure 5.1. Dose response of abalone (25-35mm) exposed to A) Cu, B) Zn, C) Hg, and D) Cd for 96h. Percent survival was measured in each test tank at 24h intervals.
5.4 DISCUSSION

The juvenile abalone was observed throughout the four days of the trial and behaviour appeared to be affected at sublethal trace metal concentrations. Abalone within control tanks displayed healthy behaviour. This was characterised by each abalone firmly adhering to the tank surface, hastily retracting into the shell when gently prodded, negligible mucus production, and active tentacle movement and response. Observations suggested that after an exposure time of only 24h, abalone exposed to the minimum concentration of each trace metal began to develop behavioural abnormalities. The observed alteration in behaviour appeared to be directly proportional to increasing exposure to metal concentrations.

5.4.1 Copper

Copper is potentially the most hazardous metals present in the marine environment. Copper is an essential metal, crucial in normal cellular function at trace concentrations. The toxicity of Cu increased over the 96h exposure period, and the health of *H. rubra* declined during exposure. The 96hLC$_{50}$ of 87µg Cu/L determined for *H. rubra* in this test is similar to the calculated 96hLC$_{50}$ of 50µg Cu/L and 65µg Cu/L reported for *H. cracherodii* and *H. rufescens*, respectively (Martin *et al.* 1977). Copper has been tested with various marine finfish and molluscs and 96hLC$_{50}$’s range from 370µg Cu/L to 9420µg Cu/L (Okazaki, 1976; Kumaragura *et al.* 1980; Devi, 1987; Devi, 1997; Karan *et al.* 1998).

5.4.2 Zinc

In comparison to Cu, *H. rubra* displayed a decreased sensitivity to Zn in the 96h short-term exposure. Like Cu, Zn is essential for normal cellular function at trace concentrations. The essential metals Cu and Zn are reported to act pathologically on respiratory systems within an organism (Spicer and Webber, 1991). Research with molluscs suggest that cellular
processes regulate excessive Zn concentrations more efficiently than excessive Cu concentrations (Anderlini, 1974; Young, 1975). Though toxic to marine organisms at elevated concentrations, Zn appears to act more slowly than Cu, and is much less harmful than Cu at equivalent concentrations (D’Silva and Kureishy, 1978). The 96hLC$_{50}$ of 1,730µg Zn/L calculated for *H. rubra* in this test is comparable to the 96hLC$_{50}$ of 1,200µg Zn/L calculated for the native abalone of Taiwan, *Haliotis diversicolor supertexta* (Liao and Lin, 2001). Zinc has been reported to produce 96h-LC$_{50}$ for marine species ranging from 580µg/L to 39,050µg Zn/L (Ahsanullah, 1976; Devi, 1987). The reduced sensitivity displayed by the majority of these bioindicator species is a function of the efficiency of cellular processes, which regulate Zn.

### 5.4.3 Mercury

In the initial 48h of exposure, Hg was more toxic to *H. rubra* than Cu. As Hg is non-essential for intracellular function, the direct toxic effects to cellular processes may have been experienced in the initial 48h of exposure. The mortality rate of *H. rubra* between the time intervals of 72h and 96h did not increase significantly in contrast to exposure to the essential metal Cu. Mercury is toxic not only to *H. rubra* but also to other aquatic species following 96h exposure. Among the trace metals, Hg is considered as one of the most toxic for its high affinity for sulfhydryl-residues of proteins (Pagliarani *et al.* 1996). Reported 96h-LC$_{50}$ for marine test species range from 64µg Hg/L to 490µg Hg/L (Ahsanullah, 1982; Devi, 1987; Devi, 1997).

### 5.4.4 Cadmium

It may be possible that toxicity of Cd in *H. rubra* may occur after prolonged exposure greater than 96h. Ahsanullah (1976) also reported that test animals were initially unaffected by Cd, but thereafter, a high proportion died in a short time. Following exposure of various
marine species to Cd, reported 96h-LC$_{50}$ include 2,600µg/L to 63,000µg/L (Ahsanullah, 1976; Brown et. al. 1984; Devi, 1987; Devi, 1997).

### 5.4.5 Behavioural Abnormalities

The amount of mucus observed produced by the gills increased with increasing metal concentration. Mucus was evident in the gill area and was also in the water column at sublethal concentrations of all trace metals tested following 48h exposure. Mucus production was most evident in *H. rubra* exposed to Cu in concentrations as low as 50µg/L. Suffocation may have been a major contributor to the death of *H. rubra* due to the gills secreting considerably more mucus than could be excreted into the surrounding water column. Trace metal toxicity has been reported to increase the oxygen diffusion distance of the gills of *H. rufescens*, inducing asphyxial hypoxia (Viant et al. 2002). An increase in mucus secretion has been demonstrated as a significant response to heightened trace metal exposure in molluscs (Scott and Major, 1972; D'Silva and Kureishy, 1978; Sze and Lee, 1995; Leung and Furness, 1999; Yorulmazlar and Gül, 2003).

Mucus acts as a barrier for the gills, isolating the animal from its environment (Davies and Hawkins, 1998). Mucus may protect the gills from trace metal exposure by forming a mucus-metal complex, which is then excreted into the surrounding water column (Martin et al. 1977). Mucus has been reported to be involved in the packing, binding and egestion of faecal material and appears to be an effective agent in depuration of trace metals by the mussels, *Perna viridis* and *Septifer virgatus* (Sze & Lee, 1995).

Sensory ability of *H. rubra* was severely affected by exposure to trace metals. The reaction time for tentacle retraction and stimulus response was significantly slower compared with abalone in control tanks. In the higher concentrations, *H. rubra* appeared to lose the ability
to withdraw tentacles when a gentle stimulus was applied. The capacity for *H. rubra* to adhere to the tank surface was also diminished. In the higher sublethal concentrations, *H. rubra* had lost all ability to hold fast. *H. rubra* that were lacking the ability to adhere had fallen from the side of test tanks, and experienced difficulty or the incapacity to right themselves when positioned on their shell. This trend was more common in the higher concentrations. *H. rubra* also exhibited the inability to draw their shell close to their foot, exposing the adductor muscle in an abnormal manner.

It may be possible that blood is shunted away from the foot and tentacles to more oxygen-dependent tissues (Donovan *et al.* 1999). This in turn would affect cellular metabolic function resulting from insufficient oxygen delivery to the tentacles, adductor muscle and foot of *H. rubra*. It can be assumed that this lack of oxygen delivery to these muscles caused *H. rubra* to experience an inability to adhere to tank surfaces. Similar decrease in muscle function attributed to metal exposure has been observed in the gastropod *Nucella lapillus* (Leung *et al.* 1999). This lack of oxygen delivery may also increase *H. rubra*’s tendency to lift the shell away from the foot and expose the adductor muscle with a decreased ability to pull the shell close to their foot. Normal energy requirements in the foot and sensory organs of the abalone could be reduced in the effort of channelling their energy resource for detoxification and preservation (Leung *et al.* 1999). Since *H. rubra*’s survival is dependent on adherence to rock surfaces and sensory awareness utilising tentacles in natural environments, reduction in muscle function following exposure to trace metals could ultimately prove fatal (Viant *et al.* 2002).

*H. rubra* have proven to be as sensitive to short-term trace metal exposure when compared to a variety of other marine species under similar environmental factors. For the majority of coastal and offshore environments, concentrations of trace metals are commonly below
“effect levels” observed in field and laboratory tests (Langston, 1990). The concentrations deemed detrimental to the survival of abalone in this short-term exposure study are at least an order of magnitude greater than concentrations in some segments of coastal waters worldwide. It may be possible to include juvenile *Haliotis rubra* as a bioindicator species for the determination of toxicological effects in the marine environment.
Chapter 6

THE BIOACCUMULATION AND DEPURATION OF TRACE METALS BY JUVENILE ABALONE, HALIOTIS RUBRA FOLLOWING LONG TERM EXPOSURE

This chapter has been submitted for publication as:


### 6.1 INTRODUCTION

Marine organisms are continuously exposed to variable concentrations of trace metals, especially in areas of developed coastlines as a consequence of anthropogenic input. Investigations of the distribution of pollutants within natural coastal areas have revealed the existence of concentration gradients (Anderlini, 1974). The concentration of metals becomes diluted with distance from the discharge point, but it is possible that metals can be transported from the point source. Sites immediately surrounding discharge points can have high trace metal concentrations as both dissolved and particulate forms, and sudden increases in metal concentration may impact biota through direct toxicity or accumulation.

Trace metals include both essential and non-essential metals. Essential metals including Cu and Zn are vital components of enzymes, respiratory proteins and certain structural elements of organisms (Depledge and Rainbow, 1990). Non-essential metals have no known biological function and include Cd and Hg. All metals, whether essential or non-essential can penetrate into the cell and produce toxic effects when in excess. Once in the organism, metals may
become associated with strong binding capacity ligands, which can result in the accumulation of the metal. Cadmium and Zn follow a carrier-mediated transport model whereby the free ion is first bound and transported intracellularly by a protein ligand. It is believed that Hg is transported across lipid membranes. Copper has the ability to enter the cell via passive/facilitated diffusion by binding of free Cu ions to transport proteins (Rainbow, 1997). Accumulation of metals within marine organisms can occur via uptake from the surrounding environment by diffusion across membranes such as gills or digestive epithelium.

In any particular organism, tissue metal concentrations reflect the amount of metal taken up into the organism, the proportion of that metal which is distributed to each tissue, and the extent to which the metal enters and is retained within the tissue (Brown and Depledge 1998). Chronic exposure to low concentrations of trace metals cause sublethal effects that affect an organisms’ potential for growth, its reproductive capacity, its ability to resist further change, and/or its effectiveness in competing with other species in the ecosystem (Harrison and Lam, 1985).

The gastropod *Haliotis rubra* (Leach) is a sedentary, benthic macroalgal grazer, inhabiting the southern Australian coastline. Within Australia, this species of abalone is appreciated for its high market value, and is collected from wild populations and also farmed within aquaculture facilities. The edible foot muscle constitutes the greatest proportion of *Haliotis* and is a large muscular organ with an extensive nerve and vascular supply (Bevelander, 1988). In order for the abalone to inflate the foot muscle, fluid intake from the external medium is required. Therefore, trace metals from seawater could be continuously bioaccumulated by abalone, and sequestered in the edible foot muscle. A common motive for selecting particular species for toxicity tests appears to be the need to investigate the potential for trace metal transfer to humans through the ingestion of seafood (Brown and Depledge, 1998).
Abalone are one of the most commercially important species worldwide, and a better understanding of their sensitivity to trace metal pollution is essential. This present study investigates the ability for *H. rubra* to accumulate Cu, Zn, Hg and Cd into different tissue compartments following prolonged exposure to individual trace metals dissolved in seawater. This study also explores the ability for *H. rubra* to depurate accumulated metals once transferred to clean seawater.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Animals

Blacklip abalone (*Haliotis rubra*) of a standardized shell size (45.7 ± 2.6mm) were obtained from Ocean Wave Seafoods, (Victoria, Australia) and initially acclimatised for 48h in ambient conditions (18°C, pH 8.0, 87% DO) with a 12h photoperiod. Each experiment consisted of eighty abalone per tank with replicates of a control tank and thee treatment tanks. Abalone in exposure tanks were fed an artificial abalone feed (Adam & Amos) at a rate of 2% body weight every second day prior to night fall. The waste material was removed without delay the following morning. Artificial abalone feed usually consists of a mineral mix that constitutes approximately 1-3% of the total feed. These include a mix of manganese, iron, copper, zinc and cobalt (Sales and Britz, 2002). Since all abalone including controls were fed identically, only exposure of trace metals via the water pathway has been considered. Accumulation via food would also be included in the tissue concentrations of abalone reported in this series of experiments. The natural diet for abalone in the field is algae which in uncontaminated sites may contain less trace metals than the artificial diet used in these experiments, but in contaminated sites trace metal accumulation via the food pathway may well be significant.
6.2.2 Experimental Set-up

Initial short-term exposure of juvenile abalone was performed to establish sublethal concentrations for each metal. Individual trace metal exposures were carried out in a continuous flow-through system. In this system, stock solutions of individual trace metals were added to a mixing chamber, which supplied the aerated, exposure tanks for each treatment concentration. The chamber was connected to a perfusor pump (Watson Marlow 505S) that generated a constant flow of 500mL/min. The outlet was connected to a 70L glass test aquarium. In this system, the aqueous test solution was renewed approximately 6 times a day. The control tanks were supplied continuously with fresh seawater maintained at the same flow rate as those in the exposure tanks. The individual metal concentrations in the water were tested by Atomic Absorption Spectroscopy (Varian SpectAA 220) and recovery of trace metals from water samples was between 92 and 109% when tested on five separate days.

The final trace metal concentrations tested were 1, 5 & 25µg Cu/L (CuSO$_4$.5H$_2$O); 20, 100 & 500µg Zn/L (ZnSO$_4$.7H$_2$O); 0.5, 5 & 50µg Hg/L (HgCl$_2$); and 4, 20 & 100µg Cd/L (CdCl$_2$.2H$_2$O). Abalone were exposed to individual trace metals in solution for 28 days. After 28 days, clean seawater (without additional metals) was supplied to the abalone for a further 28 days. At each of the time intervals (0, 2, 7, 14, 21, 28, 42 and 56 days), ten randomly selected abalone from each treatment tank were removed from the exposure tank. The length of each individual abalone, taken as the maximum diameter across the shell, was measured. Abalone were also weighed with and without the shell, then cooled in -20°C and dissected. Abalone were not fed for 24h prior to sampling.

6.2.3 Abalone Tissue Samples
The separate tissue groups were analysed to determine metal content following exposure. These were the mantle tissue; the visceral mass, which included the heart, kidneys, digestive gland and gonad (excluding gills); and the edible foot muscle, which included the columellar muscle, snout, and buccal mass. The gills were not analysed for metal accumulation as they were utilised to determine the activity of the enzyme Na\(^+\),K\(^+\)-ATPase under trace metal stress (Chapter 7). Each separate tissue group were individually separated and blotted on filter paper to remove adhered water and haemolymph. Individual tissue groups were then wrapped in aluminium foil, dipped in liquid nitrogen and stored at -80°C until ready for analysis.

### 6.2.4 Metal Analysis

The wet weight of each tissue component was measured and dried to constant weight at 60°C. Once constant dry weight was achieved, tissues were reweighed to obtain dry weight. Each tissue sample was digested in 3 parts concentrated nitric acid and 1 part sulphuric acid slowly to 80°C, after which the samples were made up to volume with distilled water. Final volumes were 5mL for the edible foot muscle and visceral tissues, and 2mL for mantle tissue. Zn, Cd and Cu were analysed by flame atomic absorption spectrophotometry (Varian SpectrAA 220) with background correction. Hg concentrations were determined by cold vapour atomic absorption spectroscopy. All samples were performed with standards and a blank, and accuracy of the methodology was checked with dogfish liver (DOLT-3). The recovery of metals in reference samples (n=6) was within 94% of the certified values in the reference material. Sample spikes and blank spikes recoveries were between 91 and 111%.

### 6.2.5 Statistical Analysis

Distribution rates (%) of each individual metal in the mantle, visceral mass and edible foot muscle were calculated from ratios of absolute amounts of the metals contained in the three compartments (Ikuta, 1987). The bioconcentration factor (BCF) for each metal was
calculated after 28 days exposure for each tissue, by the final tissue concentration (µg/g) divided by the concentration in seawater (µg/mL). After 28 days exposure, the tissue concentration did not asymptote or plateau, so the BCF was treated with caution. Results from each replicate treatment were statistically analysed and as there was no difference between replicates, results were pooled and are discussed for 20 abalone per treatment. Statistical analysis of pooled replicates for each treatment were log transformed and subjected to Tukey-Kramer analysis using the statistical software package, GraphPad InStat V.3. Groups were considered significantly different from each other if p<0.05. All results are presented as dry weights.

6.3 RESULTS

The results from this study clearly show *H. rubra*’s ability to accumulate appreciable quantities of Cu, Zn, Hg and Cd from the water column. The accumulation of these trace metals showed an increasing trend in all tissue compartments with exposure time. Concentrations of trace metals used in this series of experiments were well below the toxicity threshold (96hLC₅₀) of 3,700µg Cd/L, 87µg Cu/L, 173µg Hg/L and 1,730µg Zn/L (Gorski and Nugegoda, 2006b) determined for *H. rubra* of the same age.

There was 100% survival in all control tanks for each metal tested throughout the duration of this study. The mean whole body weight of the abalone, excluding the gills was 2.07 ± 0.7g. The edible foot muscle comprised the largest proportion of body weight of *H. rubra* in this study, with an average weight of 1.48 ± 0.6g; the viscera accounted for 0.5 ± 0.18g, and the mantle 0.08 ± 0.02g of the total weight (without shell) of the abalone. The total tissue concentration (µg) for each of the metals tested are presented in Figure 6.1. The bioconcentration factors (BCF) for each metal at 28 days is presented in Figure 6.2. The trend for accumulation and depuration in each tissue compartment with time and concentration is
presented in Figure 6.3 to 6.6. The distribution rate in the tissues of each metal following exposure for 28 days is displayed in Figures 6.7 to 6.10. The regression equations in this study for the conversion of dry weights to wet weights, where y=dry weight, and x=wet weight are: 

\[
y = 0.2228x - 0.0664, \text{ for the foot muscle; } y = 0.2729x - 0.0095, \text{ for the visceral mass; and } y = 0.1408x - 0.0018, \text{ for the mantle.}
\]

6.3.1 Edible Foot Muscle

6.3.1.1 Copper

The edible foot muscle of *H. rubra* contained 7.7 ± 3.5µg Cu/g (Figure 6.3a) and accounted for 18% of total Cu in control animals (Figure 6.7). There was significant accumulation of Cu in the foot muscle following 7 days exposure to 1µg Cu/L and 5µg Cu/L to reach a maximum Cu concentration of 17.8µg/g (av. 0.4µg/g d\(^{-1}\)) and 29.5µg/g (av. 0.8µg/g d\(^{-1}\)), respectively after 28 days exposure. Exposure to 25µg Cu/L caused significant accumulation in the initial 2 days to produce maximum Cu accumulation of 36.8µg/g (av. 1.038µg/g d\(^{-1}\)) after 28 days. When clean seawater was returned, loss of Cu from *H. rubra* foot muscle after 14 days was significant, and at 28 days depuration there was a 92%, 96%, and 92% loss of accumulated Cu following exposure to 1, 5 and 25µg Cu/L, respectively.

6.3.1.2 Zinc

The foot muscle of *H. rubra* contained 27.5 ± 4.4µg Zn/g (Figure 6.4a), accounting for 13% of the total Zn within control animals (Figure 6.8). Significant accumulation occurred after 7 days exposure to 20µg Zn/L, until a maximum concentration of 108.4µg/g (av. 2.9µg/g d\(^{-1}\)) at
Muscular tissue exposed to 100µg Zn/L experienced greatest accumulation in the initial 2 days exposure. After 28 days exposure to 100µg and 500µg Zn/L, the concentration in the foot was 193.5µg/g (av. 5.9µg/g d$^{-1}$) and 278.4µg/g (av. 9µg/g d$^{-1}$), respectively. Depuration for 14 days following exposure to 20µg, 100µg and 500µg Zn/L significantly reduced the concentration of Zn in the foot muscle by 53%, 62% and 50% respectively. Depuration for 28 days indicated no further loss of 20µg Zn/L, while the Zn concentration in the foot muscle exposed to 100µg Zn/L had increased by 12% after 28 days depuration. The foot muscle exposed to 500µg Zn/L had lost 79% of accumulated Zn.

**6.3.1.3 Mercury**

The edible foot muscle of control *H. rubra* contained 0.03 ± 0.02µg Hg/g (Figure 6.5a), the highest proportion of Hg (63%) when compared to the other tissues compartments (Figure 6.9). Exposure to 0.5µg Hg/L and 5µg Hg/L resulted in an increase in Hg accumulation to reach a maximum of 0.36µg/g (av. 0.012µg/g d$^{-1}$) and 0.32µg/g (av. 0.01µg/g d$^{-1}$) respectively, after 28 days exposure. Exposure of *H. rubra* to 50µg Hg/L caused the edible foot muscle to significantly accumulate Hg in the initial 2 days exposure, and further increase to reach a maximum concentration of 4.0µg/g (av. 0.14µg/g d$^{-1}$). Depuration of Hg after exposure to 0.5µg Hg/L resulted in 63% and 75% loss of Hg from the edible foot muscle after 14 and 28 days, respectively. Depuration following 5µg Hg/L for 14 days resulted in a 3% increase, followed by a 42% reduction in Hg content after 28 days in clean seawater. Loss of 10% and 42% of accumulated Hg following exposure to 50µg Hg/L occurred after 14 days and 28 days depuration, respectively.

**6.3.1.4 Cadmium**

The edible foot muscle contained 0.05 ± 0.02µg Cd/g (Figure 6.6a), 3% of total Cd found in *H. rubra* controls (Figure 6.10). Exposure to 4, 20 and 100µg Cd/L indicated significant
accumulation following initial exposure. At 28 days exposure to 4, 20 and 100µg Cd/L, maximum Cd concentrations in the edible foot muscle were 0.54µg/g (av. 0.02µg/g d⁻¹), 0.67µg/g (av. 0.034µg/g d⁻¹), and 5.93µg/g (av. 0.2µg/g d⁻¹), respectively. There appeared to be no significance when comparing the accumulation of 4 and 20µg Cd/L at the specific time intervals, but significance was evident in the accumulation of 100µg Cd/L when compared to the controls. Loss of accumulated Cd from the edible foot muscle following exposure to 4µg Cd/L and 20µg Cd/L occurred in the initial 14 days after transfer to clean seawater with a loss of 47% and 34%, respectively. This was followed by minimal further loss after 28 days. Depuration for 14 days and 28 days resulted in a loss of 46% and 65%, respectively of the accumulated Cd after exposure to 100µg Cd/L.

6.3.2 Mantle Tissue

6.3.2.1 Copper

The mantle contained the highest proportion of natural Cu (69%) (Figure 6.7), as indicated by control H. rubra containing 30 ± 6.1µg Cu/g (Figure 6.3b). Mean Cu concentrations in the mantle did not show significant variation from the control until 28 days exposure to 1µg Cu/L when the maximum Cu concentration was 50.9µg Cu/g (av. 0.8µg/g d⁻¹). Exposure to 5µg Cu/L indicated significant accumulation after 21 days exposure to reach 59.8µg/g (av. 1.1µg/g d⁻¹) at 28 days. Accumulation of 25µg Cu/L was significant after 2 days exposure and continued to reach a maximum concentration of 95.6µg Cu/g (av. 2.3µg/g d⁻¹) at 28 days. Depuration from the mantle tissue indicates a significant loss of 79% and 71% of accumulated Cu in the initial 14 days following exposure to 1 and 5µg Cu/L respectively. Further loss after 28 days depuration was not significant. Abalone mantle exposed to 25µg Cu/L lost 58% and 76% of accumulated Cu after 14 days and 28 days, respectively.

6.3.2.2 Zinc
The mantle accounted for 22% of the total Zn in *H. rubra* (Figure 6.8), containing 47 ± 12µg Zn/g in control abalone (Figure 6.4b). Accumulation of 20µg Zn/L in the mantle was not significantly different to control concentrations until 14 days exposure. There was a significant increase in Zn concentration to reach a maximum of 105.8µg Zn/g (*av.* 2.1µg/g d⁻¹) at 28 days. Accumulation of 100µg Zn/L in the mantle tissue increased significantly from 2 days to 7 days to reach a maximum of 370.1µg Zn/g (*av.* 11.6µg/g d⁻¹) after 28 days exposure. In the initial 21 days exposure, mantle concentrations were higher after exposure to 100µg Zn/L compared to 500µg Zn/L. The accumulation of Zn doubled from 21 days to 28 days when exposed to 500µg Zn/L and reached a maximum of 559µg Zn/g (*av.* 18.3µg/g d⁻¹).

Depuration for 14 days indicated significant loss of Zn from the mantle tissue after exposure to 20µg Zn/L, to produce 53% and 55% less Zn than measured in the control. After 14 days and 28 days depuration, there was loss of 62% and 66% of accumulated Zn, respectively following exposure to 100µg Zn/L. The mantle tissue exposed to 500µg Zn/L appeared to lose 83% of the maximum accumulated Zn after 14 days depuration, and further depuration for 28 days resulted in Zn concentrations 13% less than measured in the control mantles.

### 6.3.2.3 Mercury

Initial concentration of Hg measured in the control mantle was 0.01 ± 0.02µg Hg/g (Figure 6.5b), and accounted for 25% of the Hg in control animals (Figure 6.9). Exposure to all concentrations of Hg caused significant accumulation in the initial 2 days. After 21 days exposure to 0.5µg Hg/L, significant accumulation occurred to reach a maximum Hg concentration of 0.58µg Hg/g (*av.* 0.02µg/g d⁻¹). Exposure to 5µg Hg/L resulted in the mantle to contain 2.58µg Hg/g (*av.* 0.02µg/g d⁻¹) after 28 days. Following exposure to 50µg Hg/L, the Hg content increased to a maximum concentration of 67.04µg Hg/g (*av.* 2.4µg/g d⁻¹) after 28 days. Depuration for 14 days and 28 days resulted in a significant loss of 78% and 91% of accumulated Hg after 0.5µg Hg/L, respectively. Decrease in accumulated Hg after exposure
to 5µg Hg/L resulted in 45% and 53% loss after depuration for 14 days and 28 days, respectively. Depuration of the mantle exposed to 50µg Hg/L for 14 days produced a 5% decrease, yet the greatest depuration of Hg occurred after 28 days in clean seawater resulting in a 74% decrease.

6.3.2.4 Cadmium

The mantle accounted for 27% of the total Cd present in *H. rubra* (Figure 6.10), with the controls containing 0.38 ± 0.1µg Cd/g (Figure 6.6b). Significant difference was observed in the accumulation of 4µg/g Cd in the mantle after 7 days exposure, to reach a maximum Cd concentration of 3.9µg Cd/g (av. 0.1µg/g d⁻¹) at 28 days. After only 2 days exposure, Cd had significantly increased in the mantle following exposure to 20µg Cd/L and 100µg Cd/L, and reached a maximum Cd concentration of 5.8µg Cd/g (av. 0.1µg/g d⁻¹) and 31.5µg Cd/g (av. 1.1µg/g d⁻¹), respectively after 28 days. Following exposure to 4µg Cd/L, there was 20% and 74% loss of Cd after 14 days and 28 days depuration, respectively. Loss of Cd at 14 days and 28 days depuration following exposure to 20µg Cd/L resulted in a 38% and 55% a reduction, respectively. The rate of depuration of accumulated Cd at 100µg Cd/L was gradual with a 38% loss after 28 days.

6.3.2 Visceral Mass

6.3.3.1 Copper

The accumulation of Cu was most pronounced in the visceral tissue at each of the thee exposure concentrations compared to accumulation in the mantle and foot muscle. The visceral mass accounted for 14% of the total Cu concentration within *H. rubra* (Figure 6.7),
containing 5.9 ± 1.2µg Cu/g (Figure 6.3c). Mean Cu concentrations in the visceral mass was significantly different from the control after only 2 days exposure to all concentrations. Accumulation of Cu in the visceral tissue exposed to 1 and 5µg Cu/L reached a maximum concentration of 95.6µg Cu/g (av. 1.2µg/g d⁻¹) and 159.7µg Cu/g (av. 5.5µg/g d⁻¹), respectively after 28 days. The greatest accumulation of Cu in the visceral mass occurred after exposure to 25µg Cu/L with a maximum concentration of 276.4µg Cu/g (av. 9.7µg/g day⁻¹) after 28 days. Loss of 55%, 66% and 49% of accumulated Cu in the visceral mass of abalone occurred at 14 days depuration following exposure to 1µg, 5µg, and 25µg Cu/L, respectively. Depuration of Cu from the visceral mass for 28 days following accumulation of 1µg Cu/L, 5µg Cu/L and 25µg Cu/L reduced the concentration in the visceral tissue by 92%, 79% and 80%, respectively.

### 6.3.3.2 Zinc

The visceral mass contained the highest proportion of Zn (65%) as indicated by control abalone (Figure 6.8), with a concentration of 140.6 ± 43µg Zn/g (Figure 6.4c). The visceral mass exposed to 20µg Zn/L experienced an initial decrease in Zn concentration following exposure for 7 days, followed by a significant increase after 14 days. Zn continued to increase in the viscera until 28 days exposure to a maximum concentration of 437.8µg Zn/g (av. 10.2µg/g d⁻¹). Significant accumulation occurred in the visceral mass exposed to 100µg Zn/L and 500µg Zn/L after 7 days, reaching a maximum concentration of 835µg Zn/L (av. 24.8µg/g d⁻¹) and 877µg Zn/g (av. 26.3µg/g d⁻¹), respectively after 28 days exposure. There was 32% loss of Zn from the visceral mass following exposure to 20µg Zn/L after 14 days depuration, with further significant reduction of 94% occurring after 28 days depuration. Depuration after 14 days indicated a significant reduction in 58% and 90% after maximum accumulated Zn to 100µg Zn/L and 500µg Zn/L respectively. After 28 days depuration, the
concentration of Zn in the visceral mass at 100µg Zn/L and 500µg Zn/L had reduced by 59% and 88% respectively.

### 6.3.3.3 Mercury

The accumulation of Hg was greatest in the visceral mass compared to the mantle and foot muscle. The visceral mass control contained 0.006 ± 0.004µg Hg/g (Figure 6.5c) and accounted for 15% of total Hg (Figure 6.9). Exposure to 0.5µg Hg/L produced an increase in Hg content until a maximum concentration of 0.37µg/g (av. 0.013µg/g d⁻¹) at 28 days exposure. There was a significant increase in Hg content in the initial 2 days exposure to 5µg Hg/L and 50µg Hg/L to reach a maximum Hg content of 2.4µg Hg/g (av. 0.084µg/g d⁻¹) and 42.4µg Hg/g (av. 1.5µg/g d⁻¹) at 28 days, respectively. Significant decrease in Hg in the visceral mass was observed after 14 days depuration with a 92% loss of maximum Hg, and no further loss occurred after 28 days. Depuration of 5µg Hg/L for 14 days resulted in a 14% loss in maximum Hg accumulated, while 28 days depuration resulted in a 63% loss. Following maximum accumulation of 50µg Hg/L at 28 days, 25% and 33% of Hg was lost at 14 days and 28 days, respectively.

### 6.3.3.4 Cadmium

The accumulation of Cd was greatest in the visceral mass compared to the mantle and foot muscle. The viscera contained the highest proportion of Cd (73%) as indicated by control abalone (Figure 6.10), and contained 1.2 ± 0.28µg Cd/g (Figure 6.6c). Significant difference in bioaccumulation of Cd was not evident until 21 days exposure to 4µg Cd/L, to reach a concentration of 5µg Cd/g (av. 0.5µg/g d⁻¹) after 28 days. Visceral mass of abalone exposed to 20µg Cd/L experienced significant accumulation after 7 days, until 28 days when the concentration was 27.4µg Cd/g (av. 0.9µg/g d⁻¹). There was a significant increase in Cd in the visceral mass after 7 days exposure to 100µg Cd/L and increased to 258.1µg Cd/g (av.
9.2µg/g d$^{-1}$) after 28 days exposure. After exposure to 4µg Cd/L, depuration for 14 days and 28 days resulted in a loss of 70% and 81% of accumulated Cd, respectively. Depuration for 14 days and 28 days resulted in a loss of 44% and 65%, respectively following exposure to 20µg Cd/L. Depuration for 14 days and 28 days after exposure to 100µg Cd/L resulted in a 5% and 30% reduction in maximum Cd accumulation, respectively.
Figure 6.1: Whole body concentration (µg) in *Haliotis rubra* after exposure to Cu, Zn, Hg and Cd for 28 days followed by 28 days depuration in clean seawater.

Concentrations = Cu A) 1µg/L, B) 5µg/L, C) 25µg/L; Zn A) 20µg/L, B) 100µg/L, C) 500µg/L; Hg A) 0.5µg/L, B) 5µg/L, C) 50µg/L; Cd A) 4µg/L, B) 20µg/L, C) 100µg/L.
Figure 6.2: Bioconcentration factor in each of the tissue compartments after 28 days exposure to Cu, Zn, Hg and Cd. Concentrations of each metal are in µg/L.
Figure 6.3: Accumulation of Cu (μg/g dry wt. ± SE, n=20) in the A) edible foot muscle; B) mantle; and C) viscera after exposure for 28 days to 1, 5 and 25μg Cu/L followed by 28 days depuration in clean seawater.
Figure 6.4: Accumulation of Zn (µg/g dry wt. ± SE, n=20) in the A) edible foot muscle; B) mantle, and C) viscera after exposure for 28 days to 20, 100, and 500 µg Zn/L followed by 28 days depuration in clean seawater.
Figure 6.5: Accumulation of Hg (µg/g dry wt. ± SE, n=20) in the A) edible foot muscle; B) mantle, and C) viscera after exposure for 28 days to 0.5, 5 and 50µg Hg/L followed by 28 days depuration in clean seawater.
Figure 6.6: Accumulation of Cd (µg/g dry wt ± SE, n=20) in the A) edible foot muscle; B) mantle, and C) viscera after exposure for 28 days to 4, 20 and 100µg Cd/L followed by 28 days depuration in clean seawater.
Figure 6.7: Distribution (%) of Cu in the mantle, viscera, and edible foot muscle after 28 days exposure to Cu at (A) 1µg/L, (B) 5µg/L and (C) 25µg/L followed by 28 days depuration in clean seawater.
Figure 6.8: Distribution (%) of Zn in the mantle, viscera and edible foot muscle after 28 days exposure to Zn at (A) 20µg/L, (B) 100µg/L and (C) 500µg/L followed by 28 days depuration in clean seawater.
Figure 6.9: Distribution (%) of Hg in the mantle, viscera and edible foot muscle after 28 days exposure to Hg at (A) 0.5µg/L, (B) 5µg/L, and (C) 50µg/L followed by 28 days depuration in clean seawater.
Figure 6.10: Distribution (%) of Cd in the mantle, viscera and edible foot muscle after 28 days exposure to Cd at (A) 4µg/L, (B) 20µg/L and (C) 100µg/L followed by 28 days depuration in clean seawater.
6.4 DISCUSSION

6.4.1 Copper

Our study has highlighted that *Haliotis rubra* can efficiently bioaccumulate Cu. The uptake of Cu in all tissue compartments did not appear to reach saturation, so it may be assumed that further exposure would have caused additional tissue accumulation of Cu. The whole body content of Cu within *H. rubra* was 21.1µg increasing to a maximum of 197.5µg after 28 days exposure to 25µg Cu/L. Ikuta (1987) indicated that maximum accumulation of Cu in the viscera and foot muscle of the abalone, *Haliotis discus* did not occur until at least 90 days exposure to concentrations similar to those used in this present study. The most probable mechanism of Cu accumulation in the tissues was via the blood stream. The blood of abalone contains haemocyanin as the oxygen carrying protein, which is rich in Cu (Martin et al. 1977; George and Coombs, 1975). Copper may have been readily absorbed from the water column by the blood stream in *H. rubra* and become distributed to the tissue components. Abalone may be unable to efficiently remove Cu from the blood stream at an appropriate rate to prevent the distribution and deposition of excess Cu into organs and tissues. The importance of blood for Cu transport through abalone has also been suggested by Martin et al. (1977) following accumulation of dissolved Cu by *Haliotis cracherodii* and *Haliotis rufescens*. The BCF was far higher for *H. rubra* exposed to Cu than the other metals, and appears higher than that calculated for other marine molluscs (Anandraj et al. 2002; Lakshmanan and Nambisan, 1989), indicating that Cu is readily accumulated by *H. rubra* at low exposure concentrations in all tissue compartments.

Copper was efficiently transported throughout the body of the abalone following immediate exposure, and readily deposited within the viscera>mantle>foot. Copper is associated with the cytosol (Romeo and Gnassia-Barelli, 1995; Kaland et al., 1993) and is uniformly distributed and in higher concentration within tissues that are more vascular, e.g. digestive
tissue (Hyne et al. 1992). Numerous studies have investigated the Cu content in abalone species and it appears the visceral tissue contains a large proportion of accumulated Cu (Won, 1973; Bryan et al. 1977; Anderlini, 1974; Ikuta, 1987; Hyne et al. 1992; Viant et al. 2002). Studies have indicated that the visceral and digestive tissues of marine molluscs experience greater accumulation of Cu than both the mantle and foot (George and Pirie, 1980; Lakshmanan and Nambisan, 1989; Kaland et al. 1993).

Even though Cu was efficiently accumulated at all exposure concentrations, the results from the depuration in this study indicate that when H. rubra were once again exposed to clean seawater, they can efficiently remove excess Cu. Within 14 days depuration, there was a significant loss of Cu from viscera>mantle>foot. This trend of efficient depuration of Cu has also been seen in other molluscs (Viarengo, 1987; Romeo and Gnassia-Barelli, 1995; Gnassia-Barelli et al, 1995; Anandraj et al 2002). It appears that Cu has a biological half life of ≤14 days in the visceral tissue and edible foot muscle of H. rubra. Viant et al. (2002) suggested that exposure to Cu could increase metallothionein synthesis, increasing the ability for Cu to be detoxified by chelators such as glutathione and metallothioneins, allowing for the efficient removal of Cu from cellular processes.

The concentration of Cu in coastal environments in which Haliotis spp. inhabit worldwide ranges from 0.47-76µg Cu/L (Tarazona et al. 1991; Fabris et al. 1999; Lee et al, 1996; Apte et al. 1998; Carpenter et al. 1991; Stauber et al. 2005). Australian and South African water quality guidelines have specified 0.3µg Cu/L and 1.4µg Cu/L in marine waters, respectively to be safe concentrations for the protection of aquatic food species (ANZECC and ARMCANZ, 2000; DWAF, 1996). USEPA (1999) have specified 3.1µg Cu/L as the highest concentration in surface waters to which an aquatic community can be exposed indefinitely (four days) without resulting in an unacceptable effect. Australian Food Standards Code allow
a maximum permitted concentration (MPC) of <70µg Cu/g wet wt. within tissue for the consumption of seafood by humans (ANZFA, 1999). This present study indicates if *H. rubra* is exposed to 1µg Cu/L in seawater for 21 days, which is less than the accepted concentration in Australian waters; the tissue concentration in the edible foot muscle will exceed the MPC.

### 6.4.2 Zinc

Zn is an essential trace metal at low concentrations, playing an integral role in enzyme cofactors and metalloenzymes. This study has indicated that Zn can be effortlessly absorbed from the dissolved phase in the water column and efficiently distributed to the tissues of *H. rubra*. The whole body concentration of Zn in *H. rubra* was 103.8µg and increased to a maximum of 829µg within 28 days exposure to 500µg Zn/L. The trend for accumulation of Zn by *H. rubra* was viscera>mantle>foot, with the viscera accumulating at least 2-fold and 3-fold more Zn than the mantle and foot respectively. The increasing accumulation of Zn in the visceral mass and the ability for *H. rubra* to absorb such high concentrations may be attributed to the presence of metalloenzymes in this organ binding the Zn. The activity of metalloenzymes such as protease and amylase (Cox, 1962; Anderlini 1974; George and Coombs, 1975) are dependent on the presence of Zn in their active sites.

Similarly to Cu, concentrations of Zn appear to be higher in the viscera than the foot muscle and mantle in other abalone species (Bryan *et al.* 1977; Hyne *et al.* 1992; Anderlini, 1974), and marine molluscs (George and Pirie, 1980; Lakshmanan and Nambisan, 1989). The concentration of Zn in the viscera of *H. rubra* after laboratory exposure was of the same magnitude as *H. rufescens* and *Haliotis tuberculata* collected from natural populations in USA and France (Anderlini, 1974; Bryan *et al.* 1977).
The behaviour of *H. rubra* was affected after 5 days exposure to the environmentally unrealistic exposure concentration of 500µg Zn/L. The feeding rate of abalone decreased and abalone appeared lethargic throughout the exposure. This was characterised by abalone raising their shell to expose their adductor muscle, and the gills appearing uncharacteristically pale. These characteristics may be due to the Zn binding sites becoming saturated, causing obvious signs of toxicity. Liao *et al.* (2002) have reported that concentrations in excess of 300µg Zn/L may disrupt the calcium uptake by the gills, leading to hypocalcaemia and death. D'Silva and Kureishy (1978) observed behavioural changes in the mussel, *Mytilus viridis* after prolonged exposure to 200µg Zn/L. The decrease in mantle Zn concentration in the initial 2 days exposure to 500µg Zn/L exposure followed by the subsequent increase after 7 days may have been a mechanistic ability for *H. rubra* to remove Zn from the body to an extent where essential Zn is depleted in cellular processes.

The foot of *H. rubra* accumulated Zn efficiently at the lowest concentration in this study. The foot muscle of abalone have been reported to contain high concentrations of malate dehydrogenase, which is a metalloenzyme involved in utilizing stored fat as an energy source (George and Coombs, 1975). Even with the increasing concentration in the water column, *H. rubra* appeared to be able to keep the overall proportion of Zn away from the foot muscle. *H. rubra* appeared to regulate the accumulation in the mantle and foot muscle to a greater extent then the viscera.

The behaviour of Zn in *H. rubra* supports the suggestion that Zn appears to be metabolically regulated in abalone (Anderlini 1974; Bryan 1977) and other marine invertebrates (Phillips and Rainbow, 1994; Chan, 1988; Kaland *et al.* 1993; Nugegoda and Rainbow, 1995; Müller *et al.*1998). Laboratory based experiments exposing *Haliotis diversicolor supertexta* to Zn within the water column produced a significant increase in the total body Zn concentration,
followed by efficient loss of accumulated Zn (Lin and Liao, 1999). This study has indicated that *H. rubra* can readily accumulate Zn more efficiently than other marine invertebrate species (Nugegoda and Rainbow, 1995; Canli and Furness, 1993), possibly due to less effective depuration methods.

Depuration in clean seawater allowed for the efficient removal of Zn from the mantle tissue. The depurated Zn may have moved back into the blood stream and into the visceral tissue. The rapid depuration of the accumulated Zn in the viscera may be a result of membrane-limited granules in the kidney binding the Zn and excreting these granules in the urine or faeces. This phenomenon is a common occurrence and has been noted in the mussel, *Mytilus edulis* (George and Pirie, 1980), and ensures that excess Zn is isolated from the tissue fluids that are in contact with cellular processes. Depuration in clean seawater following exposure to the unrealistically high concentration of 500µg Zn/L improved the health of the abalone, and the abalone re- commenced feeding within 4 days. The foot muscle appeared to lose accumulated Zn efficiently in the initial stages of depuration but the remaining Zn may have been tightly bound in the foot. The edible foot displayed a biological half-life of >28 days for 20 and 100µg Zn/L, and <28 days after exposure to 500µg Zn/L.

Zinc concentrations within coastal waters are extremely variable, and have shown to vary from 0.47-3000µg Zn/L in extreme cases (Fabris and Monahan, 1999; Ferguson, 1983; Lee *et al.*, 1996; Mikulic *et al*. 1994; Stauber *et al*. 2005). Concentrations of Zn in Taiwanese waters, in which abalone are farmed have ranged from 60-300µg Zn/L (Lin and Liao, 1999; Lee *et al*. 1996). Water quality guidelines specified for the protection of marine species varies between countries. Australian and South African guidelines specify 7µg and 2µg Zn/L, respectively as maximum concentrations for the protection of marine species (ANZECC and ARMCANZ, 2000; DWAF, 1996). North American guidelines specify 81µg Zn/L as the
maximum concentration of Zn in surface waters to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect (USEPA, 1999). Australian standards specify the MPC of Zn in seafood to be 150µg/g wet weight (ANZFA, 1999). Exposure to 20µg Zn/L for 7 days produced Zn concentrations comparable to MPC in the edible foot muscle.

6.4.3 Mercury

Mercury is capable of bioaccumulating rapidly under elevated conditions within the tissue of *H. rubra*. The accumulation of Hg within the mantle and viscera was 17-fold and 11-fold greater than the edible foot muscle, and followed the bioaccumulation trend of mantle>viscera>foot. The total Hg content increased from 0.02µg to a maximum of 54.82µg after 28 days exposure to 50µg Hg/L. Mercury was effectively accumulated in all tissues immediately following commencement of exposure. As suggested by Canli and Furness (1993) and indicated in this present study the non-essential metal Hg cannot be regulated and accumulation occurs in proportion to the water concentration. Research by other authors investigating Hg has indicated that Hg is readily bioaccumulated in abalone. Won (1973) has shown that the concentration of Hg was similar in the viscera and foot of *Haliotis discus hanoi* collected from natural populations in Korea. Walker (1982) indicated that the Hg content in the viscera of *H. rubra* was 2.2 times that of the foot. Anderlini (1974) found that the trend for accumulation of Hg in *H. rufescens* collected along the Californian coastline was digestive tissue>mantle>foot muscle.

*Haliotis rubra* was successful at keeping a large proportion of the accumulated Hg away from the edible foot muscle at the higher exposure concentrations. Canli and Furness (1993) suggested that the level of Hg in tissues remains constant because detoxifying storage tends to occur in one particular site. The mantle appeared to be the major site for bioaccumulation of
Hg and possibly acted as a storage compartment, removing the accumulated Hg from more vital tissues. The BCF was greatest for the mantle at the highest concentration of 50µg Hg/L in this study, indicating the mantle has an efficient absorption system. The accumulation of Hg in the mantle may have been a mechanism that *H. rubra* may implement to remove the metal from the tissue and possibly partition within the crystalline matrix of the shell. The mantle is a sheet of loose connective tissue containing muscle fibres and rich supply of nervous and vascular elements covered by an epithelium, and is responsible for the production of the organic layer of the shell. Calcareous skeletons of organisms have the ability to accumulate and concentrate trace metals to levels several orders of magnitude above those found in their environments (Chinchón *et al.* 2000). The shell could act as a toxic dump to remove toxic chemicals from the metabolically active tissue and therefore effectively eliminate these chemicals from the food chain (Walsh, 1994).

The trend for Hg depuration in *H. rubra* exposed to 0.5 and 5µg Hg/L was viscera>mantle>foot, and foot>mantle>viscera for 50µg Hg/L. Removal following exposure to the higher concentrations appeared more difficult in all the tissue compartments. Gregory *et al.* (2002) and Anandraj *et al.* (2002) both demonstrated that Hg can be efficiently removed once accumulated in soft tissue; though after depuration, Hg levels can still be 100 times that in the control. In the foot muscle, which had the least efficient depuration, Hg had a biological half-life in the foot muscle of >28 days in the higher concentrations, and concentrations still exceeded the control.

Mercury is listed as an extremely toxic metal, and concentrations within coastal waters are regulated at exceptionally low concentrations. The concentration of Hg has been reported at 0.0002-0.075µg Hg/L in coastal waters (Fabris *et al.* 1999; Beiras *et al.* 2002). Australian and South African water quality guidelines have specified 0.1µg Hg/L and 0.04µg Hg/L
respectively in coastal waters to be a safe concentration for the protection of aquatic food species (ANZECC and ARMCANZ, 2000; DWAF, 1996). USEPA (2000) have specified 0.94µg Hg/L as the highest concentration of Hg in surface waters to which an aquatic community can be exposed indefinitely (4 days) without resulting in an unacceptable effect. National food standards within Australia allow a maximum concentration of <0.5µg/g wet weight Hg within seafood for consumption by humans (ANZFA, 1999). Results in this present study have indicated that if *H. rubra* is exposed to 0.5µg Hg/L in seawater for as little as 2 days, the tissue concentration in the edible foot muscle will exceed the MPC. This indicates that if the water quality guidelines are breached, the Hg entering the marine environment will be easily bioaccumulated within the tissue of *H. rubra*.

### 6.4.4 Cadmium

The order of Cd accumulation in the body components determined in this study was viscera>mantle>foot muscle. The accumulation of Cd within the viscera was at least 4-fold and 25-fold greater than the mantle and edible foot muscle, respectively. The visceral tissue was the most prominent site for uptake, and this trend has been observed in a variety of marine invertebrates (George and Pirie, 1980; Canli and Furness 1993; Blackmore and Wang, 2002). Accumulation of Cd following exposure to 20 and 100µg Cd/L indicates efficient uptake immediately following the commencement of exposure. It appears that *H. rubra* may have attempted to regulate the concentration of Cd after prolonged exposure following the initial spike in tissue concentration. Research by other authors has highlighted the viscera of abalone to contain a large proportion of accumulated Cd (Won, 1973; Anderlini, 1974; Vattuone *et al*., 1976; Bryan *et al*., 1977; Ikuta, 1987). Anderlini (1974) investigated the Cd content in the tissue of *H. rufescens* collected from the coastline of California and determined that the digestive gland contained 184 - 1163µg Cd/g dry wt., the mantle 2.8 - 12.8µg Cd/g
dry wt, and the foot muscle 0.17 - 0.53µg Cd/g dry wt. These concentrations in *H. rufescens* are comparable to concentrations determined in *H. rubra* tissue for this present study.

Cadmium may become trapped within organs, particularly in the kidney to become firmly bound within cellular components such as metallothioneins or metal-binding granules, and limit the opportunity to be released (Gnassia-Barelli *et al*., 1995; Viarengo, 1993). Romeo and Gnassia-Barelli (1995) have suggested that Cd might be associated with soluble proteins such as metal-binding proteins at the beginning of exposure, but after longer exposure, the Cd may be displaced to the particulate fraction composed of membranes, or accumulated in intracellular granules.

The depuration of Cd from *H. rubra* following exposure to 4 and 20µg Cd/L followed the trend viscera>mantle>foot. Depuration of Cd following exposure to 100µg Cd/L indicated loss in the foot<mantle<viscera. Depuration of Cd appears to be more pronounced following 14 days in clean seawater. Further depuration appeared to have a limited effect on the release of accumulated Cd. The slow rate of loss may be due to the formation of strong metal complexes with cellular components. Gnassia-Barelli *et al*. (1995) did not report any loss of accumulated Cd in the clam *Ruditapes decussates* after 8 days depuration following exposure to 150µg Zn/L.

The removal of Cd from tissue appears to be much more difficult than Zn. Zinc is an essential metal while Cd is not required for normal cellular process, hence pathways for depuration of Cd in molluscs may not be developed. Cd and Zn show a structural similarity, and Cd will compete with Zn for binding sites within cellular processes. An increase in Cd has been suggested to be paralleled with a decrease in Zn content, suggesting that the protective system
of the organism against Cd is stronger than the binding capacity of Zn under duress (Nugegoda and Rainbow, 1995; Müller et al. 1998).

The BCF established for *H. rubra* was high in the viscera after exposure to all concentrations in this study. The BCF determined in this study for Cd is similar to the concentrations reported in marine invertebrates by other authors, ranging from 22 to 3,160 (George and Coombs, 1975; Olesen and Weeks, 1994; Gnassia-Barelli et al. 1995; Müller et al., 1998). *H. rubra* displayed a far more efficient uptake of Cd compared with marine invertebrates (Nugegoda and Rainbow, 1995; Romeo and Gnassia, 1995; Blackmore and Wang, 2002). At concentrations similar to those used in this study, Ikuta (1987) determined that maximum accumulation of Cd in the viscera and foot muscle of *H. discus* did not occur until at least 90 days. Ikuta (1987) determined accumulation of Cd after 30 days exposure to 20µg Cd/L to be 5 to 6-fold less in the viscera and foot muscle than the concentrations determined for *H. rubra* in this study.

Cadmium is reported as an extremely toxic metal, yet the Cd has not been shown to be exceedingly toxic to *H. rubra*. The concentration of Cd ranges from 0.03-72µg Cd/L in coastal waters (Tarazona et al, 1991; Abdullah and Mustafa 2002; Fabris et al. 1999; Apte et al. 1998). Australian and South African water quality guidelines have specified 0.7µg Cd/L and 0.17µg Cd/L respectively, in coastal waters for the protection of aquatic food species (ANZECC and ARMCANZ, 2000; DWAF, !996). USEPA (2000) have specified 8.8µg Cd/L as the highest concentration of Cd in surface waters to which an aquatic community can be exposed for an extended period of time (4 days) without deleterious effects. National food standards within Australia allow a MPC of <2µg/g Cd within tissue for the consumption of seafood by humans (ANZFA, 1999). Results in this present study have indicated that if *H.
*H. rubra* is exposed to 20µg Cd/L in seawater for 28 days, the tissue concentration in the edible foot muscle will exceed the MPC.

This study clearly demonstrates the ability *H. rubra* to bioaccumulate trace metals without resulting in mortality. Such accumulation of trace metals seen in this present study may not be acutely toxic to *H. rubra* but they may have the potential to interrupt the organism’s ability to maintain itself. Accumulation of trace metals by *H. rubra* in the viscera always exceeded that in the foot. The viscera included the digestive gland and kidneys, and the higher metal accumulation may have been attributed to the presence of metallothioneins or metal-binding granules within these organs, and this is an opportunity for further study. Consideration should be given to the apparent sensitivity of *H. rubra* to sublethal trace metal exposure in the establishment of water quality and seafood quality standards established throughout coastal environments where human inputs of trace metals occur.
Chapter 7

ATPASE ACTIVITY IN THE GILLS OF THE BLACKLIP ABALONE,

HALIOTIS RUBRA IS DEPRESSED FOLLOWING EXPOSURE TO

SUBLETHAL TRACE METAL CONCENTRATIONS

This chapter has been accepted for publication in a peer reviewed journal.


Comparative Biochemistry and Physiology, Part A.

7.1 INTRODUCTION

In all aquatic animals, the gills provide an extensive contact surface with the surrounding environment. Gills are the primary site of gas exchange, acid-based regulation, and ion transfer (Randall, 1990), and any change in the water quality can alter their structure and functional ability. Exposure to aquatic pollutants has the ability to affect the structure of the gills, in such a way to compromise normal gill activity. One particular suite of pollutants that can have a deleterious impact on marine organisms is the trace metals (Harris et al. 1996; Rainbow, 1990). Trace metals are introduced into marine environments from both natural and anthropogenic sources, through anthropogenic inputs of metals far exceed natural inputs as a result of industrialisation.

Metals such as Zn and Cu are essential micronutrients that play a crucial role in the cellular processes of an organism. Once the concentrations of essential metals exceed levels that the organism can manage, the normal metabolic and physiological activities of the organism
begin to suffer. Other metals such as Hg and Cd have no known cellular function and are considered to be extremely toxic. Whether essential or not, when in excess, metals have the ability to block functional groups of enzymes, displace essential cellular metals and enzymes, and/or modify the conformation of biomolecules.

ATPases are a group of enzymes that influence and regulate the movement of cations through cellular membranes, and play an essential role in maintaining the ionic balance of the gills of aquatic animals (Spencer et al. 1979; Parvez et al. 2006). This group of enzymes are responsible for hydrolysing adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate (Pi). The sodium, potassium-activated ATPase (Na\(^+\),K\(^+\)-ATPase) is an integral enzyme located in the plasma membrane. This enzyme facilitates the active transport of Na\(^+\) and K\(^+\) across the cell membrane against their electrochemical gradients and allows for the passive transport of Cl\(^-\). Other ATPase enzymes including Na\(^+\)-ATPase, Mg\(^{2+}\)-ATPase and Ca\(^{2+}\)-ATPase, are also fundamental to the active transport of cations and essential for the integrity of cellular processes.

Adjustment of ATPase activity in the gill epithelium is an important mechanism by which marine animals can adapt to differing environmental concentrations of metals (Mayer-Gostan & Lemaire, 1991). Waterborne toxicants impact the membrane fragility of the gills, and the gill ATPases can be used as a meaningful toxicological tool (Haya et al. 1983; Stagg et al. 1992; Rahman et al. 2000). Trace metals are known to alter ATPase activity in gills of aquatic animals (Haya et al. 1983; Hansen et al. 1992; Pérqueux et al. 1996; Bianchini et al. 1999; Postel et al. 1998; Tkatcheva et al. 2004). To our knowledge, there is no published literature on the response of this group of enzymes in abalone gills following sublethal exposure to trace metals, and the applicability of using ATPases as a biomarker of metal toxicity.
Abalone are invertebrate molluscs that inhabit both temperate and tropical marine waters worldwide. There are in excess of 100 species of abalone worldwide. Abalone populations in Australia, South Africa, North America and Asia support important recreational and commercial fisheries. In recent years abalone populations have declined due to exploitation by recreational and commercial fishing, increased prevalence of disease and pollution of coastal waters. Impacts of both acute and chronic metal toxicity on various stages of abalone development have been widely documented (Martin et al. 1977; Hunt and Anderson, 1989; Liao and Lin, 2001; Viant et al. 2002; Gorski and Nugegoda, 2006a; Gorski and Nugegoda, 2006b). This present study investigates the impact sub-lethal concentrations of the selected trace metals Cu, Zn, Hg and Cd have on ouabain sensitive ATPase activity in the gills of the abalone, Haliotis rubra. Following exposure, the ability for ATPase activity to recover while H. rubra were depurated in clean seawater was also assessed. Copper, Zn, Hg, and Cd represent four of the most extensively studied inorganic contaminants and were chosen to investigate the impact of both essential and non-essential metals on the enzyme activity in the gills of H. rubra.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Juvenile Haliotis rubra aged 2 years (30-40mm length) were transported from an abalone culture facility to Victorian Institute of Marine Science (Queenscliff, Victoria) and initially acclimatised for 48h. The physicochemical parameters of the exposure tanks were maintained (19 ± 1°C; 87 ± 3% DO; 35ppt; pH 8.1 ± 0.1) with a 12h photoperiod for the duration of the assay. Aquarium water was oxygenated by aeration. Abalone in exposure tanks were fed 2% of their body weight every second day at 18:00 hours. The feed utilized was an artificial pellet (Adam & Amos Abalone Foods Pty. Ltd. South Australia), commonly used for the
culture of *H. rubra* in farms throughout southern Australia. The waste and faecal material was removed early the next morning to minimize the absorption of metals in the feed and eliminate ingestion as a possible uptake mechanism by the abalone. Abalone were not fed for 24h prior to sampling.

### 7.2.3 Experimental Set-up

Short-term exposure assays were initially performed with juvenile *H. rubra* to establish sublethal concentrations for each metal. Individual trace metal exposures were carried out in a continuous flow-through system. In this system, stock solutions of individual trace metals were added to a mixing chamber, which supplied the exposure tanks for each treatment concentration. The chamber was connected to a perfusor pump (Watson Marlow 505S) that generated a constant flow of 500mL/min. The outlet was connected to a 70L glass test aquarium. In this system, the aqueous test solution was renewed approximately 6 times a day. The control tanks were supplied continually with fresh seawater maintained at the same flow rate as those in the exposure tanks. The individual metal concentrations in the water were tested by Atomic Absorption Spectroscopy (Varian SpectrAA 220).

Each experiment (n=1) consisted of three treatment tanks and a control tank tested in replicate, and each tank contained eighty abalone. Abalone were exposed to individual trace metals in solution for 28 days followed by 28 days depuration in control seawater. The nominal trace metal concentrations tested were 1, 5 & 25µg Cu/L (CuSO$_4$.5H$_2$O); 20, 100 & 500µg Zn /L (ZnCl$_2$); 0.5, 5 & 50µg Hg /L (HgCl$_2$); and, 4, 20 & 100µg Cd /L (CdCl$_2$.2H$_2$O).

Ten randomly selected abalone were removed from each replicated control and treatment tank, cooled in -20°C, and dissected at 0, 2, 7, 14, 21, 28, 42 and 56 days. Gills were excised and placed on filter paper to remove excess water and haemolymph. Individual gills from
each abalone were wrapped in aluminium foil and dipped in liquid nitrogen, and stored at -80°C until ready for analysis.

**7.2.4 ATPase analysis**

Na⁺,K⁺-ATPase activity was determined in individual abalone gill tissue using the method of Mayer-Gostan and Lemaire (1991) as modified by Hartl et al. (2001). This assay is based on the rate of orthophosphate (Pi) accumulation in the reaction medium as the product of the enzymatic hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) + Pi (Hartl et al. 2001). The measured activity is expressed as inorganic phosphorous liberated per mg protein per hour (µmol Pi/mg protein h⁻¹). The liberated Pi was used as the indirect measure for the ATPase activity.

When ready for analysis, the gills from individual abalone were thawed on ice and placed into ice-cold homogenizing buffer at a ratio of 1 part tissue:5 part buffer. The buffer consisted of 25mM NaCl, 1mM Dithiothereitol, and 1mM HEPES-Tris (pH 7.4). The gills were homogenized (IKA Works® Ultra-Turrax T25 Basic) for 10-15 sec. The homogenate was then centrifuged for 10min at 2212g at 4°C. The resulting supernatant was drawn off and centrifuged again at 2212g for 30min at 4°C. The supernatant containing cellular debris was discarded and the final pellet was gently rinsed thee times with ice-cold 0.3M sucrose. The pellet containing plasma membrane and mitochondrial fragments was resuspended in ice-cold buffered reaction medium containing 100mM NaCl, 0.1mM EDTA, 5.0mM MgCl₂ and 20mM HEPES-Tris (pH 7.4), to a final volume of 1mL. The resuspension was vortexed for 5-10sec to ensure even distribution within the reaction medium and was incubated for 5min on ice.
A series of inorganic phosphate standards (potassium dihydrogen phosphate) ranging from 1 to 20µM was prepared in the ice-cold buffered reaction medium. Triplicate 10µl of each standard and the reagent blank of ice-cold buffered reaction medium were individually pipetted into microplate wells. For each individual sample, 10µl sub-samples were pipetted in duplicate into microplate wells. Immediately following addition of sample to wells, 50µl of 1mM ouabain buffered reaction medium was added to one duplicate well, and 50µl of 3mM ATP buffered reaction medium was added to the other duplicate well. All assays were performed in triplicate and run with separate ATP and ouabain buffer blanks. Each microplate was incubated at 30°C on a gentle plate shaker for 30min.

Following incubation, the enzyme reaction was terminated by the addition of 150µl of the colour reagent stock (5.72% ammonium molybdate in 6N HCl, 2.32% polyvinyl alcohol, 0.0812% malachite green and distilled water in a ratio of 2:2:4:1) to each individual well containing either standard or test sample. Thirty minutes after addition of the colour reagent, absorbance was measured on a microplate reader (BioRad 680XR) at 620nm. Na⁺,K⁺ activated ATPase activity was calculated by subtracting the measured quantity of inorganic phosphate liberated in the presence of 1mM ouabain from the measured activity in the absence of ouabain (total ATPase) (Hartl et al. 2001). The phosphate liberated from the ATP and ouabain buffer blanks were subtracted from the samples containing the gills to eliminate the Pi liberated by the blanks. The protein content of the homogenate was measured using modifications of Bradford (1976).

### 7.2.5 Statistical analysis

ATPase activity in the gills of *H. rubra* for each trace metal with exposure time and dose are expressed as means ± SE. Statistical differences between treatment and control groups were determined by analysis of variance (ANOVA) with the Tukey-Kramer post hoc test used for
multiple comparison performed with GraphPad Instat® statistical software. The significance of results was ascertained at p<0.05.

### 7.3 RESULTS

The ouabain sensitive Na\(^+\),K\(^+\)-ATPase activity measured in the gills of control *H. rubra* was 28.2 ± 2 µmol Pi·mg protein\(^{-1}\)·h\(^{-1}\). ATPase activity in the gills of *H. rubra* after exposure to Cu, Zn, Hg and Cd are presented in Fig 7.1. The recovery of trace metals from water samples was not less than 92%.

#### 7.3.1 Copper

Exposure of abalone gills to 1 and 5 µg Cu/L produced a significant decrease in ouabain sensitive Na\(^+\),K\(^+\)-ATPase activity throughout the 28 days exposure compared to the control activity. Depuration for a further 28 days resulted in recovery of the Na\(^+\),K\(^+\)-ATPase activity. Exposure to 25µg Cu/L resulted in an initial increase in Na\(^+\),K\(^+\)-ATPase activity that continued for 7 days. Na\(^+\),K\(^+\)-ATPase activity significantly decreased after at 14 days exposure and Na\(^+\),K\(^+\)-ATPase activity remained suppressed until abalone were placed in clean seawater. After 28 days depuration, the Na\(^+\),K\(^+\)-ATPase activity of gills exposed to 25µg Cu/L had increased to 68% of control activity.

#### 7.3.2 Zinc

Gills of *Haliotis rubra* exposed to 20µg Zn/L showed a significant decrease in Na\(^+\),K\(^+\)-ATPase activity after 14 days, followed by a gradual recovery to 95% of control activity after 28 days exposure. There was a subsequent decrease in activity after 14 days depuration, followed by a recovery at 28 days depuration when Na\(^+\),K\(^+\)-ATPase activity was 85% of the control. Exposure to 100µg Zn/L produced an initial significant decrease in activity that
remained constant for 21 days, after which time recovery was evident. Full recovery of Na⁺,K⁺-ATPase activity in gills occurred after 28 days depuration. Exposure to 500µg Zn/L resulted in a 89% reduction in Na⁺,K⁺-ATPase activity after the initial 2 day exposure. Recovery of Na⁺,K⁺-ATPase activity in the gills had commenced after 7 days, and after 28 days exposure to 500µg Zn/L, the enzyme activity was 64% of controls. After 28 days exposure to 500µg Zn/L, the abalone had perished. Depuration for 28 days following exposure to 500µg Zn/L increased the Na⁺,K⁺-ATPase activity to 84% of controls.

### 7.3.3 Mercury

Mercury appeared to inhibit the activity of Na⁺,K⁺-ATPase to a greater degree than Cu, Zn and Cd. Gills exposed to 0.5µg Hg/L experienced a continued decrease in activity for 21 days after which time recovery of Na⁺,K⁺-ATPase activity was evident. Depuration of abalone for 28 days following exposure to 0.5µg Hg/L increased Na⁺,K⁺-ATPase activity to 40% of that of the control. Exposure of gill tissue to 5 and 50µg Hg/L significantly decreased the Na⁺,K⁺-ATPase activity after 2 days exposure. A further decrease of Na⁺,K⁺-ATPase activity was measured in gills exposed to 5µg Hg/L for 7 days. It was not until 28 days exposure at 5µg Hg/L that the Na⁺,K⁺-ATPase activity appeared to recover. Na⁺,K⁺-ATPase activity in gills exposed to 50µg Hg/L remained significantly low for 14 days. After 21 days exposure to 50µg Hg/L, Na⁺,K⁺-ATPase activity had decreased to 96% of control. At 14 days depuration, the activity was <95% of control until 28 days depuration when the Na⁺,K⁺-ATPase activity experienced a significant recovery.

### 7.3.4 Cadmium

The effect of Cd on gill Na⁺,K⁺-ATPase activity was significantly different that of the control in the initial 7 days exposure. The Na⁺,K⁺-ATPase activity began to recover from 4µg Cd/L
exposure following a significant decrease initially after 2 days to produce activity levels comparable to control after 28 days exposure. After 28 days depuration following exposure to 4 \( \mu \text{g Cd/L} \), the \( \text{Na}^+\text{,K}^+\)-ATPase activity in \textit{Haliotis rubra} gills was greater than the control. \( \text{Na}^+\text{,K}^+\)-ATPase activity recovered from exposure to 20 \( \mu \text{g Cd/L} \) after 14 day depuration. The lowest \( \text{Na}^+\text{,K}^+\)-ATPase activity was recorded following 2 days exposure at 100 \( \mu \text{g Cd/L} \). Recovery from 100 \( \mu \text{g Cd/L} \) was slower than that observed at 4 and 20 \( \mu \text{g/L} \), yet recovery of \( \text{Na}^+\text{,K}^+\)-ATPase activity had occurred after 14 days depuration.
Figure 7.1: Comparison of ATPase (µmol Pi·mg protein⁻¹·h⁻¹) activity in the whole gill of *Haliotis rubra* after exposure to (A) Cu, (B) Zn, (C) Hg, and (D) Cd for 28 days followed by 28 days depuration in clean seawater (mean ± SE, n=20). Common letters shared represent no significant difference in ATPase activity (p<0.05).
Our results have demonstrated that trace metals have an inhibitory effect on the Na⁺,K⁺-ATPase activity in the gills of *H. rubra*. Abalone gills appeared to be at the top end range of Na⁺,K⁺-ATPase activity compared with a variety of marine invertebrate species. Results of Na⁺,K⁺-ATPase activity in the gills of other marine invertebrates range from 1.2-30.1 µmol Pi mg protein⁻¹ h⁻¹ (Holliday, 1985; Haya *et al.* 1983; Borgatti *et al.* 2003; Pagliarani *et al.* 2006). The indication of high Na⁺,K⁺-ATPases in the gills of control abalone in this study may be a distinct characteristic of the young age of the test animals in this experiment. At various stages of growth and development, enzyme levels may be higher or lower than those of adults, and sometimes unique enzymes may exist at particular developmental stages (NRC, 1993).

Ouabain is a known inhibitor of Na⁺,K⁺-ATPase activity (Motais and Isaia, 1972; Postel *et al.* 1998; Andres *et al.* 2002) and within this current assay ouabain inhibited >90% of the ATPase activity present in *H. rubra* gills. The centrifuge techniques used in this study isolated both the mitochondria and plasma membrane. The high Na⁺,K⁺-ATPase measured in *H. rubra* gills may also be due to the measurement of other ATPases, such as Ca²⁺-ATPase, Na⁺-ATPase and Mg²⁺-ATPase expressed in the presence of ouabain (Motais and Isais, 1972; Parvez *et al.* 2006). Commonly associated with the mitochondrial fraction (Haya *et al.* 1983), Mg²⁺-ATPase may have contributed to the high ATPase activity measured in the gills of *H. rubra*. Mg²⁺-ATPase is responsible for the transport of Mg²⁺ across the gill epithelium and is found associated with Na⁺,K⁺-ATPase (Reddy *et al.* 1992; Parvez *et al.* 2006). Pagliarani *et al.* (2006) reported the gills of mussels to be particularly rich in Na⁺-ATPase activity compared to Na⁺,K⁺-ATPase. The gills of *H. rubra* may also similarly contain greater Na⁺-ATPase enzyme activity than other species such as mammals and fish contributing to the measured ATPase activity.
Aquatic pollutants exert a biological effect on the ATPase system by partitioning the enzyme complex to potentially cause an allosteric change and decreased ATPase activity (Reddy et al. 1992). This ultimately affects the transfer of ions in the cell. Copper exerted a dose dependent inhibitory effect on ATPase activity in the gills of *H. rubra* for the duration of exposure. This inhibitory effect was also observed in the gills of crabs exposed to Cu (Hansen et al. 1992). Initial exposure to the highest Cu concentration resulted in a stimulation of ATPase activity. As a consequence, this may have enhanced the movement of Cu into the cell. The inhibition of the ATPase activity by Cu remained consistent throughout the exposure regime. This continued decreased activity may have been a homeostatic compensatory mechanism to reduce the amount of Cu entering the cell and an adaptive change to ATPase activity repaired once water quality returned to normal. The higher exposure concentration may have triggered deleterious effects in the abalone gills, significantly reducing the activity of the enzyme for the duration of exposure, causing the activity to be impaired in such a way to reduce the recovery in the 28 days depuration.

Mercury produced the most significant decrease in ATPase activity compared with that produced by the other three metals tested. Mercury is a non-competitive inhibitor of ATPases (Andres et al. 2002), disrupting the structure and function of numerous important proteins, and impairing energy production in cells. The ATPase activity in the abalone gills immediately suffered a significant depression, and further exposure to Hg continued to decrease gill ATPase activity. Mercury disturbs ionic and osmotic regulation, and has been implicated in disturbing Na transport by directly affecting ATPase activity (Pagliarani et al. 1996; Péqueux et al. 1996), and it may be possible that Hg acted with a direct toxic action to the ATPase activity in *H. rubra* gills. This may explain the powerful alterations of enzyme
activities reported in marine species following exposure to Hg (Pagliarani et al. 1996; Pérqueux et al. 1996; Andres et al. 2002; Burlando et al. 2004).

The ATPase enzyme substrate may have been changed in such a way as to limit any recovery following Hg exposure. A significant decrease in activity and the lack of recovery has been observed in aquatic animals (Verma et al. 1983), and resulted in increased accumulation of chloride ions in the gills, further exacerbating the toxicological effect. Mercury has also proven to inhibit aquaporins, the proteins responsible for the movement of water across cell membranes (Anon, 2006). It may be possible that Hg inhibited the mode of aquaporins and the movement of water, and as a result depressed the metabolic rate of the cell and the activity of the Na\(^+\),K\(^+\)-ATPases.

The time dependent trend for ATPase inhibition was similar in H. rubra gills exposed to both Zn and Cd. The non-essential metal Cd has the ability to mimic Zn in biological functions, and these two metals have a structural similarity. It has been suggested that Zn and Cd may quickly occupy the active sites of the ATPase activity, competing with the essential ions involved in ATPase activity (Haya et al. 1983; Pivovarova and Lagerspetz, 1996; Postel et al. 1996; Bianchini et al. 1999). This may have resulted in a significant dose dependent decrease in activity observed in the initial exposure to both Zn and Cd, possibly changing the ability of H. rubra gill cells to utilize the ATPase enzyme efficiently.

A similar decrease in Na\(^+\),K\(^+\)-ATPase activity has also been observed in the gills of marine invertebrates following exposure to Zn (Haya et al. 1983; Pagliarani et al. 1996; Bianchini et al. 1999). It appears that H. rubra was unable to regulate Zn at the highest exposure concentration and exposure to 500\(\mu\)g Zn/L may have caused the gills to become saturated. Essential metal such as Zn and Cu exhibit their toxicity once regulatory mechanisms within a
cell are compromised. The higher exposure concentration of Zn was toxic to a small proportion of *H. rubra* and signs of lethargy and behavioural alterations in the later period of exposure was evident in those that survived. Haya *et al.* (1983) also reported alterations in the behaviour of lobsters after exposure to Zn. Subsequent death of the abalone may have been due to increased energy consumption involved in the mobilization, detoxification and excretion of Zn (Bianchini *et al.* 1999). *H. rubra* appeared to recover following depuration and it may be suggested that this environmentally unrealistic concentration of 500µg Zn/L is the tolerance threshold for this species. At this concentration, Zn may not have caused irreversible changes in the enzyme structure allowing for recovery of ATPase activity. Previous authors have indicated that exposure of marine invertebrates to Zn at higher concentrations to irreversibly alter ATPase activity (Haya *et al.* 1983; Bianchini *et al.* 1999).

Similarly to Hg, Cd has been suggested to be a non-specific inhibitor of ATPase activity through the impact of Cd on ATPase activity was not as dramatic as Hg. Prolonged exposure of *H. rubra* gills to Cd resulted in a time dependent recovery in enzyme activity, possibly by the non-essential metal being detoxified and sequestrated within metallothioneins and lysosomes. *Haliotis rubra* gills exposed to Cd recovered completely after the initial 14 days depuration to the extent that there was increased ATPase activity compared with control activity at this time. Because enzymes are regenerated mainly by *de novo* synthesis, when levels are depressed a large amount of enzyme must be produced (Sancho *et al.* 1997). This may have occurred to the ATPase activity during depuration after Cd exposure.

This study demonstrates that exposure to water-borne trace metals has a direct effect on ATPase activity in the gills of *H. rubra*. As indicated by Parvez *et al.* (2006) the active transport mechanisms for the absorption of nutrients and essential ions may be affected by the inhibition of the ATPase activity within the gills, resulting in intracellular increases of ionic
concentrations. In the case of *H. rubra* exposed to Cu, Zn and Cd, ATPase activity appeared to recover once *H. rubra* were allowed to depurate, possibly indicting that ATPase activity in the gills of this species did not undergo an irreversible change. Recovery within the gills may have occurred by homeostatic regulation; increasing the activity and turnover rates of the enzyme to compensate for reduced ATPase activity; and/or, detoxification of metals by sequestering the excess within metallothioneins and lysosomes. In the case of *H. rubra* gills exposed to Hg, the ATPases were still significantly inhibited following depuration, indicating that Hg caused irreversible damage to the ATPase activity in the gills. This assay may be a useful biomarker of exposure to trace metals and other environmental stressors in abalone populations.
Chapter 8

GENERAL DISCUSSION

8.1 Toxicity of Trace Metals to *H. rubra*

Initial investigations assessing the effects of trace metals on biota involved simple toxicity assays to evaluate the lethal doses to particular species of interest (Chapman, 1995b; Fisher and Hook, 2002). Over the past decade, increased focus was applied to the biochemical, physiological and cellular impact of both chronic and acute exposure of the suite of trace metals produced in the era of industrialisation (ANZECC and ARMCANZ, 2000). As knowledge grew about the trace metals, so too did knowledge of the impact selected metals can have on marine organisms.

Over the years, toxicological assays have been performed on many vertebrate and invertebrate marine species. Almost every genus populating marine environments have been tested in one form or another (for review see Furness and Rainbow, 1990). Frequently utilised species include mussels, oysters, and a variety of finfish species and these are now commonly utilised as bioindicators of metal toxicity (Phillips and Rainbow, 1990). This research has underpinned the derivation of water quality criteria and standards aimed at protecting aquatic communities (CCME, 1991; DWAF, 1996; ANZECC and ARMCANZ, 2000; USEPA, 2000).

Despite four decades of research on trace metals in the marine environment, there are few reports of their effects on abalone. This research evaluated the toxicity of trace metals to the abalone, *H. rubra* at the various life stages integral to the development. The results of this study suggest that the abalone, *H. rubra* is as sensitive or potentially more sensitive to metal
toxicity than commonly utilised invertebrate bioindicator species (see Chapter 3 and 4). The metals tested were also far more inhibitory to the youngest stage of *H. rubra* tested, the veliger larvae in this thesis (Chapter 3 and 4) when compared with concentrations of metals that produced mortality in the juvenile *H. rubra* (Chapter 5). However, metal concentrations that were inhibitory to larvae also exerted subcellular effects in juvenile *H. rubra* after chronic exposure (Chapter 7). This thesis has demonstrated that of the metals tested, Cu and Hg were more toxic to *H. rubra* than Fe, Zn, Cd and Pb.

The essential metals Cu, and the non-essential metal Hg (Brown and Depledge, 1998), exerted greater toxicity than Zn and Cd, especially on the early life stages of *H. rubra* development (Chapter 3 and 4). Toxicity of Cu to the early larvae (Chapter 3) was observed at concentrations comparable to the water quality guidelines for Cu established for United States of America and Canada (Table 1.1). The EC$_{50}$ observed for development from embryo fertilisation to veliger larvae was comparable to the Cu concentration inhibiting metamorphosis. Acute toxicity to juvenile *H. rubra*, approximately 2 years of age was expressed at concentrations an order of magnitude greater than the concentration resulting in larval toxicity (Chapter 5). Short-term exposure of juveniles to the higher concentrations resulted in an 8-fold increase in toxicity between 24h and 96h exposure to Cu. Chronic exposure for 28 days to Cu concentrations comparable to those utilised for larval exposure resulted in significant bioaccumulation of Cu into the tissue compartments (Chapter 6). The viscera accumulated more Cu than the mantle and edible foot muscle. This accumulation of Cu did not appear to reach saturation in the time utilised for exposure. ATPase enzyme activity within the gills was significantly depressed at Cu concentrations that resulted in tissue accumulation (Chapter 7). When juvenile abalone were allowed to depurate following exposure to Cu, the accumulated Cu was efficiently lost from the tissue and enzyme function appeared to be repaired.
The non-essential Hg was more toxic to the developed veliger larvae (Chapter 4) than to the earlier development of the fertilised embryo to the veliger stage of larval development (Chapter 3). Metamorphosis of the larvae into the adult form was significantly affected by Hg at concentrations similar to Cu. It was demonstrated that even though Hg produced a slightly greater EC$_{50}$ than Cu, exposure of the fertilised embryo to the higher concentrations of Hg resulted in complete cessation of embryo development. This may be due to the lipid solubility of Hg, and the ability of Hg to be absorbed by cells of any organism (Depledge et al. 1994). Short-term exposure of juveniles to Hg indicated that compared to Cu, Hg was not as toxic during the 96h exposure (Chapter 5). The concentration lethal to juvenile _H. rubra_ was halved between 24h exposure and 96h exposure and was twice that of the Cu concentration lethal to juvenile abalone. Chronic exposure of juvenile _H. rubra_ for 28 days resulted in significant accumulation within all tissue compartments, with the mantle accumulating more Hg than the viscera and the edible foot muscle. ATPase enzyme activity was severely depleted in the gills by Hg, which is a non-specified inhibitor. Following chronic exposure to Hg, depuration resulted in limited recovery of ATPase enzyme activity, and limited loss of the accumulated Hg within the tissues (Chapter 7).

Marine organisms can potentially regulate concentrations of the essential metal Zn (Phillips and Rainbow, 1990; Depledge et al. 1994). Fertilised embryos were the most sensitive stage of _H. rubra_ presented in this thesis and concentrations that produced abnormalities in veliger larvae were 6-fold and 2-fold higher than Cu and Hg respectively. Zinc exhibited less toxicity on the later stage of larval development than on embryo development (Chapter 4). Zinc appeared more readily available to the embryo compared to the older larvae that had advanced development (Chapter 3). Exposure of juvenile _H. rubra_ to Zn resulted in a significantly reduced toxicity compared to Cu and Hg, with mortality produced at concentrations 20-fold and 10-fold less than Cu and Hg respectively (Chapter 5). Bioaccumulation of Zn by _H.
rubra was higher within the viscera compared to the other tissue compartments, and this appeared to become saturated following exposure to the unrealistic concentration of 100µg Zn/L (Chapter 6). Efficient depuration of accumulated Zn occurred except from tissues of abalone exposed to 500µg Zn/L. It could be suggested from this thesis that 500µg Zn/L may be the threshold concentration for extended exposure of H. rubra to Zn. This is further supported by what could potentially have been an irreversible change in enzyme activity in the gills of H. rubra exposed to 500µg Zn/L (Chapter 7). ATPase enzyme activity was altered following chronic exposure to Zn, yet depuration resulted in the efficient recovery in activity with the exception of exposure to 500µg Zn/L, which may have resulted in an irreversible change to the structure of the enzyme or its ability to function appropriately.

Of the metals extensively tested within this research study, H. rubra proved relatively insensitive to Cd in each of the development stages investigated. Cd toxicity has been reported to increase within environments of decreased salinity, thus displaying far less toxicity within marine environments (ANZECC and ARMCANZ, 2000). This non-essential metal exerted its toxicity most prominently on the older veliger larvae of H. rubra, inhibiting ciliary beating and delaying settlement and metamorphosis at concentrations at least 100-fold greater than Cu and Hg (Chapter 4). Fertilised embryos appeared relatively insensitive to dissolved Cd concentrations (Chapter 3). The Cd concentration determined as the EC50 for fertilised embryo development to veliger larvae (Chapter 3) was higher than the Cd concentration deemed lethal to juvenile H. rubra after short-term acute exposure (Chapter 5). This may indicate that H. rubra can possibly regulate excess Cd, or Cd was unavailable to biological processes during short-term exposure. Bioaccumulation of Cd was significantly higher in the viscera than the other tissue components (Chapter 6). It appears H. rubra was able to regulate chronic exposure to Cd at the lower concentrations utilised in the chronic assay and appeared unable to regulate 100µg Cd/L after prolonged exposure. This was also
the case for ATPase enzyme activity within the gills of *H. rubra*. Activity was depressed but it appears that once *H. rubra* was allowed to depurate, activity was once again returned to normal (Chapter 7).

In the initial stages of developing the framework for this thesis, Pb and Fe were two metals that were considered for investigation. However, as indicated in Chapter 3, exposure of fertilised embryos demonstrated that only environmentally unrealistic concentrations affected normal development. Pb and Fe flocculated out of solution, and were confined as particulate matter at the base of test containers. To further test the toxicity of both these metals, short-term toxicity tests employing range finding concentrations indicated that toxicity to juvenile *H. rubra* did not occur at 10,000µg/L, and it was difficult to keep the metals in solution. Therefore, considering also that concentrations affecting both larvae and juvenile *H. rubra* were unrealistic within the dissolved phase of a marine environment, Pb and Fe were not further investigated in this research study.

### 8.2 Environmental Significance

Environmental protection should usually act on the side of caution. Trace metals are available to *H. rubra* via both the dissolved and particulate phase. The behaviour of metals in natural waters is a function of the substrate sediment composition, the suspended sediment composition, and the water chemistry. Dredging activities within harbours and shipping channels have the ability to displace trace metals partitioned with the sediments releasing metals that were partitioned with organic matter back into the water column and hence making them bioavailable from the dissolved water phase (Harris *et al.* 1996).
Organisms are most commonly exposed to a mixture of metals in the environment, however data is limited on the behaviour of mixtures of metals from controlled experimental work (Boening 2000). Interactions can occur when two or more trace metals, or pollutants are applied simultaneously to living organisms, the combined effect may result in any of the following: (1) the addition of toxic effect of one chemical to the other (non-interactive or additive action); (2) the toxic effects caused by the mixture being significantly less than the sum of the toxic effects of the separate constituents (antagonisms); and (3) the toxic effects caused by the mixture may significantly exceed the sum of effects of the separate constituents (synergism) (Otitoloju, 2003).

This thesis only considered the effects of water-borne dissolved trace metals on \textit{H. rubra}. Food pathways can also be another parameter that can lead to increased toxicity of trace metals to \textit{H. rubra}. Since adult \textit{H. rubra} feed on drift algae, ingestion of contaminated algae can also be another mechanism of trace metal intake by abalone. This is an avenue for further research beyond the current results highlighted in this thesis. Research in this thesis has identified that larvae in the pelagic form, as well as older \textit{H. rubra} are susceptible to dissolved concentrations on metals within the water column. Luoma and Rainbow (2005) have suggested a model that calculates net trace metal bioaccumulation by biota as a result of a balance among thee mechanisms: uptake rate from diet, uptake rate of dissolved forms, and loss rates.

\section*{8.3 Overall Conclusions}

Abalone have difficulty recruiting into slightly to moderately disturbed systems such as ecosystems lying adjacent to a metropolitan areas (McShane \textit{et al.} 1986). The absence of abalone populations in highly disturbed areas, such as shipping ports and sections of harbours serving coastal cities, suggests that abalone may be adversely affected by pollution levels.
The impact of trace metal toxicity on populations of *H. rubra* can be compared to impact on other species of *Haliotis*. Similar sensitivities have been reported for other abalone species. Various stages of other *Haliotis spp.* have been investigated and the collated results are comparable to effects measured on *H. rubra*.

This thesis has revealed that the early life stages of *H. rubra* are the most sensitive phase of development when exposed to dissolved concentrations of trace metals. *H. rubra* larval studies in this thesis indicate that if larvae encountered a plume of trace metals at concentrations deemed effective in this thesis, the survival rate of a population of *H. rubra* would be severely reduced. A population can still potentially be viable after being affected by trace metal exposure, yet intrinsic compromise to the survival and success of longevity is inevitable. Those trace metal concentrations deemed as the 48h LOEC to fertilised embryo development to veligers and also to inhibit normal metamorphosis in the exposed populations may ultimately prove detrimental to the population recruitment of *H. rubra* following exposure. Those concentrations of trace metals that subsequently reduce the potential for proliferation of *H. rubra* populations, but allow the organism’s self-sustenance throughout the entirety of its lifecycle and remain viable to continue the genetic diversity, are not yet known.

Results have demonstrated that exposure of juvenile *H. rubra* to 1µg Cu/L, 0.5µg Hg/L, 20µg Zn/L and 20µg Cd/L for 28 days resulted in accumulation within the edible foot potentially exceeding the maximum permissible concentration (MPC) specified by the Australian government for consumption by humans (ANZFA, 1999). The concentrations deemed effective at inhibiting *H. rubra* development are comparable to background concentrations identified in some marine coastal waters of the world (See Table 1.1). Maximum permissible concentrations need to be established at least an order of magnitude below the level at which significant risk occurred (Fisher and Hook, 2002). If the most sensitive life stage of *H. rubra*
determined in this thesis is used as an indicator of metal toxicity, then the maximum regulatory concentrations for water quality guidelines to protect *H. rubra* in natural marine waters should at least be 0.05µg Cu/L, 4µg Zn/L, 0.05µg Hg/L, and 62µg Cd/L.

This thesis highlights that the sensitivity of *H. rubra* to Cu and Hg may need to be considered by the regulators when revising or amending the water quality guidelines for Australian waters. It has also been established by this research that *Haliotis rubra* displays sensitivity similar to that of other *Haliotis* spp. to trace metal exposure. This suggests that the results of this thesis could be a benchmark for the revision of marine water quality guidelines worldwide, such as those in the United States, South Africa and Canada, to ensure that detrimental effects on inshore *Haliotis* populations are prevented and future generations preserved.
Chapter 9
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