EVALUATION OF METALLOTHIONEIN AS AN ECOTOXICOLOGICAL BIOMARKER IN *NUCELLA LAPILLUS* AND *LITTORINA LITTOREA*

KENNETH MEI-YEE LEUNG

Presented in candidature for the degree of Doctor of Philosophy, to the Institute of Biomedical and Life Sciences, University of Glasgow.

March, 2000

In Memory of JAMES HENRY SCOTT

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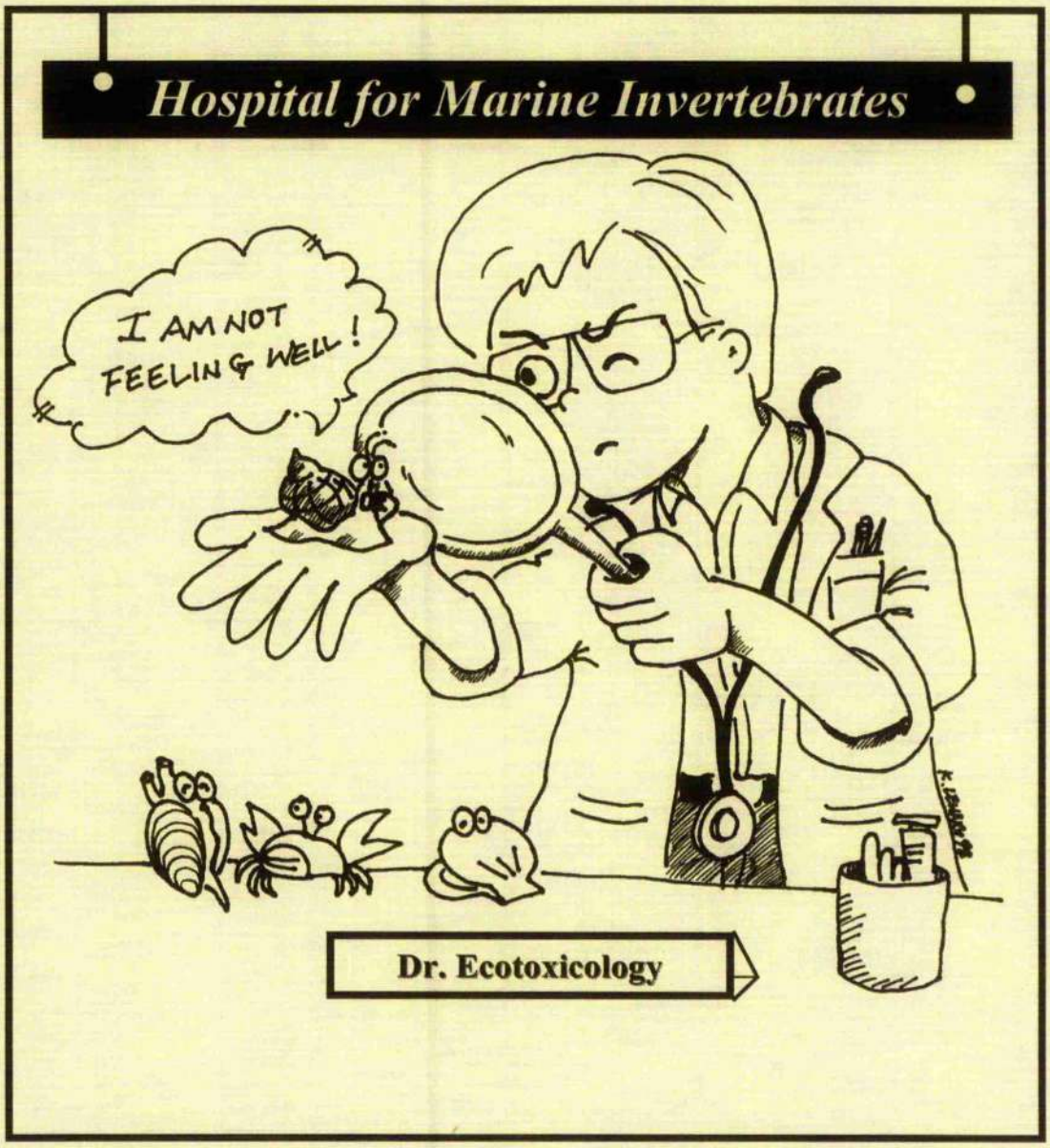
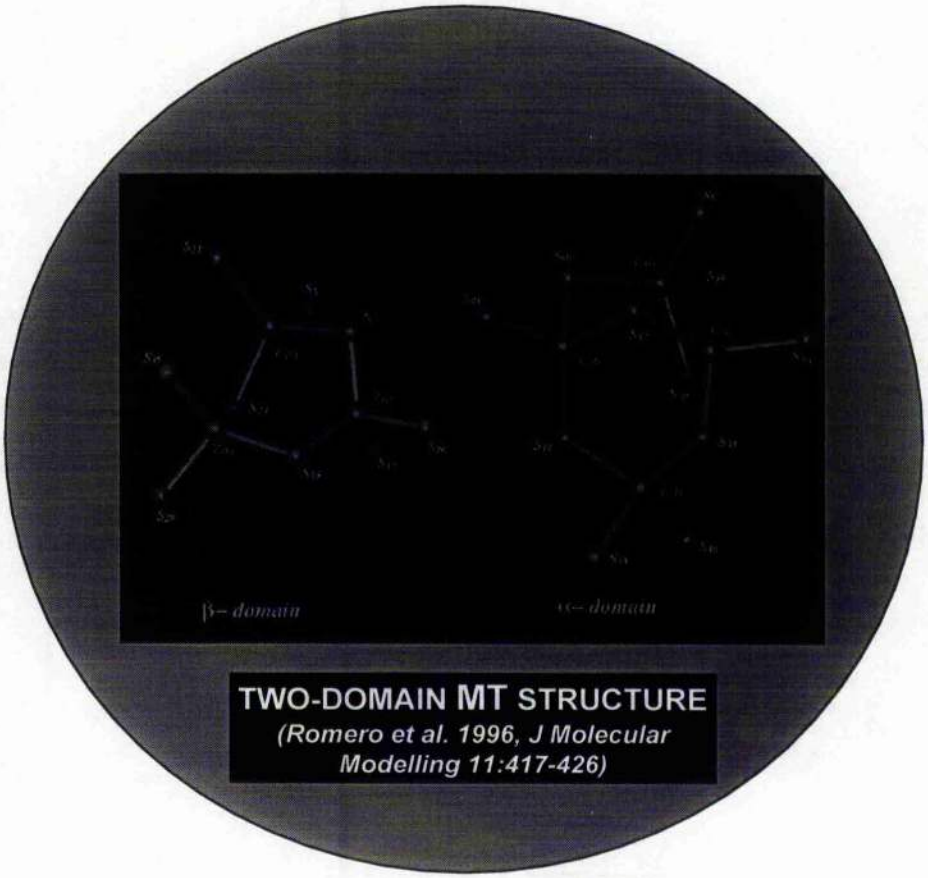


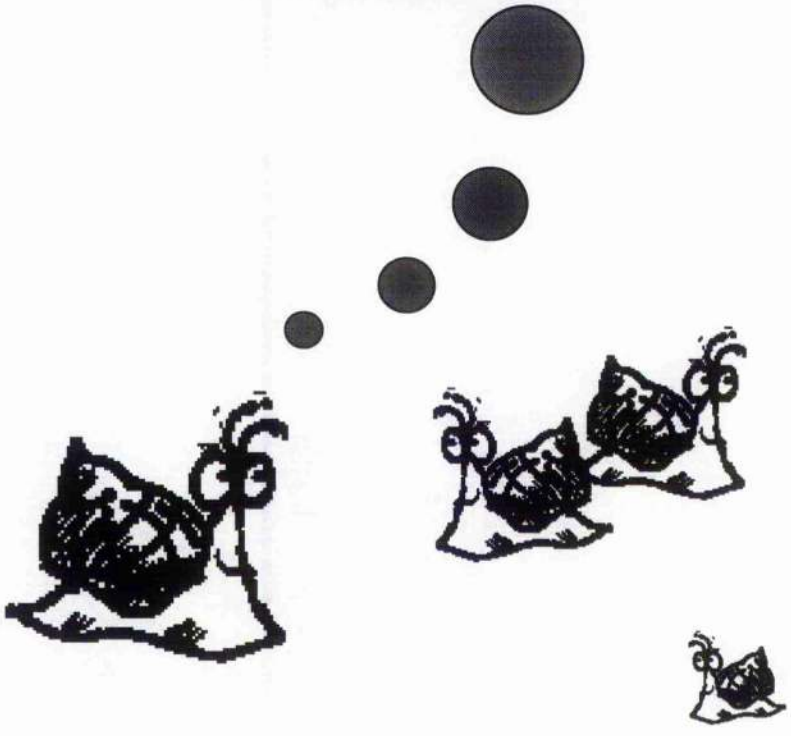
"I don't know what I may seem to the world, but, as to myself, I seem to have been only like a boy playing on the sea shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."
Sir Isaac Newton (1642-1727)

Source: Carey J. (1995) The Faber Book of Science, f. f., London. pp. 30-34.



TWO-DOMAIN MT STRUCTURE

(Romero et al. 1996, J Molecular Modelling 11:417-426)



Candidate's Declaration

I declare that the work recorded in this thesis is entirely my own, unless otherwise stated and that it is of my own composition. No part of this work has been submitted for any other degree.

Kenneth Mei-Yee Leung

March 2000

Dedication

This thesis is dedicated with love and respect to my parents,

Lam and Kam-Chu Leung

Acknowledgements

I am greatly indebted to John Swire and Sons Ltd. for providing me with a James Henry Scott Scholarship that made this study possible. I would like to thank Sir Edward Scott (Director of John Swire and Sons Ltd.), Mr Graham Docherty and Ms Winnie Wong for their caring, friendship, wonderful arrangement and hospitality.

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Although I could not include the research results obtained from Iceland in this thesis (most samples await metal and biochemical analyses), this trip to Iceland was one of the most important milestones in my PhD study. I am especially grateful for the TMR research grant (as a part of BIOICE project from EC) that allowed me to stay and work at the Sandgerdi Marine Centre (SMC), and carry out laboratory and field studies during August to November 1999. I am grateful for the enthusiastic support provided to me by Mr Gudmundur and Prof Jorundur Svavarsson. In particular, Prof Svavarsson patiently taught me how to identify of the various stages of imposex in dogwhelks, generously offered financial support for my field trips in Iceland (sampling of dogwhelks from South to the very North - Grimsey Island) and collaborated for the project. Thanks are also due to all colleagues and lovely people at Sandgerdi, especially Halldor, Reynir, Oli, Stebbi, Binna, Ragnar, Stuart and Ann, for their great help and wonderful friendship.

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Contents

Summary

1) Metallothioneins (MTs) are frequently proposed as biomarkers for metal exposure and toxicity in molluscs. However, various biotic and abiotic factors influencing the rate of MT synthesis, are not well understood. The objectives of this study are to investigate the effects of biotic factors (size, sex, growth rate, nutritional state, prey type) and abiotic factors (temperature, Cd or oxidative exposure) on MT induction in *Nucella lapillus* and *Littorina littorea*, and to evaluate the usefulness of applying MT as a monitoring tool. In this study, total MTs in tissue samples were quantified using the silver saturation method.

2) Induction of MT was monitored in *N. lapillus* during and after exposure to Cd. *N. lapillus* were exposed to 500 μg Cd l^{-1} (2.2% of 96h LC_{50}) for 60 d and then placed into clean seawater for 110 d. The concentration of MT in the whole animal increased during the exposure period, peaked at Day 70, and then declined gradually. The half-life of MT was ca. 40 d. Cd concentration increased throughout the period of exposure and while in clean seawater, levelling off only after Day 120, indicating that Cd concentration could not be regulated by *N. lapillus*. Highest MT induction and Cd accumulation were found in the Leiblein gland of *N. lapillus*, suggesting that measurement of MT induction in this tissue may prove useful as a sublethal biological response to Cd contamination.

3) The combined effects of Cd and water temperature on the oxygen consumption rate (MO_2) and biochemistry of fasted *N. lapillus* were investigated. Inhibition of MO_2 by Cd increased with increasing temperature and decreasing animal size. Cd exposure caused significant reductions in glycogen concentrations in *N. lapillus* at both temperatures (5 & 10°C). Cd-exposed *N. lapillus* showed significantly higher MT concentrations in the Leiblein gland at 10°C but not at 5°C, indicating that MT synthesis is temperature dependent. Reduction in MO_2 may be directly linked to Cd-induced mucus production, structural damage to gills and reduction in oxygen carrying capacity of haemocyanin. However, metabolic depression, including low MO_2, glycogen stores and activity in Cd-exposed *N. lapillus*, may be a strategy to minimise the uptake and toxicity of Cd, and energy expenditure to spare energy reserves for detoxification and maintenance.

4) The influences of nutritional state and prey type on the survival, growth, Cd accumulation, MT induction and glycogen stores in *N. lapillus* were studied. Prolonged starvation and Cd exposure synergistically reduced the survivorship of *N. lapillus*, but feeding could help *N. lapillus* to combat Cd toxicity and minimise mortality. Extended fasting also caused tissue wastage, leading to higher concentrations of Cd and MT in tissues, whereas fed animals increased in weight and had lower Cd and MT concentrations because of the tissue dilution effect. Prey type significantly affected growth rate of *N. lapillus* and indirectly influenced Cd accumulation, MT induction and glycogen stores. Eating mussels promoted better growth and higher glycogen reserves than eating barnacles. Individual growth rate decreased with increasing Cd accumulation. Cd-exposed survivors grew faster and consumed more than control animals, implying that these survivors may have better fitness and greater tolerance to Cd toxicity.

5) Investigation of the effect of hydrogen peroxide (H_2O_2), and the combined effect of H_2O_2 and Cd on MT induction and condition index (CI) in *N. lapillus* was conducted. Exposure to either Cd or H_2O_2 alone induced synthesis of MT or MT-like proteins in *N. lapillus*. Exposure to high H_2O_2 (1000 ppm) alone or combined with Cd, and exposure to Cd (0.50 ppm) or H_2O_2 (2.0 ppm), resulted in significant weight loss, indicated by a reduction of CI. However, CIs of *N. lapillus* exposed to 0.5 ppm Cd + 2.0 ppm H_2O_2 or 0.25 ppm Cd + 2.0 ppm H_2O_2, were similar to that of the control suggesting that Cd antagonistically reduces toxicity caused by H_2O_2 since Cd-induced MT may have a protective function against hydroxyl radicals.

6) The influences of animal size on concentrations of metals and MT in resident *L. littorea* were investigated. Different sizes of *L. littorea* were collected from four areas (Dunoon, Gourock, Largs and Loch Fyne) in the Firth of Clyde, Scotland. Concentrations of MT, Cd and Zn (μg g^{-1} dry soft-body weight) generally decreased with an increase in size of *L. littorea*. MT concentrations were better correlated with Cd than with Zn or Cu concentrations. Nevertheless, MT and the metals in *L. littorea* (μg $individual^{-1}$) increased significantly with increasing size. Concentrations of MT and the metals among the sampling areas were compared at a standardised soft-body weight. The problems and differences in using different variables for size-standardisation were discussed.

7) The marine environments of Gourock and Largs were contaminated with significantly higher TBTs, Pb and Zn than Loch Fyne, as indicated by the results of imposex indices, and metal concentrations in transplanted Chelex® 100 and *Mytilus edulis*. However, the concentrations of metals and MT in *N. lapillus* displayed a very different pattern. These differences can be attributed primarily to differences in *N. lapillus* growth rate between sites. Largs *N. lapillus* grew slowly, whereas Gourock animals grew faster, and had higher CI and RNA/protein ratio in the foot muscle than those in Loch Fyne, especially at small sizes. Differences in growth rate may be due to differences in prey availability, predation pressure, and/or genotype. Fast growing Gourock animals showed lower metal concentrations because of a tissue dilution effect. In contrast, higher levels of MT, Cd and Cu in *N. lapillus* from Largs can be attributed to their growth rate being slower than the rate of metal accumulation. Slow growing animals in Loch Fyne had relatively high MT, Cd, Pb and Zn although Loch Fyne has been regarded as a clean reference site. These results demonstrate that inter-site differences in growth rate can confound the use of *N. lapillus* as biomonitors of metals.

8) In conclusion, quantification of MTs in biomonitors is a promising monitoring tool for indicating metal bioavailability, and to a lesser extent for toxicity. However, water temperature and biological data including animal size, growth rate, condition index, food type and availability, and reproductive status are essential for interpretation of MT data, and should be incorporated into the monitoring program. The recent advancement in analytical methods for quantifying different MT-isoforms in tissue samples of molluscs will enhance the usefulness of MTs as biomarkers of trace metal contamination in the near future.

CHAPTER 1

General Introduction

ECOTOXICOLOGY

Following post-war industrialisation, the global population has increased markedly since 1930, from 2 billion to over 6 billion at present, and it may grow to 8.9 billion by 2050 (United Nations, 1998). Due to rapid development and extensive use of synthetic chemicals, and a significant increase in waste disposal and sewage discharges, there has been a growing concern about the harmful effects of chemicals upon human health as well as upon natural ecosystems, especially in developed countries. In 1969, the term 'ecotoxicology' was first introduced by a toxicologist, Truhaut, who recognised the importance of investigating the fate and toxic effects of natural or artificial substances on living organisms in ecosystems (Truhaut 1975, 1977). This relatively new branch of science also includes studying the interaction of these substances with the physical environment in which these organisms live.

Ecotoxicology is a young science compared to mammalian toxicology, whose long history evolved from pharmacology (Rand et al. 1995). The differences between classic mammalian toxicology and ecotoxicology have been summarised by Rand (1991) and are listed in Table 1.1. Ecotoxicology, unlike mammalian toxicology, is concerned with toxic effects of chemicals upon diversified species within an ecosystem including invertebrates (they cover 95% of overall diversity in animal kingdom). Interaction between chemicals and/or environmental factors (e.g. salinity, temperature) which may influence the accumulation and toxicity of the chemicals must be considered; and fundamental mechanisms of the toxic effects may differ among species. All these uncertainties and knowledge gaps within the context of ecotoxicology, demand research studies to improve our present understanding of ecological risk of the chemicals in the environment, and to allow precise prediction for the future that will be beneficial to environmental management.

Table 1.1. Comparisons between mammalian toxicology and ecotoxicology

Mammalian toxicology	Ecotoxicology*
Objective: to protect humans from exposure to toxic substances and materials at concentrations which are or may be associated with adverse effects	Objective: to protect populations and communities of many diverse species from exposure to toxic substances and materials at concentrations which are or may be associated with adverse effects
Must almost always rely on animal models (e.g., rat, mouse, guinea pig, rabbit) since experimentation with human is not feasible	Can experiment directly on species of concern (although there may be uncertainty on whether the most appropriate "indicator" or "sensitive" species is used)
Species of interest (man) is known; thus degree of extrapolation is more certain	Not able to identify and test all species of concern; thus, degree of extrapolation is uncertain. Organism responses and toxicity may be different in more complex natural systems because of bioavailability of chemical, organic matter concentrations and other environmental interactions
Test organisms are homeothermic or warm-blooded (body temperature is relatively uniform and nearly independent of environmental temperature); thus, toxicity is predictable	Test organisms (aquatic) live in a variable environment and most are poikilothermic or cold-blooded (body temperature varies with the environmental temperature), birds and aquatic mammals being the exception; thus toxicity may not be sufficiently predictable
The dose of a test chemical usually can be measured directly and accurately, and may be administrated by a number of routes. However, unless "absorbed dose" measurements are made via tissue dosimetry, the typical LD50 (e.g. oral bolus) estimate is an external or exposure dose	The external or exposure "dose" is known in terms of the chemical's concentration in a medium (typically water, but also sediment and/or food) and the length of exposure to it; the actual "absorbed dose" is often determined now experimentally using bioconcentration, bioaccumulation and metabolism studies
Extensive "basic" research has been conducted; emphasis has been on understanding mechanisms of toxic action	Much less "basic" research has been conducted, as emphasis has been on measuring toxic effects and generating media-based threshold concentration data, with an eye toward regulatory needs. More recently, emphasis has been on mechanisms of action and structure-activity relationships
Test methods are well developed, their usefulness and limits well understood	Many commonly used test methods are relatively new and some are formalised (standardised). However, their usefulness in many cases at predicting field impacts and protecting natural ecosystems is often uncertain

*Organisms can include aquatic and terrestrial species including plants, invertebrates, fish, birds, and mammalian wildlife. Adapted from Rand (1991).

BIOMONITORING OF AQUATIC ENVIRONMENTS

In the late 1960s and early 1970s, researchers began to study the possibilities of using organisms to monitor conservative contaminants in aquatic ecosystems (Butler 1969, 1971, 1973; Haug et al. 1974; Phillips 1976a, b). Later, the organisms employed in such monitoring programmes were termed as 'biomonitors' in order to avoid the confusion with the term 'bioindicators' which indicates the structure and health of a community in an ecosystem by their presence or absence (Phillips and Rainbow 1993). Phillips (1990) refined the concept and basic criteria for an ideal biomonitor species.

The use of biomonitors to quantify the degree of contamination of aquatic environments has a number of theoretical and practical advantages over the analysis of either natural waters or sediments in monitoring programmes (Phillips and Rainbow 1993). Firstly, most biomonitors exhibit contaminant concentrations, which permit relatively simple measurement compared to the techniques for water analysis. Secondly, biomonitors may provide a time-integration capacity as their accumulated concentrations of contaminants reflecting an average of the short-term temporal fluctuations in contaminant abundance in the ambient waters. Thirdly, the most important advantage is that the bioavailabilities of the contaminants are of most concern and can be measured directly using biomonitors.

One of the most widely known developments in biomonitoring is the "Mussel Watch Program" in the USA and later throughout the World. This biomonitoring programme covered radionuclides, heavy metals, stable isotopes of metals, and hydrocarbons in addition to pesticides (Goldberg et al. 1983; Martin 1985; Phillips and Rainbow 1993; Phillips 1995) which allowed us to map the contamination profiles of coastal areas in a global scale. However, much early work in ecotoxicology or biomonitoring was mainly concerned with the detection and determination of chemicals in samples of animals and plants, and seldom investigated the effects of chemicals upon individual organisms, let alone effects upon populations or communities (Walker et al. 1996). Although improvements in analytical techniques facilitated the detection of very low concentrations of chemicals in biota, establishing the biological significance of these residues was a more difficult matter in the 1970s and early 1980s.

ECOTOXICOLOGICAL BIOMARKERS

Fortunately, significant advancements in medical sciences, physiology, biochemistry and molecular biology have occurred during the last 20 years that provide new tools and wider scope for ecotoxicology. In theory, any detrimental effects of chemical exposure will initially manifest themselves at the cellular level where measurement should be made for the greatest sensitivity (Haux and Forlin 1988). Therefore, subcellular or biochemical responses have the advantage that they are specific to a

given group of contaminants and that it is possible to relate the toxic effect to essential cellular processes (Depledge and Fossi 1994).

Ecotoxicological biomarkers have been defined as 'xenobiotically-variations in cellular or biochemical components or processes structures, or functions that are measurable in a biological system or sample' (NRC 1987), or broadly defined as 'biological responses to a chemical or chemicals that give a measure of exposure and sometimes, also, of toxic effect' (Depledge and Fossi 1994). The use of biomarkers in environmental pollution assessment enables monitoring of stress responses ranging from the biomolecular/ biochemical to the population and community levels (Lagadic et al. 1994). The responses of animals to chemical stress segregate along gradients of both toxicological and ecological relevance and of response time (Adams et al. 1989). The biomarker approach was developed initially to chart the responses of individual organisms to increasing pollutant exposure and stress (Depledge 1993; Fig. 1.1). This approach raises the possibility of determining where an organism is located on this continuum and so potentially offer a warning of early, reversible pollution-induced departures from health (Depledge 1993; Depledge and Fossi 1994; Fig. 1.1).

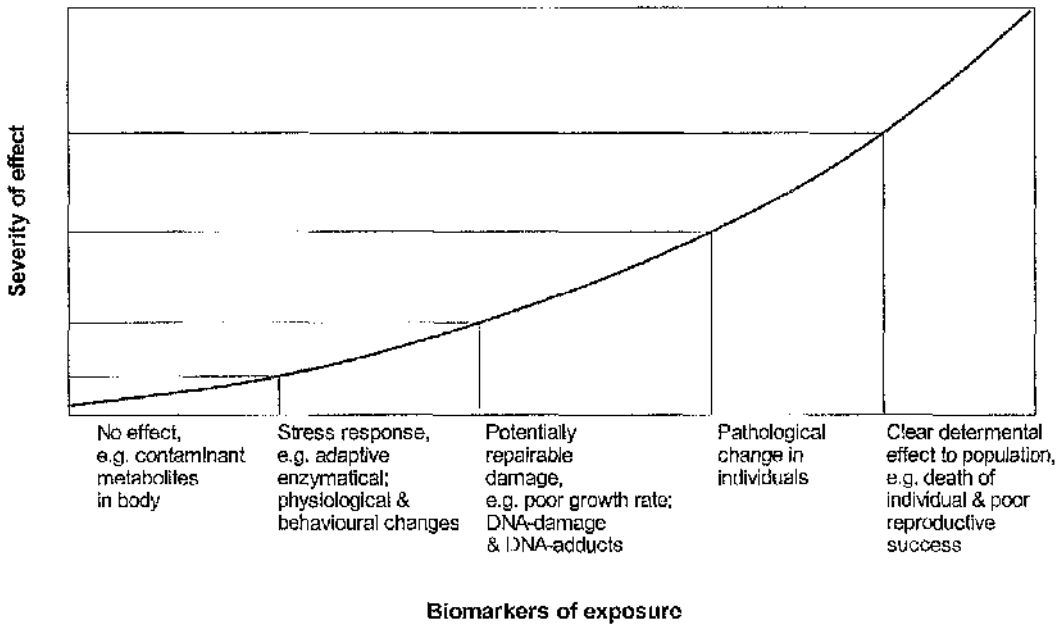

Figure 1.1. The use of biomarkers to assess individual and population stress. (Adapted from Depledge, 1993)

Ecotoxicological biomarkers include molecular, biochemical, physiological, behavioural and pathological stress responses and can be divided into two main groups: general and specific biomarkers. General biomarkers can only indicate that organisms have been exposed to any kind of stress such as heat shock proteins (Mayer et al. 1992). Specific biomarkers are able to indicate exposure and toxic effects caused by specific stressors, for example, metallothionein (MT) generally increases following metal exposure (Benson et al. 1990) and acetyl cholinesterase is inhibited following exposure to organohalogen pesticides (Mayer et al., 1992). Although these biomarkers can indicate an exposure to stress or a specific pollutant, they contribute little to the prediction of the direct consequences for the organism or population. Therefore, a particular biomarker response should, if possible, be related to a given degree of impairment of growth or reproductive output or energy utilisation which directly affects the survivorship of the organism and which can be attributed to exposure to a known amount of the specific pollutant (Depledge and Fossi 1994; Depledge et al. 1995; Fig. 1.2). However, direct linkage of biomarkers with change at population and/or community levels, is poorly documented (Lagadic et al. 1994). Therefore, this invites investigation of the possible linkages or relationships among different levels of biomarkers, for example, an inverse correlation between MT and glycogen stores has been shown in sea bass *Dicentrachus labrax* exposed to Cd (Cattani et al. 1996).

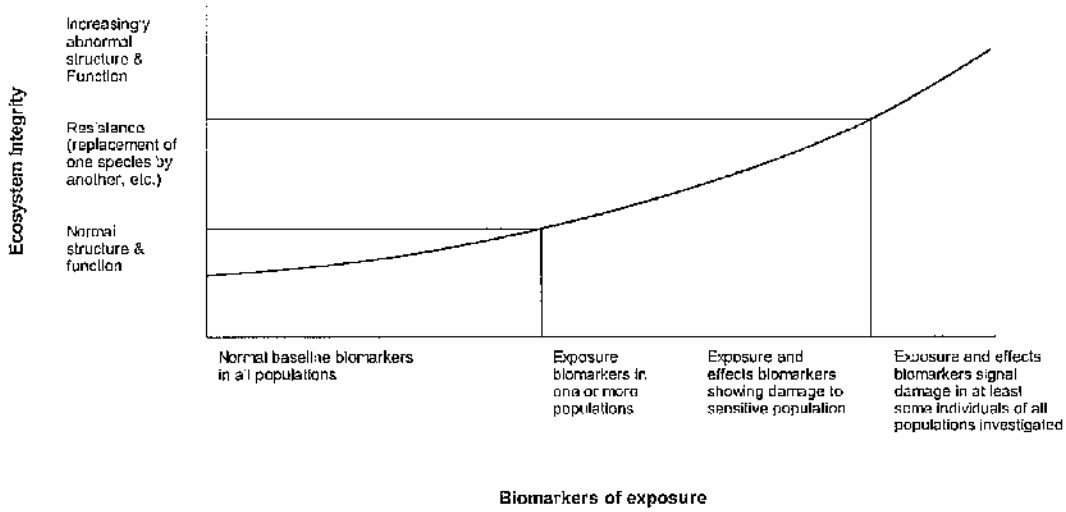

Figure 1.2. The use of biomarkers to assess ecosystem integrity. (Adapted from Depledge and Fossi, 1994)

Many biomarker responses are transient; however, some biomarker responses may persist for weeks or months with continued exposure and others may be detectable throughout the lifetime of the organism (Depledge and Fossi 1994). For example, if a biomarker occurred but then receded despite continued exposure or adverse effects, it may be misinterpreted and lead to the conclusion that the population and indeed the ecosystem were unaffected. To understand the persistence of biomarker response is therefore important to ecotoxicological study.

METALLOTHIONEIN

At present, pollution by metals in the marine environment is generally monitored by measuring the levels of selected metals in biomonitors. However, some of these metals measured may be in the form of a detoxified store, not biologically available or toxic to the animals. For example, a high concentration of Zn is accumulated by barnacles as pyrophosphate-based granules, which are stored permanently in tissues below the midgut, and are not bioavailable or toxic to the barnacles (Pullen and Rainbow 1991). Thus, measurement of metal concentration alone cannot indicate stresses or toxic effects caused by metals.

Metallothioneins (MTs) are a group of low molecular weight, cysteine-rich and heat-stable proteins, which are induced by and bind Cu, Zn, Cd, Hg, Ag and Au[*] (Table 1.2; Roesijadi 1992; Carpenè 1993; Roesijadi 1996; Aspholm and Hylland 1998; Dallinger et al. 1997, Nordberg 1998). The major functions of MT in marine invertebrates include regulation and storage of essential elements such as Cu and Zn, and detoxification of toxic metals such as Cd and Hg by sequestering these metals and inhibiting interactions with sensitive cellular components (Carpenè 1993; Roesijadi 1992, 1996), although there are several other cellular functions of MTs observed in mammals such as serving as an antioxidant (Table 1.3; Nordberg 1998). Two different models for detoxification of toxic metals by molluscs have been proposed by Roesijadi (1996) and Dallinger et al. (1997), respectively (Fig. 1.3). The first model involved Zn/Cd exchange mediated by Zn-MT (Roesijadi 1996), while the second model is based on the idea that specific isoform of MT is induced directly by the corresponding element such as Cd-MT for Cd or Cu-MT for Cu (Table 1.3; Dallinger et al. 1997).

[*] Ag, Au, Cu and Hg are Class B metals, tend to bind with sulphur or nitrogen ligands; Zn and Cd are borderline metals (for details, see Nieboer and Richardson 1980).

Table 1.2. Characteristics of metallothioncin

1.	Soluble intracellular protein
2.	Low molecular weight 6-10kDa, 60-68 amino acids
3.	Cysteine-rich (20-30%), no aromatics or histidine
4.	Unique amino acid sequence
5.	Tertiary structure/metal clusters
6.	High metal content: Cd, Zn, Cu, Hg; 5-10% w/w (or 7 atoms per mole of protein)
7.	Absorption spectra characteristic of metal-thiolate complexes (mercaptides): absorption 250 nm (Cd), 225 nm (Zn), 275 nm (Cu), 300 nm (Hg)
8.	Induced synthesis by Cu, Zn, Cd, Hg, Ag and Au
9.	No disulfide bonds
10.	Heat stability
11.	Consisting of a number of isoforms

Table 1.3. Function of metallothionein

1.	Transport of metals
2.	Detoxification of metals
3.	Protection from metal toxicity
4.	Free radical scavenger
5.	Storage of metals
6.	Metabolism of essential metals
7.	Immune response
8.	Genotoxicity and carcinogenicity

After Nordberg (1998)

In general, MT induction responses follow exposure to trace metals such as Cu, Zn, Cd, Hg and Ag (George 1990; Benson et al. 1990). Cellular toxicity may result if the rate of metal influx into the cell exceeds the rate of MTs synthesis and/or the maximum level of these proteins produced by the cell (Viarengo 1985; Giulio et al. 1995). Therefore, it has been suggested that MTs can be used as indicators of exposure to the MT-inducing metals (Rer 1999; Viarengo et al. 1999). The concentration of MT not only reflects the bioavailable fraction of these metals present in the organism but also indicates the potential toxicity of these metals if the MT concentration is closer to or higher than the threshold value (Fig. 1.4). Nonetheless, an empirical value of such a

threshold for MT may vary among different species, and has yet to be established for marine invertebrates.

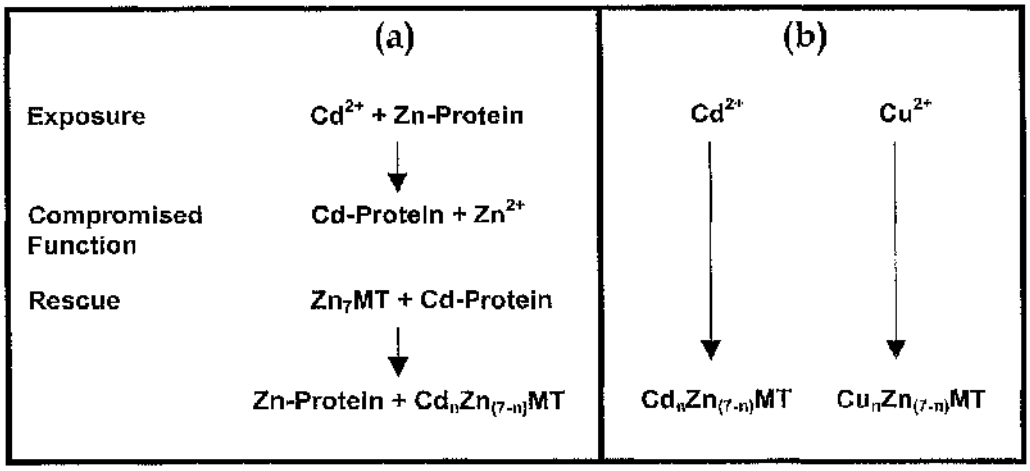

Fig. 1.3. Proposed models for detoxification of toxic metals by metallothionein: (a) model of Roesijadi (1996) and (b) model of Dallinger et al. (1997).

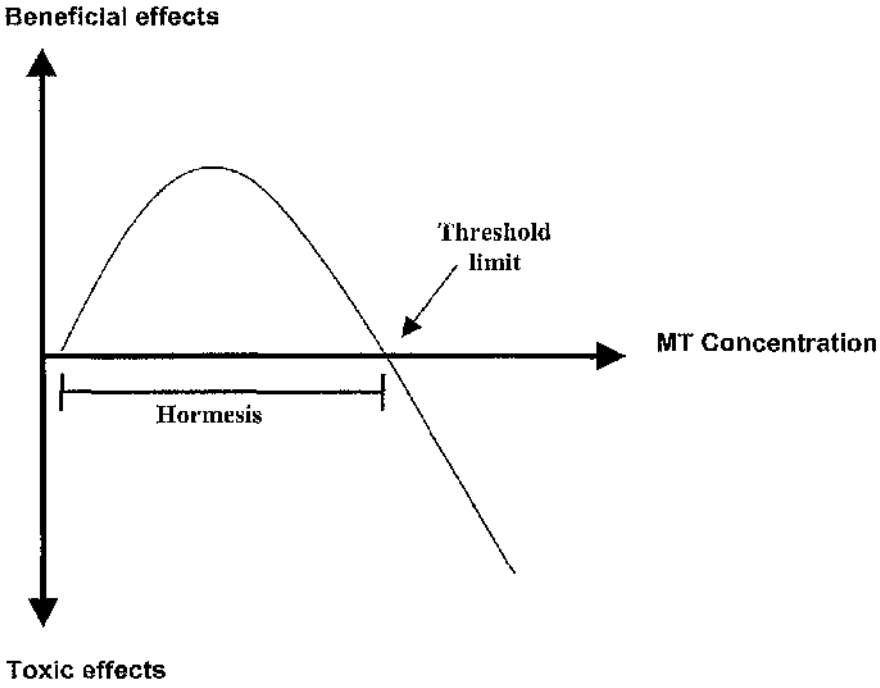

Fig. 1.4. A schematic diagram illustrating the relationship between the concentration of metallothionein in tissues and biological responses (Y-axis) of an animal exposed to MT-inducing metals. Hormesis is the production of beneficial effects in an organism at low exposures and adverse effects at high exposure to a given chemical (i.e. MT or a trace metal) (Rozman & Doull 1999).

Metallothionein Like Proteins

Although many aquatic invertebrate species possess proteins that have features consistent with the MTs (Table 1.2), those from only a few species have been purified and characterised to the extent that has permitted analysis at the structural level (Roesijadi 1992). The structure and configuration of metal binding proteins found in marine invertebrates may differ from the MTs (Table 1.2), with differences in molecular weight, and amino acids composition, particularly the amount of cysteine. Therefore, some authors have suggested that the term MT-like proteins (MTLPs) should be used for those metal binding proteins which exhibit similar properties to the MTs, but have not been structurally characterised (Pavicic et al. 1993; Bordin et al. 1997; Mouneyrac et al. 1998, 2000). In the present study, the term MTLP is used to describe a MT-like protein in dogwhelk *Nucella lapillus* induced by exposure to hydrogen peroxide in water.

Methods for the Quantification of Metallothionein

Classical techniques for the quantification of MT's include metal-saturation assays, polarography, and immunological assays, both radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (Lobinski et al. 1998; Nordberg 1998). Each of these methods has advantages and disadvantages. Metal saturation assays are easy to conduct. These techniques are based on the competitive displacement and the subsequent determination of the initially MT-bound metal (usually Cd and Zn) by a metal with higher affinity to MT (usually Hg and Ag), and depend sometimes on assumption of speculative stoichiometry and lack selectivity with regard to the individual MT-isoforms.

Differential pulse polarography (DPP) has been widely used to quantify MT in marine molluscs (e.g. Bebianno and Langston 1989; Bebianno and Machado 1997; Aspholm and Hylland 1998). However, this electrochemical technique is based on the quantification of MT-fraction via sulfhydryl groups, and may suffer from interferences by other redox systems present in the sample and fail to give information both on the initial metal content and on the isoform composition (Lobinski et al. 1998). The unmatched absolute detection limits by immunological assays (RIA or ELISA) are often observed, because of the difficulties encountered in raising high titers of antibodies, by their length, by an inability to quantify individual MT isoforms in a mixture, and by an

inability to provide information on the original metal composition (Lobinski et al. 1998).

There are new methods still under development such as ELISA incorporated with separation techniques, e.g. isoelectric focusing for quantifying different MT-isoforms (Nordberg 1998); a comprehensive approach based on the coupling of a high resolution separation technique (e.g. size-exclusion chromatography or anion-exchange and reverse-phase HPLC) with an element or species selective detection technique (e.g. inductively coupled plasma emission spectrometry (ICP-ES) or ICP-mass spectrometry (Dallinger et al. 1997; Lobinski et al. 1998). Although all these new techniques may provide detailed information on individual MT isoforms, including metal composition and improve accuracy of MT quantification, they required expensive instruments and trained personnel to operate.

The Silver Saturation Method

The silver saturation method (Scheuhammer and Cherian 1986, 1991) is one of the most simple and cheap techniques for quantifying total MT in tissue samples and is suitable for laboratory comparative physiological and biochemical study. This technique has been demonstrated to be useful and satisfactory to quantify the amount of Cd-MT in crayfish *Procambarus clarkii* and *Artemia* spp. after exposure to sublethal Cd (Martinez et al. 1993; Del-Ramo et al. 1995). In the present study, the silver-saturation method was employed with some minor modifications. Firstly, bovine red blood cell haemolysate was used instead of human red blood cell haemolysate. Secondly, in order to improve the accuracy of the results, an external calibration or standard addition of purified horse kidney MT was made to determine the amount of MT in the sample by the calibration curve rather than calculating the MT values based on the stoichiometric assumption.

Metallothionein in Marine Molluscs

As methods for quantification of MT, especially DPP, have become available to marine biologists, the number of studies on MT induction in marine molluscs, both in laboratory and field, have greatly increased. In Table 1.4, some of the results obtained in various molluscs over the last decade are compiled. All the data were obtained using the DPP technique with mammalian MT as standard, because of the absence of

reference materials specifically made for MT of molluscs. These data indicate that the concentrations of MT in these molluscs are dependent on species, tissue type, animal size, and contamination levels of trace metals, as well as seasons in the field (Table 1.4).

Indeed, variations in MT concentrations as a result of season, reproductive cycle, stressors (including temperature, the presence of organic contaminants, steroid hormone levels, tissue injury) and other factors, might hinder the use of this metalloprotein as a monitoring tool (Phillips and Rainbow 1993; Giulio et al. 1995). For example, Olsson et al. (1987) observed fluctuations in Zn levels associated with MT in female trout during the annual reproductive cycle. Other factors such as stress response, cold, and hypoxia can also induce MT and MT-like proteins (Benson et al. 1990). Therefore, before MT can be used effectively in environmental monitoring and risk assessment, the effects of these factors on MT induction should be established; and its physiological and biochemical functions in the particular biomonitor species under normal conditions must be understood (Giulio et al. 1995).

Metallothionein in Dogwhelk and Periwinkle

The dogwhelk *Nucella lapillus* and periwinkle *Littorina littorea* are common prosobranch molluscs that live on the rocky intertidal shores of Europe and North America. They are commonly used as biomonitors for trace metal contamination (Ireland and Wooton 1977; Gibbs et al. 1991; Evans et al. 1996; O'Leary and Breen 1997). Metallothionein, which has been shown to be present in *N. lapillus* and *L. littorea* respectively, can be induced by exposure to waterborne Cd (Nöel-Lambot et al. 1978, Bebianno & Langston 1989, Bebianno et al. 1992). The biochemistry and biology of Cd-induced MT in *L. littorea* have been extensively studied and are well understood (Langston and Zhou 1986, 1987; Bebianno et al. 1992; Bebianno and Langston 1995, 1998), however, similar knowledge of MT in *N. lapillus* has yet to be established.

Table 1.4. Metallothionein in various marine molluscs. The concentrations are given for the dry weight of whole soft tissues unless otherwise stated.

Species	MT concentration (mg g^{-1})	Source
Gastropods		
Littorina littorea	18.5 (digestive gland)	Langston et al. (1989)
	11.93 (digestive gland); 2.55 (remaining tissues)	Bebianno & Langston (1989)
	8.4 ± 1.3 (digestive gland); 3.3 ± 0.6 (remaining tissues)	Bebianno et al. (1992)
	2.35 ± 0.41 (gills); 3.84 ± 0.55 (kidney)	Bebianno & Langston (1995)
Littorina saxatilis	15.45 (digestive gland)	Bebianno & Langston (1989)
Nucella lapillus	5.29(digestive gland); 1.95 (remaining tissues)	Bebianno & Langston (1989)
Patella vulgata	21.3 (digestive gland); 1.69 (remaining tissues)	Bebianno & Langston (1989)
Bivalves		
Cerastoderma edule	4.55	Bebianno & Langston (1989)
Crassostrea gigas	5-7 (digestive gland)	Imber et al. (1987)
Donax vitatus	6.37	Bebianno & Langston (1989)
Macoma balthica	0.85 ± 0.28 to 7.81 ± 0.19 (depending on size & seasons)	Bordin et al. (1997)
	0.8-12.2 (dependent on size)	Amiard-Triquet et al. (1998)
Mytilus edulis	2-3	Langston et al. (1989)
	2.43; 8.04 (digestive gland)	Bebianno & Langston (1989)
	2.75	Bebianno & Langston (1991)
	5.0-30.0 (digestive gland; depending on size)	Amiard-Triquet et al. (1998)
Mytilus galloprovinciallis	2.1 ± 0.4 (wet wt; digestive gland)	Pavicic et al. (1993)
	2.81 ± 0.66	Bebianno & Langston (1992)
	3.9-13.0 (depending on the degree of metal contamination)	Bebianno & Machado (1997)
Ostrea edulis	0.08 (gonad); 0.04 (gut); 0.01 (muscle)	Alonso & Martin-Mateo (1996)
Ruditapes decussatus	4.29 -6.34 (depending on size)	Bebianno & Langston (1989)
	2.05 ± 0.41; 1.97 (gills); 4.7 (digestive gland); 1.31 (remaining tissues)	Bebianno et al. (1993)
	2.45 ± 0.38 (digestive gland); 1.03 ± 0.22 (gills); 1.96 ± 0.72 (remaining tissues)	Bebianno et al. (1994)
Tridacna crocea	0.08 ± 0.019 to 0.138 ± 0.025 (wet wt, kidney)	Duquesne & Coll (1995)

RATIONALE OF THIS PROJECT

This general introduction highlights the need to understand and assess the biological effects of contaminants of the marine environment using a biomarker approach in biomonitor species. Measurement of the biomarker responses such as MT concentration may subsequently provide information on the toxic effects of metal contaminants on populations from which the individuals have been sampled. However, the MT concentration may be affected by various biotic and abiotic factors, and its responses may vary among different species. Before applying MT as a monitoring tool, the effects of these factors should be fully understood. Furthermore, the relationship and linkage between MT and other biomarkers (e.g. growth, condition index and glycogen stores) are also important and worth exploring, since any association between MT and other biomarkers could enhance the usefulness of MT as a biomarker for toxic effects of metal contaminants.

The rationale behind using Cd as a model element in the present study lies primarily in the fact that Cd is a toxic, non-essential metal (Phillips 1980) and has been shown to induce MT synthesis in various marine invertebrates (Roesijadi 1992). In this study, the characteristics of Cd-induced MT in *N. lapillus*, including the time/response relationships, the longevity and magnitude of the response of Cd-induced MT synthesis have been investigated under controlled laboratory conditions. Various biotic factors (size, sex, growth rate, nutritional state, prey type) and abiotic factors (temperature, Cd or oxidative exposure) on MT induction by *N. lapillus* have also been examined. Further, field experiments using *N. lapillus* and *L. littorea* as biomonitors, have been conducted to validate the laboratory results, and in addition, the effect of animal size on the MT concentrations in wild populations of *L. littorea* has also been examined.

OBJECTIVES AND STRUCTURE OF THE THESIS

The principal aims of this thesis are to investigate the effects of various biotic and abiotic factors on MT induction in two common intertidal gastropods *N. lapillus* and *L. littorea* under laboratory and field conditions, and to evaluate the use of MT as an ecotoxicological biomarker of trace metal contamination and toxicity in these gastropod molluscs.

Each of the chapters (**Chapter 2** to **Chapter 7**) in this thesis has been submitted separately as a scientific paper. These chapters can therefore be read in isolation without the need for cross-reference and the lists of references are presented in the style of the journals to which they were submitted.

The first part of the thesis (**Chapter 2** to **Chapter 5**) consists of laboratory experiments on the effects of Cd and other factors (exposure and depuration, temperature, nutritional state and prey type, oxidative stress) upon MT induction and other biomarker responses of *N. lapillus*. **Chapter 2** reports an acute toxicity study of Cd on *N. lapillus*, and half-life of MT and Cd in fasted *N. lapillus* after being exposed to 0.5 ppm Cd for 60 days and then depurated for 110 days. The concentrations of MT and various metals were also determined in various tissues throughout this time course study, in order to find out the tissues providing the greatest sensitivity and consistency for MT measurement. **Chapter 3** investigates the combined effects of Cd and water temperature on oxygen consumption, Cd accumulation, MT induction and glycogen store in fasted *N. lapillus*. Since it was noted that oxygen consumption and glycogen store significantly decreased in Cd-exposed individuals at 10°C, further investigations on behavioural responses, ultra-structure of gills, and oxygen carrying capacity of haemolymph were conducted with a view to understanding the mechanisms causing the metabolic depression by Cd and establishing any association between MT and other biomarker responses.

During sampling in the field, it was observed that food type and variations in food availability between sites could be essential factors, affecting the growth of *N. lapillus*. As any change in growth rate may eventually affect the concentration of metals in the biomonitor (Phillips and Rainbow 1993; Langston and Spence 1995), nutritional state or diet associated changes in biomass may influence the metal concentrations and thus affect MT concentration. Therefore, **Chapter 4** describes the interacting effects of nutritional state or prey type and chronic Cd exposure on the survival, growth rate and concentrations of Cd, MT and glycogen of *N. lapillus* which were either fasted or fed with barnacles, mussels or Cd-dosed mussels. Recent research in mammalian toxicology has suggested that MT can also be induced by oxidative stress (e.g. H_2O_2) and MT may play a significant role as an antioxidant (Sato and Bremner 1993). If this were true, the use of MT as a specific biomarker for trace metals would be doubtful. This uncertainty invited **Chapter 5** to answer, for the first time (*in vivo* study), whether

exposure to hydrogen peroxide can induce MT in *N. lapillus* and whether Cd-MT has a protective role as an antioxidant.

The second part of this thesis comprises two biomonitoring studies in the Firth of Clyde, west Scotland (i.e. **Chapter 6** and **Chapter 7**). The effects of animal size on MT induction and metal accumulation by *L. littorea* are presented in **Chapter 6** and the problem of using different variables for size-standardisation is also discussed. **Chapter 7** illustrates the effects of *in situ* growth rate on concentrations of metals, MT, glycogen, RNA in *N. lapillus* and provides further evidence that growth rate can be a confounding factor to the use of *N. lapillus* as biomonitors for trace metals and MT. To consider the biotic and abiotic factors investigated in this study and reported in literature, the usefulness and problems of using MT for monitoring the bioavailability and toxicity of metals are discussed and summarised **in Chapter 8**.

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CHAPTER 2

Induction of Metallothionein in Dogwhelk *Nucella lapillus* During and After Exposure to Cadmium*

ABSTRACT

Induction of metallothionein (MT) was investigated in a common biomonitor, the dogwhelk *Nucella lapillus* (shell length: 27.7 ± 1.4 mm; wet tissue weight: 667 ± 196 mg) during and after exposure to cadmium (Cd) under controlled laboratory conditions (10 ± 1°C and 34 ± 1 ‰ salinity). The dogwhelks were exposed to 500 μg Cd l^{-1} (2.2% of 96h LC_{50}) for 60 days and then placed into clean seawater for 110 days. MT concentration in whole animal increased during the exposure period, peaked at Day 70, and then declined gradually. Half-life of MT was ca. 40 days. MT concentration increased very significantly with increasing Cd concentration ($r = 0.74$, $n = 24$, $P<0.001$). Nevertheless, Cd concentration increased throughout the period of exposure and while in clean seawater, levelling off only after Day 120, indicating that Cd concentration could not be regulated by *N. lapillus*. Throughout the study, MT and Cd concentrations in gills, Leiblein gland, kidney, digestive gland and gonad tissues increased gradually. Highest concentrations of MT and Cd were found in the Leiblein gland. Measurement of MT induction in the Leiblein gland of *N. lapillus* may therefore prove useful as a sublethal biological response to Cd contamination.

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INTRODUCTION

Metallothioneins (MTs) are low molecular weight cysteine-rich metal-binding proteins, which normally bind with d^{10} metal ions, such as Zn^{2+}, Cd^{2+}, Hg^{2+} and Cu^{+} (Wang *et al.*, 1996). They are present in many aquatic animal species (Rosijadi, 1993). These proteins have been considered to play a central role in regulation of tissue concentrations of essential metals (e.g. Zn and Cu) and to be involved in detoxification of non-essential highly toxic metals (e.g. Cd and Hg) (Kägi, 1991; Rosijadi, 1992; 1996). In general, MTs vary in amount according to metal exposure levels, and therefore MTs have been regarded as potential specific biomarkers of metal pollution (Benson *et al.*, 1990).

Owing to the ability of marine invertebrates to accumulate heavy metals, they have been widely used to monitor the bioavailability of such contaminants in estuarine or coastal ecosystems (Rainbow and Phillips, 1993). Following the development of methods for MT quantification (Bebianno and Langston, 1989; Scheuhammer and Cherian, 1991; Tissier and Blais, 1996; Viarengo *et al.*, 1997), induction of MT or MT-like proteins has been reported extensively in many marine invertebrates, especially in common biomonitors such as *Mytilus galloprovincialis* (Viarengo *et al.*, 1985; Bebianno & Machado, 1997); *Mytilus edulis* (Bebianno & Langston, 1991), *Littorina littorea* (Bebianno *et al.*, 1992), *Ostrea edulis* (Alonso & Maetin-Mateo, 1996), *Dreissena polymorpha* (Tissier & Blais, 1996) and *Macoma balthica* (Bordin *et al.*, 1997). A recent field study using *M. galloprovincialis* as biomonitor has also demonstrated that measurements of MT can provide an accurate indication of subtle environment increases in metal contamination (Bebianno & Machado, 1997).

However, persistence of biomarker responses (e.g. MT induction) is very important for biomonitoring (Depledge and Fossi, 1994). If a biomarker occurred but then receded despite continued exposure or adverse effects, it may be misinterpreted and lead to the conclusion that the population and indeed the ecosystem, were unaffected. For example, the half-life of MT (25 days) is much shorter than that of residual cadmium in *Mytilus edulis* (300 days) (Bebianno & Langston, 1993). It is therefore essential to determine the persistence of MT in different biomonitors. Although a number of studies have reported significant correlations between concentrations of MT and metal (including Cd, Cu and Zn), only a few studies have documented the half-life of MT in marine organisms (Bebianno & Langston, 1993). It has also been suggested

that major sites for MT induction should be identified in each biomonitor species in order to improve the sensitivity and accuracy of MT detection. For example, Bebianno and Langston (1995) have identified that the major site for MT induction in *Littorina littorea* is the kidney which is very sensitive to MT quantification and thus, more useful in biomonitoring.

The dogwhelk *Nucella lapillus* is one of the most commonly used biomonitors, especially in relation to tributytin contamination (Bailey & Davies, 1991; Gibbs *et al.*, 1991; Evans *et al.*, 1996). Metallothionein-like proteins have been identified in *N. lapillus* exposed to Cd in an early laboratory study using gel-filtration chromatography (Nöel-Lambot *et al.*, 1978). The present study aimed to determine the concentrations of MT and metals (including Cd, Cu, Zn and Fe) in whole body and various tissues of *N. lapillus*; and to estimate the half life of MT and Cd in *N. lapillus*, during and after exposure to Cd under controlled laboratory conditions.

MATERIALS AND METHODS

For an acute toxicity test, dogwhelks (shell length: 29.6 ± 2.8 mm; wet tissue weight: 601 ± 172 mg, mean ± SD) were collected from Gourock, West Scotland, in April 1997. Subsequently, dogwhelks (27.7 ± 1.4 mm; 667 ± 196 mg) were sampled at the same place in July 1997 for the sublethal experiment. In both cases, the animals were acclimated and fasted in natural seawater at 10 ± 1°C and 34 ± 1 ‰ salinity at a constant photoperiod of 12h: 12h (light: dark) for 72 h before experimentation.

A standard acute 96-h toxicity test for *N. lapillus* was performed to determine 96-h LC_{50} for Cd and to indicate an appropriate concentration of Cd used for subsequent experiments. The dogwhelks were separately exposed to nominal concentrations of < 0.01 (control in clean natural seawater), 1.25, 2.5, 5.0, 10.0, 20.0, 30.0, 50.0 mg Cd/litre ($CdCl_2$) seawater for 96 hours in 10-litre Plexiglas tanks. Two replicate groups of 20 animals were put into each concentration. In each tank, animals were kept inside a plastic cage to prevent them escaping from the seawater. The natural seawater contained < 0.01 mg/litre of Cd, Zn and Cu. Temperature and salinity were identical to those in the acclimation period. pH was kept at 7.8 ± 0.1 while dissolved oxygen was maintained at above 80% of saturation by aeration. For each tank, seawater was changed every 24 hours. Behaviour and mortality were observed and recorded twice

daily. Death was defined as a failure to respond to probing with forceps. All gastropods were fasted during the entire exposure period.

To investigate MT induction and Cd accumulation, dogwhelks (n = 360) were separately exposed to either < 0.01 mg Cd/L (control) or 0.5 mg Cd/L (Treatment). The concentration of Cd used for the treatment was selected based on results of the acute toxicity test (2.2% of 96-h LC_{50}). Three replicates were applied in each group. Each replicate contained 60 animals, which were enclosed in a plastic cage submerged into 10-litre seawater with specified Cd concentration. Seawater was changed once every four days in both control and treatment. After 60 days exposure to Cd, both of the treatment and control groups were transferred to 30-litre fibre-glass tanks with flow through seawater for another 110 days. The environmental conditions were identical to the acute toxicity test throughout the experiment (i.e. temperature: 10 ± 1°C; salinity: 34 ± 1 ‰; photoperiod: 12-h light: 12-h dark; pH: 7.8 ± 0.1; and dissolved oxygen: > 80% of saturation). For each replicate, four dogwhelks were collected at Day 1, 12, 24, 36, 60, 70, 120 and 170; dissected; pooled; and analysed for MT and metal concentrations in whole body tissue. For either control or treatment group, three dogwhelks were collected from each replicate at Day 1, 36, 60 and 170, pooled and dissected into six parts: gills, Leiblein gland, kidney, digestive gland, and gonad tissues for the MT and metal analysis.

Before dissection, shell length of each individual animal was measured with a calliper (accuracy: ± 0.5 mm). Fresh weight of whole body tissue was measured by electronic balance after blot-drying and expressed as mg. Condition index (CI) was calculated by dividing fresh weight by the shell length and expressed as milligrams per millimetre.

For determination of MT contents, tissue samples were weighed and placed in a homogenising tube with a solution of 0.25 M sucrose in 1:4 w/v ratio. The mixture was homogenised using an Ultraturax (T25 Janke & Kunkel, IKA Labortechnik) at 4°C. The homogenate (~10g) was centrifuged at 20,000g for 20 min at 4°C. Aliquots of 600 µl supernatant were analysed for MT content by the silver saturation method of Scheuhammer and Cherian (1991) with small modifications. Samples were incubated with 0.5 ml of 20 mg/litre silver solution for 20 min at 20°C to saturate the metal binding sites of MT. Excess silver ions were removed by the addition of 100 µl bovine red blood cell hemolysate to the assay tubes followed by heat treatment in a water bath (100°C for at least 10 min). The heat treatment caused precipitation of silver-bound

haemoglobin and other proteins, except for MT, which is heat stable. The denatured proteins were removed by centrifugation at 1,200*g* for 10 min. The hemolysate addition, heat treatment and centrifugation were repeated three times in each sample. Finally, the supernatant was centrifuged at 20,000*g* for 10 min. The amount of silver metal in the final supernatant fraction which was proportional to the amount of MT present was determined by atomic absorption spectrophotometry using a Philips PU9200 A.A.S with deuterium background correction. Assay tubes containing purified horse kidney metallothionein obtained from Sigma Chemicals in a range of concentrations from 2 to 20 μg underwent the same process in order to establish a calibration curve (μg Ag ml^{-1} vs. μg MT ml^{-1}) for MT quantification. The MT concentrations were expressed as micrograms per gram dry body weight.

Table 2.1. Comparison of metal concentrations (μg g^{-1} dry weight) in standard reference material DORM-1 certified by the National Research Council of Canada, and analytical results from the current study*

Metal	Certified values	Current study values (n = 8)
Cd	0.086 ± 0.012	0.076 ± 0.042
Cu	5.22 ± 0.33	4.47 ± 0.90
Fe	63.6 ± 5.3	65.2 ± 7.1
Zn	21.3 ± 1.0	26.8 ± 6.3

* Mean ± 95% confidence interval.

The water content of the homogenate of whole body tissue was obtained by weight difference after drying at 80°C for 48 h. The dry weight of the sample was then calculated as the difference between total dry weight and weight of sucrose added in the homogenate. Samples of 0.1-0.5 g dried tissue, were acid-digested in 10 ml concentrated nitric acid on a hot plate, by first soaking at 20°C for 24 h, then boiling for at least 2 h until clear solution could be obtained. Samples were diluted to 25 ml using distilled water. Metal concentrations were analysed using the Philips PU9200 A.A.S. Detection limits in the digested sample were 0.014 μg/g for Cd, 0.010 μg/g for Zn, 0.035 μg/g for Cu, and 0.010 μg/g for Fe. All metal concentrations were expressed on a dry weight basis. The reproducibility and accuracy of this method was tested by analysing a reference material, dogfish muscle (DORM-1) from the National Research Council, Canada (Table 2.1).

The 96-h LC_{50} for Cd was estimated following Møller *et al.* (1996). Differences in concentrations of MT, Cd, Cu, Zn, or Fe in whole body tissue between control and treatment were compared using two-way analysis of variance (ANOVA), with a subsequent comparison between individual means using Tukey-Kramer Multiple Comparisons Test (Zar, 1984). Correlations between concentrations of MT and various metals in whole body tissue were determined using Pearson correlation analysis and, the results were corrected with a sequential Bonferroni test (Rice, 1988). Differences in CI between groups were analysed by Analysis of Covariance (ANCOVA) using time as a covariate. Statistical significance was defined as $P < 0.05$.

RESULTS

Acute Exposure

The standard acute 96-h toxicity test for *N. lapillus* indicated that 96-h LC_{50} for Cd was 23.2 mg Cd/litre (Fig. 2.1). Secretion of mucus, egestion and closing operculum were behaviours observed in *N. lapillus* during exposure to high Cd levels (Table 2). However, no observable behavioural effect was found at 1.25 mg Cd/litre and below. A sublethal concentration of 0.5 mg Cd/litre, which was selected for the subsequent experiment based on these results, represents 2.2 % of the LC_{50} value and falls within the no observable effect range (0-1.25 mg Cd/litre).

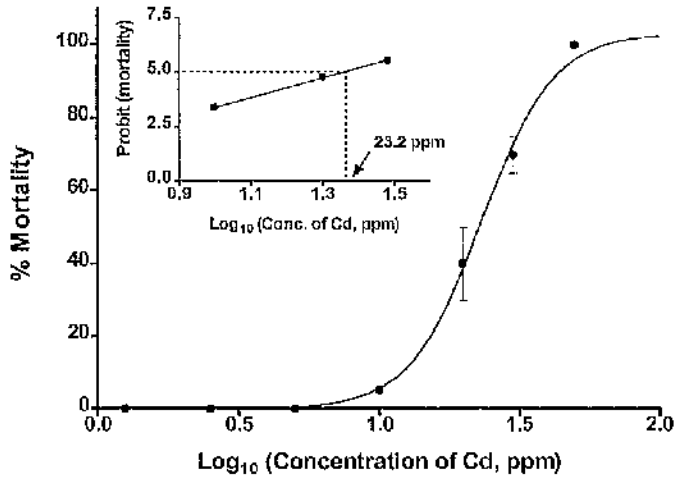

Figure 2.1. Results of the standard acute toxicity test for *Nucella lapillus*. Means and SD are provided. The value of 96-h LC_{50} for Cd is estimated using Probit Analysis and presented in the small figure.

Table 2.2. Effect of Cd on behavioural responses in *Nucella lapillus*

Concentration of Cd (ppm)	Behavioural responses			
	Day 1	Day 2	Day 3	Day 4
<0.01	N	N	N	N
1.25	N	N	N	N
2.5	N	N	N	O
5.0	N	N	O	•
10.0	N	O	•	•
20.0	O	•	•	•
30.0	•	•	•	•
50.0	•	All death	All death	All death

Note. N, normal; O, secretion of mucus, egestion and closing operculum; •, less active and less sensitive to stimulation by forceps.

Metallothionein and Metals in Whole Body Tissue

Metallothionein concentrations in whole animal tissue were significantly higher in the treatment compared with the control after Day 24 and peaked at Day 70 (3035.0 ± 239.5 μg/g dw; mean ± SD) and then declined gradually along with the depuration period ($P < 0.05$; Fig. 2.2; Table 2.3). Concentrations of MT in the control were constant throughout the study with a mean value 768.7 ± 105.4 μg/g dw (± SD), reflecting the background MT levels in this group of *N. lapillus*. Also, across individuals in the treatment group, concentrations of MT increased according to the Cd concentrations in whole body tissues ($r = 0.736$, $n = 24$, $P < 0.001$; Table 4). After depuration for 110 days (i.e., Day 170), there was a decrease in concentration of MT to 1845.8 ± 379.0 μg/g dw, although significantly higher than in the control ($P < 0.05$). Based on the rate of decline in MT concentration in clean seawater, the half-life of MT in *N. lapillus* was approximately 40 days. Nevertheless, Cd concentrations increased in the treatment and levelled off only after Day 120, indicating that Cd concentration could not be regulated by *N. lapillus* ($P < 0.05$; Fig. 2.3; Table 2.3).

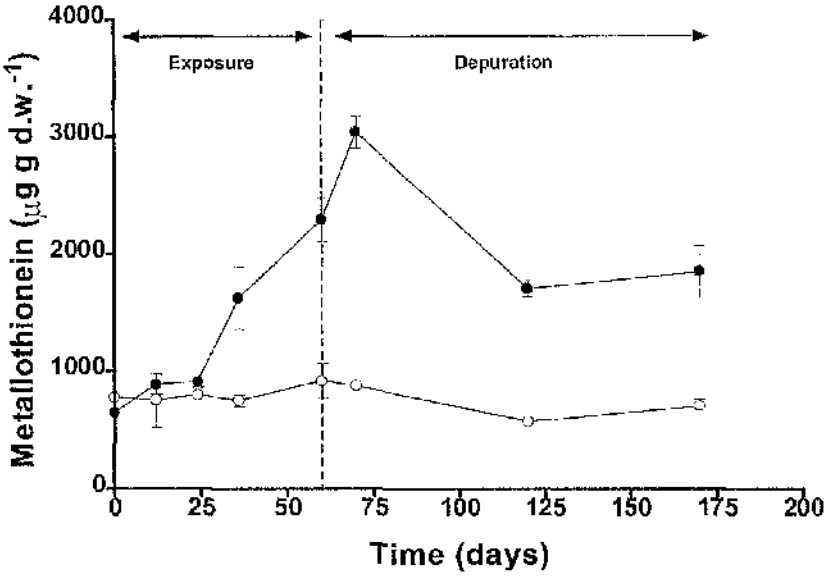

Figure 2.2. Concentrations of metallothionein within whole body tissue of *Nucella lapillus* during and after exposure to Cd (solid circle), and compared with the control (open circle). Means and SD are given (n = 3; however, each sample is a pool of four animals).

Table 2.3. Results of two-way ANOVA

Effects	F(df1,df2) value	*P* value	
Time	$F_{7,32}$ = 21.16	<0.001	***
MT	$F_{1,32}$ = 172.89	<0.001	***
MT × Time	$F_{7,32}$ = 17.80	<0.001	***
Time	$F_{7,32}$ = 42.14	<0.001	***
Cd	$F_{1,32}$ = 891.96	<0.001	***
Cd × Time	$F_{7,32}$ = 40.54	<0.001	***
Time	$F_{7,32}$ = 1.92	0.100	
Cu	$F_{1,32}$ = 0.90	0.350	
Cu × Time	$F_{7,32}$ = 0.36	0.917	
Time	$F_{7,32}$ = 2.31	0.050	
Fe	$F_{1,32}$ = 1.88	0.180	
Fe × Time	$F_{7,32}$ = 0.46	0.858	
Time	$F_{7,32}$ = 5.16	0.000	***
Zn	$F_{1,32}$ = 9.94	0.004	**
Zn × Time	$F_{7,32}$ = 3.13	0.012	*

Note. Levels of significance are indicated by asterisks (*P<0.05; **P<0.01 and ***P<0.001)

Table 2.4. Correlation matrices of time (days), MT, and individual metal concentrations (μg g^{-1} dry weight)

	MT	Cd	Cu	Fe	Zn
Control (n = 24)					
Time	-0.263	0.488	0.365	-0.103	0.416
Zn	0.195	0.725***	0.424	0.381	
Fe	0.382	0.494	0.059		
Cu	0.354	0.604*			
Cd	0.209				
Treatment (n = 24)					
Time	0.480	0.853***	0.286	-0.021	0.759***
Zn	0.227	0.688***	0.415	0.097	
Fe	-0.125	-0.036	0.130		
Cu	0.350	0.462			
Cd	0.736***				

Note. Values of correlation coefficient (r) are presented. Levels of significance are indicated by asterisks (*p<0.05 and ***p<0.001) after correction with a sequential Bonferroni test.

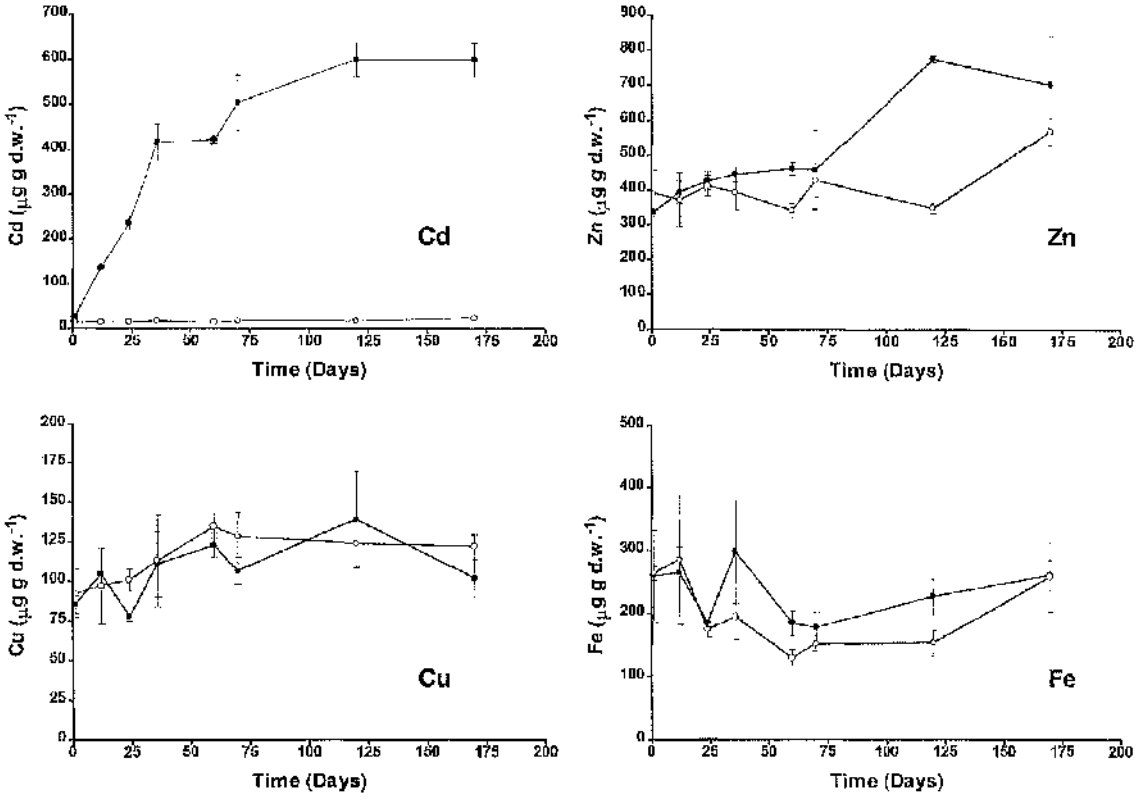

Figure 2.3. Concentrations of individual metal within whole body tissue of *Nucella lapillus* during exposure to Cd between Day 0 and Day 60, and depuration between Day 60 and Day 170 (solid circle), and compared with the control (open circle). Means and SD are presented (n = 3, however, each sample is a pool of four animals).

No significant difference in Cu and Fe concentrations within whole animal was found between the experimental and control groups (Fig. 2.3; Table 2.3). Zinc increased significantly in the treatment group during the experimental period (Table 2.4), and compared to controls, significantly higher levels of Zn were observed in the treatment group at Day 120 ($P < 0.05$; Fig. 2.3; Table 2.3). In addition, significant positive correlations were also observed between Cd and Zn concentrations in both groups (Table 2.4). These results suggest that Cd may enhance the accumulation of Zn in *N. lapillus* through MT production.

Metallothionein and Metal in Various Tissues

Comparing with the control, MT increased markedly in gills, Leiblein gland and gonad tissues 36 days after exposure to Cd (Fig. 2.4). However, a shift of MT levels was observed in the treatment at Day 60; decreases of MT were found in the gills, Leiblein gland, and gonad tissues, whereas increases of MT were observed in the kidney and digestive gland (Fig. 2.4). This shift of MT levels may be explained by the transference of Cd from the gills and Leiblein gland to these two tissues (Fig. 2.5). At the end of the depuration period, the order of the MT levels was ranked as follows: Leiblein gland > gonad > kidney = digestive gland > gills > other tissues (Fig. 2.4).

Considerably different distributions of MT were shown in the control (Fig. 2.4). Highest MT was found initially in the gonad, followed by the Leiblein gland tissues. Although there was no significant reduction of MT concentrations within whole body tissue of *N. lapillus* in the control group throughout the experimental period, lower MT levels were observed in all tissues at the end of 170 days starvation period (Fig. 2.4). This may be explained by protein catabolism occurring in *N. lapillus* to meet their energy expenditure under prolonged starvation.

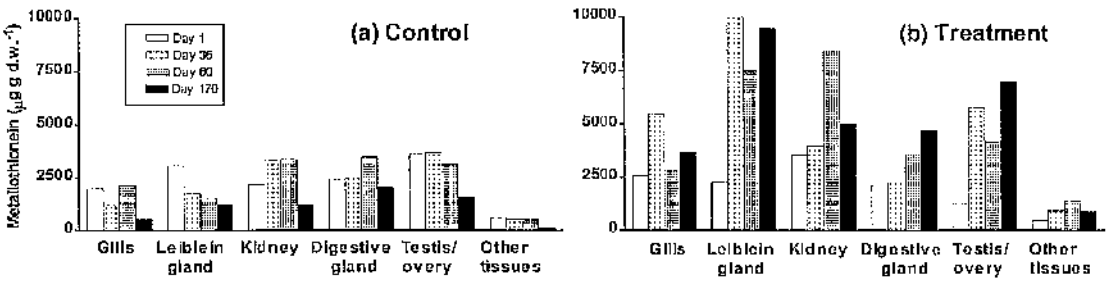

Figure 2.4. Concentrations of metallothionein in various tissues of *Nucella lapillus* during exposure to Cd between Day 0 and Day 60, and depuration between Day 60 and Day 170 (b), and compared with the control (a). Bars indicate the mean values pooled from nine animals.

Cadmium increased in all tissues of *N. lapillus* exposed to Cd throughout the study (Fig. 2.5). Highest concentrations of Cd were accumulated in the Leiblein gland in both treatment and control groups. High Cd concentrations were observed in the digestive gland of the treatment group (Fig. 2.5). At the end of the depuration period, the order of Cd concentrations in different tissues was similar in both groups (Leiblein gland > digestive gland > gonad > kidney > gills > other tissues; Fig. 2.5). Moreover, similar patterns of Cu and Fe distributions were observed in both groups, while, on the contrary, in the Leiblein gland, Zn increased in the treatment group but decreased in the control group throughout the study period (Fig. 2.5). Also, levels of Zn remained higher in the Leiblein glands compared to the control after Day 21, supporting the idea that Cd-induced MT might also bind to Zn and thus store metals in these tissues. In both groups, Cu showed highest concentrations in the digestive gland followed by the gonad, while high levels of Zn and Fe were found in the Leiblein gland generally (Fig. 2.5).

Condition Index

Variations in CI were analysed using ANCOVA, and indicated that the CI values decreased very significantly in both groups ($P < 0.001$; Fig. 2.6; Table 2.5). These results indicated that tissue weight of *N. lapillus* was lost throughout the study due to starvation. Cd exposure, however, caused a significantly greater decline of CI value during the exposure period compared to that of the controls ($P < 0.01$; Fig. 2.6; Table 2.5).

Table 2.5. Results of ANCOVA on condition index

Effects	F(df1,df2) value	*P* value	
Exposure			
Effect of Time	$F_{1, 116} = 5.41$	0.022	*
Effect of Cd	$F_{1, 116} = 12.77$	0.001	**
Interaction	$F_{1, 116} = 6.34$	0.013	*
Depuration			
Effect of Time	$F_{1, 68} = 5.29$	0.025	*
Effect of Cd	$F_{1, 68} = 1.70$	0.196	
Interaction	$F_{1, 68} = 0.73$	0.397	
Entire Period			
Effect of Time	$F_{1, 188} = 50.90$	<0.001	***
Effect of Cd	$F_{1, 188} = 11.07$	0.001	**
Interaction	$F_{1, 188} = 3.32$	0.070	

Note. Levels of significance are indicated by asterisks (*p<0.05, **p<0.01, and ***p<0.001).

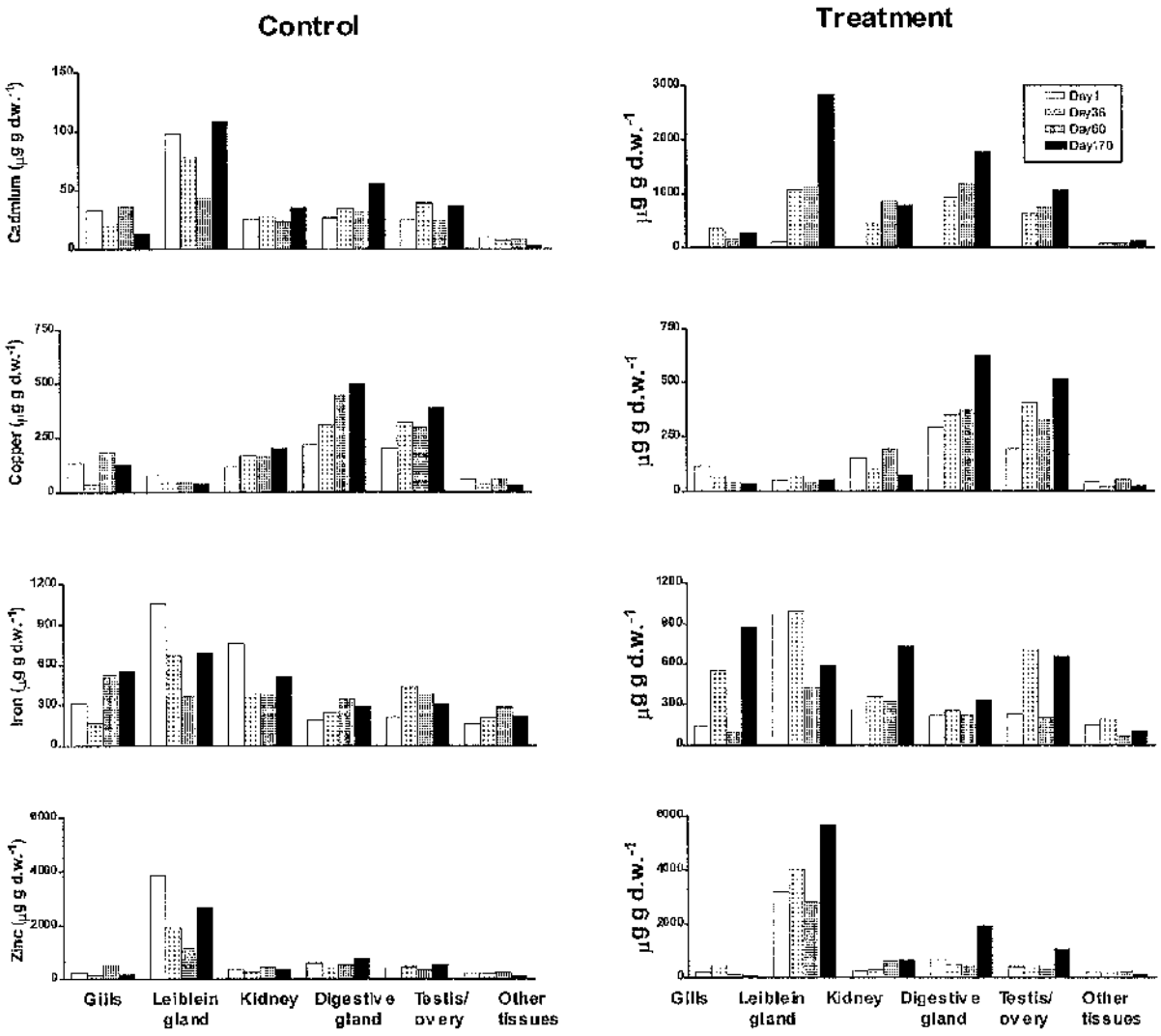

Figure 2.5. Concentrations of individual metal in various tissues of *Nucella lapillus* during exposure to Cd between Day 0 and Day 60, and depuration between Day 60 and Day 170 (right), and compared with the control (left). Bars indicate the mean values pooled from nine animals.

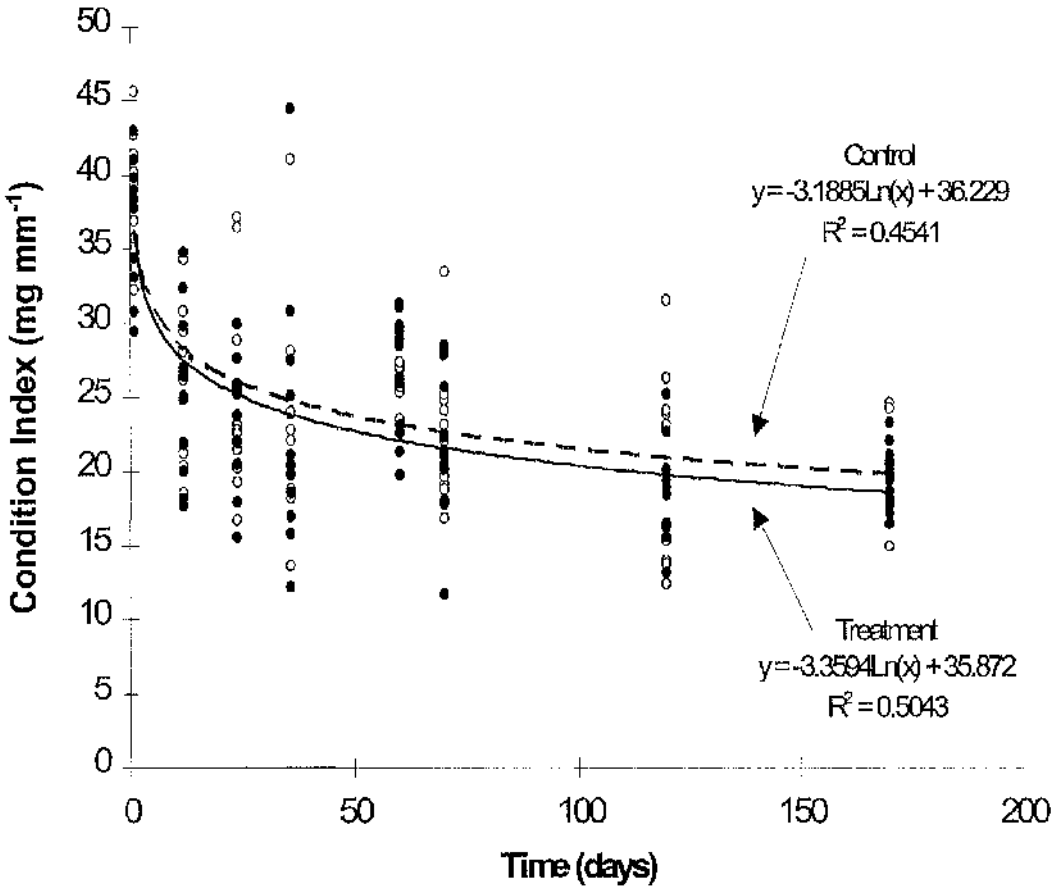

Figure 2.6. Condition Index of *Nucella lapillus* during the entire experimental period. Results are fitted with non-linear regression models. Treatment and control are indicated by closed circle (solid line) and opened circle (dashed line), respectively.

DISCUSSION

Secretion of mucus in mussels, *Perna viridis* and *Septifer virgatus*, has been regarded as an important route for removal of copper (Sze and Lee, 1995). The behavioural changes, including secretion of mucus, egestion and closing operculum, observed in *N. lapillus* during exposure to high Cd concentrations might all be considered as defence mechanisms to reduce Cd uptake. However, the changes in behavioural response in *N. lapillus* are only observed in Cd concentrations > 1.25 mg Cd/litre, suggesting that

these behavioural responses are not relevant as protection against Cd uptake at low concentrations. Therefore, it has been suggested that physiological, biochemical and molecular responses can provide better more sensitive indications of stress in invertebrates associated with the level of contamination in marine environments (Depledge and Fossi, 1994).

The present results confirm the induction of metallothionein in dogwhelks exposed to cadmium, inferred in earlier work on this species (Nöel-Lambot *et al.*, 1978). The pattern of MT induction by Cd in *N. lapillus* is similar to results obtained from laboratory studies on mussels, *M. edulis* and clams, *Ruditapes decussata* (Bebianno and Langston, 1993; Bebianno *et al.*, 1994). The half-life of MT in *N. lapillus* (40 days) is longer than that for *M. edulis* (exposure to 0.4 mg Cd/litre for 18 days with half-life 25 days; Bebianno and Langston, 1993) and *R. decussata* (exposure to 0.1 mg Cd/litre for 40 days with half-life 20 days estimated approximately in digestive gland; Bebianno *et al.*, 1994), suggesting that turnover rate of MT in *N. lapillus* is slower compared with these bivalve molluscs. In general, MT turnover rate is faster in vertebrates compared to marine invertebrates (Bebianno and Langston, 1993). Longer MT turnover rates in marine invertebrates provide longer detectable periods for the changes in MT levels due to the contaminants in marine environments, and therefore, provide further support for the use of MT as biomarkers in these species.

Although the half-life for Cd in *N. lapillus* was not calculated because of the lack of data after Day 170, it was certainly much more than 110 days (the entire depuration period in the present study). This result suggests that Cd concentration cannot be regulated by *N. lapillus*. Half-life of Cd in *M. galloprovincialis* and *M. edulis* has been estimated at approximately 120 days and 300 days, respectively (Bebianno and Langston, 1993; Viarego *et al.*, 1985). As with these mollusc species, turnover of MT in *N. lapillus* is much faster than Cd turnover.

Yang and Thompson (1996) observed that Cd-induced MT can also bind endogenous Cu and Zn in the mussel *Perna viridis*. The present results suggest that Cd may enhance the accumulation of Zn in *N. lapillus* through MT production. However, further investigation on the Zn and Cd contents in the heat stable cytosolic proteins fraction is required to test this hypothesis.

Greater induction of MT and higher accumulation of Cd in the Leiblein gland, followed by the digestive gland, gonad, and kidney, suggest that MT in these tissues is involved in the detoxification of Cd. However, of these tissues, only the Leiblein gland

can be dissected easily from the body of dogwhelk without contamination from other tissues. Bibianno and Langston (1995) have identified the problems of using digestive glands from *L. littorea* for MT quantification, including (1) the presence of heat-stable, high-molecular-weight, thiol-containing proteins in the digestive gland that may interfere with the assay for MT quantification and (2) net induction of MT in response to Cd exposure is small and difficult to detect, relative to the inherently high levels present in this tissue. For the digestive gland of *N. lapillus*, differences in MT levels between the control and treatment were also small (Fig. 2.4) and may suffer the same problems found in *L. littorea* (Bebianno & Langston, 1995). A recent study on Dab *Limanda limanda* has identified another metal-binding protein, distinct from MT, in ovaries, and although this protein can be affected by Cd exposure, it is not induced by Cd and may be involved in ovary development (Kammann *et al.*, 1996). In addition, composition and weight of the gonad vary with sex and seasonal changes, so that using the gonad of *N. lapillus* for MT measurement is not advocated. Although, MT induction in the kidney of *N. lapillus* responded very significantly 60 days after Cd exposure, MT increased in the Leiblein gland markedly 36 days after the exposure and remained at high levels even after prolonged depuration. Therefore, measurement of MT induction in the Leiblein gland of *N. lapillus* may prove useful in the determination of sublethal biological response to metal contamination.

Although, the level of MT in an organism can indicate metal exposure, and correlate directly to the level of bioavailable trace metals within the organism, the relationship between MT concentration and stress in marine invertebrates is virtually unknown. Undoubtedly, there is a need to study the relationship between MT induction and other stress-biomarker responses before using MT in a monitoring programme. The results of the present study suggest that *N. lapillus* utilises more energy reserves for detoxification mechanisms such as MT production during intoxication. Cattani *et al.* (1996) have also demonstrated that glycogen stores decrease with increasing free glucose and MT in sea bass, *Cicentrarchus labrax*, during exposure to Cd. Therefore, protection against Cd has an energy cost, which can be detected in these experiments, and supports the idea that there is a link between MT and other physiological and biochemical responses.

CONCLUSIONS

Although turnover of MT is much faster than Cd turnover in *N. lapillus*, the half- life of MT in *N. lapillus* is longer than found in bivalve molluscs. Greater induction of MT and higher accumulation of Cd in the Leiblein gland of *N. lapillus*, suggest that measurement of MT induction in the Leiblein gland may be useful as a sublethal biological response to metal contamination. It can be concluded that MT in dogwhelk is a suitable and promising biomarker, indicating the levels of bioavailable metals in the organism and reflecting the levels of metal contamination in the environment. However, more work needs to be done in order to establish relationships between MT induction and various stress responses in the organism (e.g. energy-metabolism and growth).

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CHAPTER 3

Temperature-Dependent Physiological Responses of the Dogwhelk *Nucella lapillus* to Cadmium Exposure*

ABSTRACT

The aim of this study was to investigate the combined effects of cadmium (Cd) and temperature on the physiology (oxygen consumption rate (MO_2)) and biochemistry (glycogen, metallothionein (MT) and Cd concentrations) of fasted dogwhelks *Nucella lapillus* using two Cd Concentrations (<0.01 or 500 μg l^{-1}) and two water temperatures (5 or 10°C). After 20 days of exposure, the MO_2 of dogwhelks of various sizes (12-32 mm in shell length) were measured individually. Analysis of Covariance indicated that dogwhelks exposed to Cd exhibited significantly lower MO_2 at 10°C but not at 5°C when compared with control groups at the same temperature. Multiple regression analysis showed that there were significant relationships among MO_2 (mg g^{-1} d^{-1}), temperature (t, °C) and size (W, g wet soft-body weight), expressed by the following equations: (i), Control: $MO_2 = 0.59\ exp^{(0.16 \pm 0.02)t}\ W^{-(0.61 \pm 0.07)}$ ($r^2 = 0.868$, $P < 0.001$); and (ii) Cd-exposed: $MO_2 = 0.68\ exp^{(0.10 \pm 0.03)t}\ W^{-(0.58 \pm 0.11)}$ ($r^2 = 0.592$, $P < 0.001$). Inhibition of MO_2 by Cd increased with increasing water temperature and decreasing animal size. Cd exposure caused significant reductions in glycogen concentrations in foot muscle and digestive gland at both temperatures. Cd-exposed dogwhelks showed significantly higher MT concentrations in the Leiblein gland at 10°C but not at 5°C, indicating that MT synthesis is temperature dependent. Based on these results, temperature is an important factor affecting toxicity of Cd in *N. lapillus*. Activity of dogwhelks (measured as recovery time from upside-down posture) and the oxygen carrying capacity of the haemolymph, were significantly reduced as a result of Cd-exposure at 10°C. In

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addition, mucus secretion and necrotic cells were observed on the gill surface of Cd-exposed individuals. Therefore, reduction in MO_2 may be directly linked to Cd-induced mucus production, structural damage to gills and reduction in oxygen carrying capacity of haemocyanin. However, metabolic depression, including low MO_2 and activity, in Cd-exposed *N. lapillus* may be a strategy to (i), minimise the uptake and toxicity of Cd; and (ii), minimise energy expenditure to spare energy reserves for detoxification (e.g. MT synthesis) and maintenance (e.g. repair of cellular damage). The results are discussed with reference to the use of metallothionein and glycogen as biomarkers for metal exposure and toxicity in marine molluscs.

INTRODUCTION

Marine intertidal molluscs, often used as biomonitors, experience a wide range of environmental variations in their natural environments. Factors such as temperature, salinity and tidal action may affect the uptake of trace metals and their toxicity (Phillips and Rainbow, 1993). As cold-blooded animals, behaviour and activities of marine invertebrates are heavily influenced by environmental temperature. For example, crawling activities and food consumption rates of dogwhelks, *Nucella lapillus*, are positively correlated with environmental temperature (Lawrence, 1973). Furthermore, increases in water temperature generally give rise to elevated rates of metal uptake and toxicity in marine and estuarine molluscs (McLusky et al., 1986; Phillips and Rainbow, 1993). For example, Cd and Zn accumulation by the oyster *Saccostrea echinata* increased with elevated temperature (Denton and Burdon-Jones, 1981) as did Cd accumulation in the mussel *Mytilus edulis* (Phillips, 1976).

In general, oxygen consumption rate (MO_2) decreases when molluscs are acutely exposed to trace metals such as Cu, Zn, Cd and Hg (Spicer and Weber, 1991). Cheung and Wong (1998) showed a 61-78% reduction in MO_2 in the marine subtidal gastropod *Babylonia lustosa* after exposure to 0.2 ppm Cu for 8 days. The MO_2 of *N. lapillus* declined after exposure to 0.6 ppm Cd for 14 days (Abdullah and Ireland, 1986). The MO_2 of the mud snail *Nassarius obsoletus* was reduced in relation to the concentrations of As, Ag, Cu and Zn but elevated by exposure to Cd (MacInnes and Thurberg, 1973). Although the effect of metals on MO_2 is known to be influenced both by animal size (Cheung and Wong, 1998) and water temperature, none of the previous studies

considered the combined effects of metal, temperature and size on MO_2. In general, MO_2 can be expressed by the following equation:

$$MO_2 = a\ exp^{kt}\ W^b \quad (1)$$

Where MO_2 is the oxygen consumption rate (mg O_2 g^{-1} d^{-1}); W is the wet soft-body weight (g); t is the temperature (°C); a, k and b are constants for slope, temperature and body weight, respectively. In the present study, the MO_2 of *N. lapillus* of different sizes were examined in order to establish the relationship between size, temperature and MO_2; and to investigate how Cd exposure affects this relationship.

Metallothioneins (MTs) are small molecular weight proteins rich in cysteine residues and commonly present in marine molluscs (Roesijadi, 1992; Carpenè, 1993). MTs play a central role in homeostasis of essential metals (e.g. Cu and Zn), acting as metal donors for metalloenzymes, but also having an important role in detoxification of toxic metals (e.g. Cd and Hg) and serving as antioxidants (Carpenè, 1993; Roesijadi, 1992, 1996; Wallace, 1996). Many studies have shown that quantification of MTs in biomonitors can provide an indication of subtle environmental increases in metal bioavailability and toxicity (e.g. Bebianno and Machado, 1997). However, other environmental factors such as water temperature, which can influence metal accumulation and toxicity, may affect the concentrations of MTs in biomonitors (Carpenè, 1993). In rainbow trout *Oncorhynchus mykiss*, the rate of Cd-MT induction is temperature-dependent and decreases with decreasing temperature (Olsson et al., 1996). To date, there is no similar reported study to determine whether MT induction is also temperature dependent in marine molluscs, and to determine the magnitude of any such effect.

It has been suggested that combating the poisoning effects of trace metals is metabolically costly (Calow, 1991). Barber et al. (1990) estimated that metallothionein synthesis represented approximately 5% of total metabolism in metal-challenged daphnids *Daphnia magna*. Sea bass *Dicentrarchus labrax* exposed to Cd showed a decline in glycogen reserves and increase in Cd-MT, suggesting that there is a metabolic cost in combating Cd toxicity (Cattani et al., 1996). With a view to testing this cost hypothesis in *N. lapillus*, levels of glycogen in the digestive glands and foot muscles as well as MT in the Leiblein gland and kidney were quantified.

The dogwhelk *N. lapillus* is one of the most commonly used biomonitors in Europe and North America. This intertidal gastropod has been extensively used as an indicator for imposex associated with tributyltin contamination. Cd can induce MT in various

tissues of *N. lapillus* especially in the Leiblein gland and kidney; and concentrations of Cd are closely correlated with those of MT (Leung and Furness, 1999a). The objectives of the present study include (1) study of the combined effects of Cd and temperature on the physiology (oxygen consumption) and biochemistry (glycogen and MT) of *N. lapillus;* and (2) investigation of possible mechanisms causing changes in oxygen consumption in *N. lapillus* exposed to Cd.

MATERIALS AND METHODS

Experimental Design

Different sizes of dogwhelks, *N. lapillus* were collected from Largs, Firth of Clyde, Scotland and acclimatised to circulating seawater under controlled laboratory conditions (10 ± 0.5°C and 35 ± 1‰; fasted). After 1 wk, the dogwhelks were separated into four groups. The size range was similar among all groups (12-32 mm in shell length, 0.1-1.7 g wet soft-body weight). For each group, 25 animals were kept inside a net cage and submerged in a 1 litre glass tank with 800 ml of filtered (GF/C) seawater (10 ± 0.5°C and 35 ± 1‰). The water was renewed once every 2 days. Two tanks were incubated in a water bath in order to maintain the water temperature at 10 ± 0.5°C while the other two tanks were acclimated in a stepwise manner to 5°C by decreasing 0.5-1°C d^{-1} using a water bath installed with a temperature controller. Two by two experimental design was utilised in the present study (i.e. 2 water temperatures × 2 Cd concentrations). At each water temperature (5°C or 10°C), one group was exposed to waterborne Cd at 500 μg l^{-1} (Treatment: $CdCl_2$ in filtered seawater) and the other exposed to <0.01 μg l^{-1} (Control: filtered seawater) after 1 wk of temperature acclimatisation. Water was renewed once every 2 days. No food was provided during the entire exposure period of 20 days.

Oxygen Consumption Rate

After the exposure period, different sizes of dogwhelks (n = 15 to 16) were randomly selected from each group and used for determination of their oxygen consumption rates. Epifauna were removed from the shells of dogwhelks, followed by washing with filtered seawater. Individuals were placed inside sealed respiratory chambers containing a small magnetic stirrer and oxygen electrode (Strathkelvin

Instruments, Glasgow) and acclimatised for 1 h before the measurement commenced. Each chamber was filled with 219 ml aerated filtered seawater (+ 0.5°C of the desired temperature; 35‰ salinity). All the respiratory chambers were covered with aluminium foil to avoid photosynthesis due to any epi- or endo-microalgae on the shells. The temperature of the metabolism chambers was maintained at ± 0.5°C of the desired temperature using a flow-through water bath with a temperature control device. The experimental conditions were identical to those applied during the exposure period. After acclimation, oxygen levels were monitored continuously for 2-3 h using an oxygen meter (Strathkelvin Instruments, Glasgow) connected to an online computer. A chamber without an animal was used as a control. The oxygen consumption rate was calculated and expressed as mg O_2 per g soft-body weight per day (soft-body weight was determined after dissection). At the end of the experiment, all tested animals together with the remaining animals (n = 25) were removed from their shells using a vice. The soft-bodies were weighed and then dissected into Leiblein gland, kidney, digestive glands, foot muscles and the remaining tissues. They were weighed individually and stored at -25°C to await further analysis.

Cadmium Concentration and Biochemistry

The remaining tissues were dried at 60°C for at least 96 h until constant mass was achieved. They were then digested in conc. HNO_3 for 24 h at room temperature followed by boiling for at least 2 h until a clear solution was obtained. Cd concentrations in the remaining tissues were determined using a Philips PU9200 Atomic Absorption Spectrophotometer (AAS) with deuterium background correction and expressed as microgram per gram dry tissue weight.

The weighed whole Leiblein gland or kidney was homogenised with 0.4 ml of 0.25M sucrose using an Ultraturax (T25 Janke & Kunkel, IKA Labortechnik) at 4°C. The homogenate was centrifuged at 20,000g for 20 min at 4°C. Aliquots of 300 µl supernatant were analysed for MT content using the silver saturation method described by Leung and Furness (1999a). Samples were incubated with 0.5 ml of 20 mg l^{-1} Ag solution and 0.4 ml 0.5M glycine buffer (pH 8.5) for 20 min at 20°C to saturate the metal binding sites of MT. Excess Ag ions were removed by the addition of 100 µl bovine red blood cell haemolysate to the assay tube followed by heat treatment in a water bath at 100°C for ~10 min. The denatured proteins were removed by centrifugation at 3000g for 10 min. The hemolysate addition, heat treatment and

centrifugation were repeated three times for each sample. Finally, the supernatant was centrifuged at 20,000*g* for 10 min. The amount of Ag in the final supernatant fraction, which was proportional to the amount of MT present, was determined by AAS. Assay tubes containing purified horse kidney MT obtained from Sigma Chemicals in a range of concentrations from 2 to 20 μg underwent the same process in order to establish a calibration curve for MT quantification.

For glycogen analysis, 50-100 mg of the digestive glands or foot muscle tissues were dissolved in 0.4 ml 30% KOH 90°C for 30 min. After cooling in ice, 1 ml of absolute alcohol was added to the tissue solution, mixed and kept at 4°C for 2 h. It was then centrifuged at 3,000*g* for 10 min. After removal of the supernatant, the pellet was re-dissolved in 1 ml of distilled water. Subsequently, glycogen concentrations in these solutions were determined in triplicate by utilising the anthrone reagent (Seifter et al., 1950), with comparison against multiple glycogen standards. The results of MT and glycogen were expressed as μg or mg per g of wet tissue weight.

Further Investigations

In order to investigate mechanisms causing reduction in oxygen consumption by *N. lapillus* exposed to Cd, the following experiments and analyses were conducted. Dogwhelks (32.1 ± 1.8 mm in shell length; mean ± SD) were collected from the same area and put through the same exposure regime at 10°C for 20 days and then used for behavioural studies, oxygen carrying capacity of the haemolymph and histology of the gills. For behavioural studies, each dogwhelk (n = 32) was acclimated in a tray with water depth of 15 mm for 30 min (preliminary study showed that recovery time decreased with increasing water depth (unpublished data)). It was then turned upside-down from its normal posture; and subsequent recovery time was recorded.

Thereafter, a haemolymph sample was taken from each individual (n = 10 for each treatment group). Each sample of haemolymph was taken by inserting a 25G needle of a 1ml disposable syringe into the position behind the eyes of the dogwhelk. The sample was centrifuged immediately at 2000*g* for 2 min. Aliquots of 10 μl supernatant were injected immediately into a microrespiratory chamber (RC200, Strathkelvin Instruments, Glasgow) to determine its oxygen carrying capacity according to methods described by Taylor et al. (1996). The oxygen carrying capacity of the haemocyanin was then calculated by subtraction of the physically dissolved fraction. The Cu concentration of the haemolymph was determined using AAS following dilution with

dilute HNO_3 (1% w/v). For *in vitro* studies, 45 µl haemolymph samples were collected from the control animals and added to 5 µl distilled water containing 0, 0.50 and 1.67µg Cd, respectively (equivalent to 0, 10.0 and 33.3 µg Cd ml^{-1} haemolymph). The mixed samples, in triplicates, were allowed to stand for 10 min at 25°C followed by immediate measurement of their oxygen carrying capacity using the microrespiratory chamber. The results were expressed as percentage of mean oxygen carrying capacity in the controls (solution added without Cd).

For histology, gills (n = 3) were dissected and placed in fresh 2% glutaraldehyde in seawater at pH 7.2 for 1 h. The tissue was then washed carefully in filtered seawater in order to remove mucus and any impurity, post-fixed in a solution of 2% osmium tetroxide in seawater (pH 7.2) for 1 h, dehydrated through graded acetone and critical-point dried in carbon dioxide. Each specimen was trimmed and mounted on brass stubs and sputter-coated with gold using Polaron SC515 SEM Coating System. Specimens were then examined using a Philips 500 scanning electronic microscope (SEM) from 6 to 12 kV.

Statistics

Data of MO_2, Cd, MT, glycogen and size (wet soft-body weight) were natural-log transformed. Normality and homogeneity of variances of the data were checked using the normal probability plot and Bartlett's test, respectively. Analyses of MO_2, MT and Cd data were made using analysis of covariance (ANCOVA) using wet soft-body weight as covariate. Multiple regression analyses were performed to test the significance of relationships among (1) temperature, size and Cd concentrations in the remaining tissues using MO_2 as the dependent variable; (2) temperature, concentrations of MT and glycogen in different tissues using Cd concentration in the remaining tissues as the dependent variable. Correlations between different parameters were examined using Pearson's correlation analysis. Differences in concentrations of glycogen in digestive glands or foot muscles between control and treatment at the two water temperatures were compared using two-way analysis of variance (ANOVA), with subsequent comparison between individual means using the Tukey-Kramer multiple comparisons test. The differences in oxygen carrying capacity or Cu content in the haemolymph between the control and treatment were tested using Student's t test. One way ANOVA was also employed to analyse data from *in vitro* study of Cd upon the oxygen carrying capacity of haemolymph, and followed by a

Dunnett multiple comparison test for comparing mean values obtained from each treatment against control values. For the behavioural study, a Chi square test was utilised to compare the number of dogwhelks recovered from the upside-down posture within 2 min, 2-4 min or over 4 min between the treatment and control. Statistical significance was defined as $P < 0.05$.

RESULTS

Cd Induced Reduction in Oxygen Consumption Depending on Temperature

In general, the MO_2 of dogwhelks decreased in relation to size and increased according to temperature (Fig. 3.1; Table 3.1). The results of ANCOVA indicated that dogwhelks exposed to Cd, exhibited significantly lower MO_2 at 10°C but not at 5°C, while comparing with the control groups at the same temperature. The results of multiple regression analysis (Table 3.2) showed that there were significant correlations among MO_2 (mg g^{-1} d^{-1}), temperature (t, °C) and size (W, g wet soft-body weight). The relationships were expressed by the following equations:

(i), Control: $MO_2 = 0.59\ exp^{(0.16 \pm 0.02)t}\ W^{-(0.61 \pm 0.07)}$ ($r^2 = 0.868$, $P < 0.001$); and

(ii) Cd-exposed: $MO_2 = 0.68\ exp^{(0.10 \pm 0.03)t}\ W^{-(0.58 \pm 0.11)}$ ($r^2 = 0.592$, $P < 0.001$).

By comparing these two models, the toxicity of Cd caused a decline of MO_2 in *N. lapillus* especially in small animals and at high water temperatures (Fig. 3.2). For example, for 0.1g (wet soft-body weight) animals, percentage reductions of MO_2 were 20.4% and 40.9% of the control at 5°C and 10°C, respectively, while 1.5g animals showed 13.5% and 36.0% reduction at 5°C and 10°C, respectively.

Table 3.1. Results of ANCOVA on the oxygen consumption rates (MO_2) of *Nucella lapillus*.

Parameter	$F_{df1, df2}$		Sig. of F	
MO_2 at 5 ℃				
Size effect	$F_{1,27}$=	45.149	<0.001	***
Cd effect	$F_{1,27}$=	1.440	0.241	
MO_2 at 10 ℃				
Size effect	$F_{1,27}$=	48.069	<0.001	***
Cd effect	$F_{1,27}$=	15.937	<0.001	***
MO_2 of control				
Size effect	$F_{1,47}$=	78.511	<0.001	***
Temperature effect	$F_{1,47}$=	80.161	<0.001	***
MO_2 of treatment				
Size effect	$F_{1,47}$=	30.555	<0.001	***
Temperature effect	$F_{1,47}$=	14.996	<0.001	***

Table 3.2. Results of multiple regression analyses on the relationship between MO_2 (mg g^{-1} d^{-1}), water temperature (t, °C), and size (w, g wet soft-body weight) of the control and Cd-exposed *Nucella lapillus*, respectively.

Model	B	Standard error of B	t	*P*	
Control: $MO_2 = 0.59 \cdot W^{-0.61} \cdot \exp^{(0.16t)}$ ($r^2 = 0.868$; $F_{2,28} = 92.389$, $P < 0.001$)					
Constant	-0.533	0.141	-3.787	0.001	**
Soft-body wt	-0.605	0.068	-8.861	<0.001	***
Temperature	0.158	0.018	8.953	<0.001	***
Treatment: $MO_2 = 0.68 \cdot W^{-0.58} \cdot \exp^{(0.10t)}$ ($r^2 = 0.592$; $F_{2,28} = 20.332$, $P < 0.001$)					
Constant	-0.382	0.219	-1.747	0.092	
Soft-body wt	-0.580	0.105	-5.528	<0.001	***
Temperature	0.101	0.026	3.873	<0.001	***

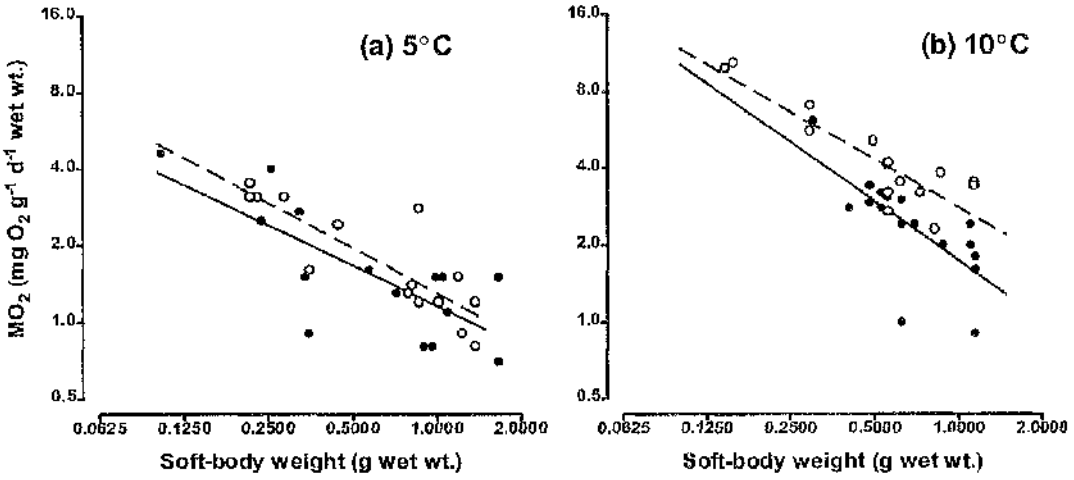

Figure 3.1. The relationship between oxygen consumption rate and wet soft-body weight of control (opened circle) and Cd-exposed (solid circle) *Nucella lapillus* at 5°C (a) and 10°C (b). Significant regression lines ($P < 0.05$) for control (dashed line) and treatment (solid line) are shown.

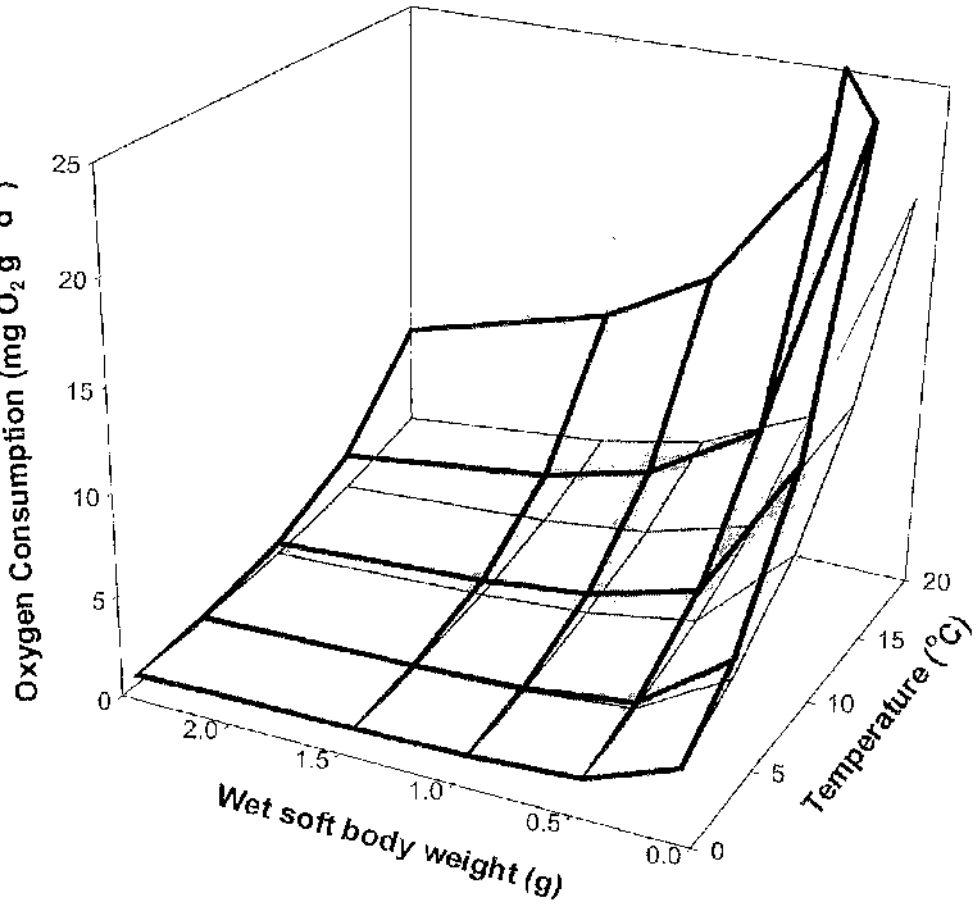

Figure 3.2. A three dimension diagram showing the relationship amongst oxygen consumption rate, water temperature and wet soft-body weight of control (upper surface) and Cd-exposed (lower surface) *Nucella lapillus*.

Cd Accumulation and MT Induction Depending on Temperature

Compared with the control groups, dogwhelks exposed to Cd showed significantly higher Cd accumulation in their remaining tissues at both temperatures (Fig. 3.3; Table 3.3). Cd concentrations in these tissues were similar between the control groups at both temperatures ($P > 0.05$). Within the treatment groups, however, *N. lapillus* accumulated significantly higher concentrations of Cd at 10°C than at 5°C (Fig. 3.3; Table 3.3), indicating that the accumulation of Cd in dogwhelks was dependent on temperature.

MT induction in *N. lapillus* was also significantly affected by temperature. As with MO_2, MT concentrations in the Leiblein gland increased significantly only in dogwhelks exposed to Cd at 10°C but not at 5°C (Fig. 3.4(a), (b); Table 3.4). Although no significant effect of Cd on Cd-MT induction in the kidney was observed at both temperatures (Fig. 3.4(c), (d); $P > 0.05$), the concentrations of MT in the kidney increased with increasing temperature and decreasing size of animal in both control and treatment (Table 3.4).

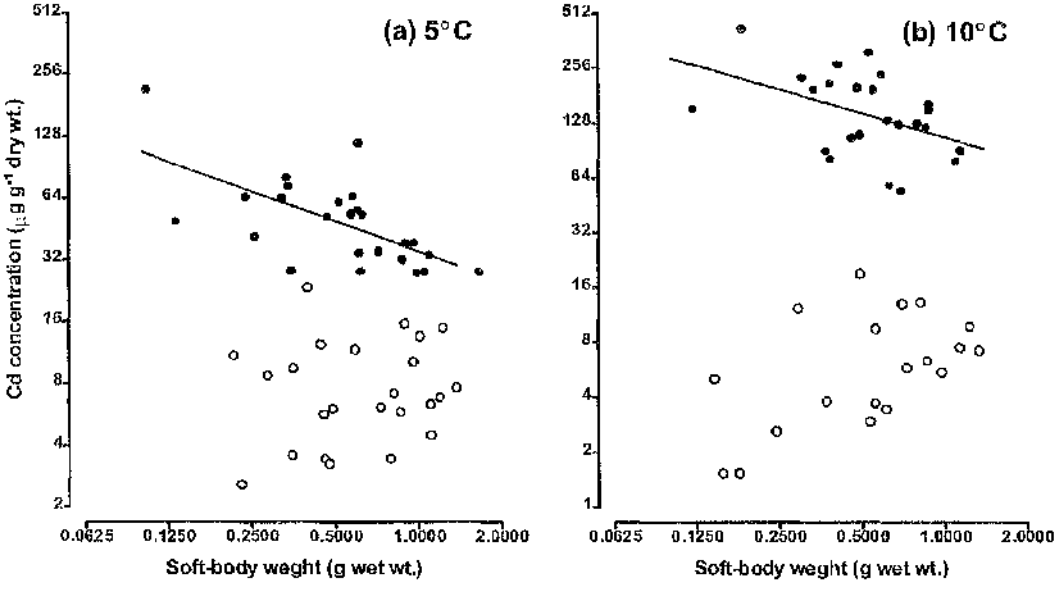

Figure 3.3. The relationship between concentration of Cd in the remaining tissues and wet soft-body weight of control (opened circle) and Cd-exposed (solid circle) *Nucella lapillus* at 5°C (a) and 10°C (b). Significant regression lines ($P < 0.05$) for treatment are shown.

Table 3.3. Results of ANCOVA on the Cd concentrations in the remaining tissues of *Nucella lapillus*.

Parameter	$F_{df1, df2}$		Sig. of F	
Cd in the tissues at 5 ℃				
Size effect	$F_{1,47}$=	3.125	0.084	
Cd effect	$F_{1,47}$=	153.578	<0.001	***
Cd in the tissues at 10 ℃				
Size effect	$F_{1,47}$=	0.211	0.648	
Cd effect	$F_{1,47}$=	291.811	<0.001	***
Cd in the tissues of control				
Size effect	$F_{1,47}$=	1.267	0.267	
Temperature effect	$F_{1,47}$=	1.238	0.273	
Cd in the tissues of treatment				
Size effect	$F_{1,47}$=	19.579	<0.001	***
Temperature effect	$F_{1,47}$=	78.082	<0.001	***

Table 3.4. Results of ANCOVA on the metallothionein (MT) concentrations in the Leiblein gland and kidney of *Nucella lapillus*.

Parameter	$F_{df1, df2}$		Sig. of F	
MT in Leiblein gland at 5 ℃				
Size effect	$F_{1,47}$=	0.051	0.822	
Cd effect	$F_{1,47}$=	0.866	0.357	
MT in Leiblein gland at 10 ℃				
Size effect	$F_{1,47}$=	32.348	<0.001	***
Cd effect	$F_{1,47}$=	11.166	0.002	**
MT in Leiblein gland of control				
Size effect	$F_{1,47}$=	0.376	0.543	
Temperature effect	$F_{1,47}$=	2.440	0.126	
MT in Leiblein gland of treatment				
Size effect	$F_{1,47}$=	11.273	0.002	**
Temperature effect	$F_{1,47}$=	16.054	<0.001	***
MT in kidney at 5 ℃				
Size effect	$F_{1,47}$=	48.024	<0.001	***
Cd effect	$F_{1,47}$=	2.984	0.091	
MT in kidney at 10 ℃				
Size effect	$F_{1,47}$=	120.050	<0.001	***
Cd effect	$F_{1,47}$=	3.669	0.062	
MT in kidney of control				
Size effect	$F_{1,47}$=	45.44	<0.001	***
Temperature effect	$F_{1,47}$=	4.510	0.040	*
MT in kidney of treatment				
Size effect	$F_{1,47}$=	115.951	<0.001	***
Temperature effect	$F_{1,47}$=	55.004	<0.001	***

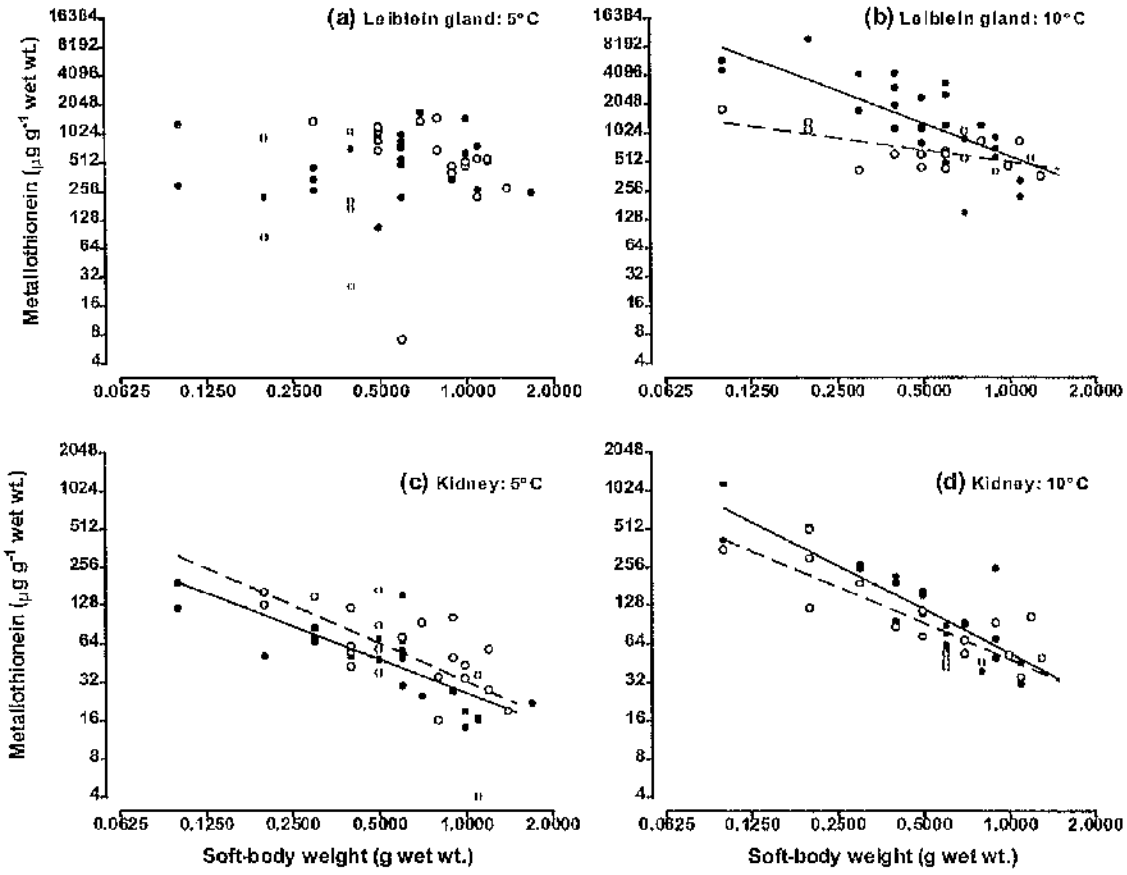

Figure 3.4. The relationship between concentration of MT in the Leiblein gland (a, b) or kidney (c, d), and wet soft-body weight of control (opened circle), and Cd-exposed (solid circle) *Nucella lapillus* at 5°C and 10°C. Significant regression lines ($P < 0.05$) for control (dashed line) and treatment (solid line) are shown.

Cd Induced Reduction in Glycogen Reserves

As there was no significant correlation between size and glycogen concentration ($P > 0.05$), mean values of the glycogen concentrations were used for comparisons. The dogwhelks exposed to Cd had a significantly lower average glycogen concentration in the foot muscles at 10°C (Fig. 3.5(a); ANOVA: $F_{1,96} = 8.434$, $P < 0.01$) and in digestive glands at 5°C (Fig. 3.5(b); $F_{1,96} = 12.450$, $P < 0.01$) than the controls. Significantly higher concentrations of glycogen were observed in the foot muscles of both control and treated dogwhelks at 10°C compared to those at 5°C (Fig. 3.5(a); $F_{1,96} = 10.590$, $P < 0.01$), but not in the digestive glands ($F_{1,96} = 0.184$, $P > 0.05$). Percentage reduction of glycogen content in foot muscle by Cd were 17.5% and 29.2% of the control at 5°C and 10°C,

respectively, while reductions in digestive gland glycogen were 44.7% and 21.4% at 5° C and 10°C, respectively. Correlation analyses carried out for each temperature revealed that glycogen in the muscles or digestive glands decreased with increasing concentration of Cd in the remaining tissues (Fig. 3.6; foot muscles: r = -0.291, P<0.05 (at 5°C) and r = -0.498, P < 0.001 (at 10°C); digestive gland: r = -0.546, P < 0.001 (at 5°C) and r = -0.367, P < 0.05 (at 10°C)). Further ANCOVA using the concentration of Cd in the remaining tissues as covariate, reconfirmed that the concentration of glycogen in these tissues was significantly influenced by the Cd concentration in the remaining tissues; and glycogen concentration in the foot muscles was also affected significantly by temperature (Fig. 3.6(a); Table 3.5). These results suggested that Cd toxicity caused significant depletion of glycogen energy storage in *N. lapillus*.

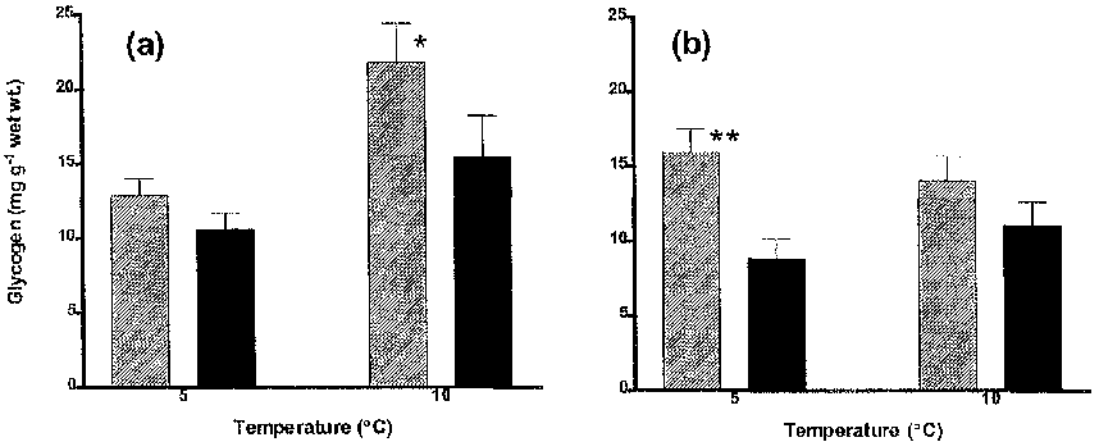

Figure 3.5. Concentrations of glycogen in the foot muscle (a) and digestive gland (b) of control (shaded bar) and Cd-exposed (filled bar) *Nucella lapillus* at 5°C and 10°C. Values are mean ± SD. Significant different means between the control and treatment are indicated by asterisks (*P < 0.05 and **P < 0.01).

Table 3.5. Results of ANCOVA on the glycogen concentrations in the foot muscles and digestive glands of *Nucella lapillus*, using concentration of cadmium in the remaining tissues as covariate.

Parameter	$F_{df1, df2}$		Sig. of F	
Glycogen in the foot muscles				
Effect of Cd concentration in the tissues	$F_{1,47}$=	17.602	<0.001	***
Effect of Temperature	$F_{1,47}$=	13.716	<0.001	***
Glycogen in the digestive gland				
Effect of Cd concentration in the tissues	$F_{1,47}$=	20.355	<0.001	***
Effect of Temperature	$F_{1,47}$=	1.195	0.277	

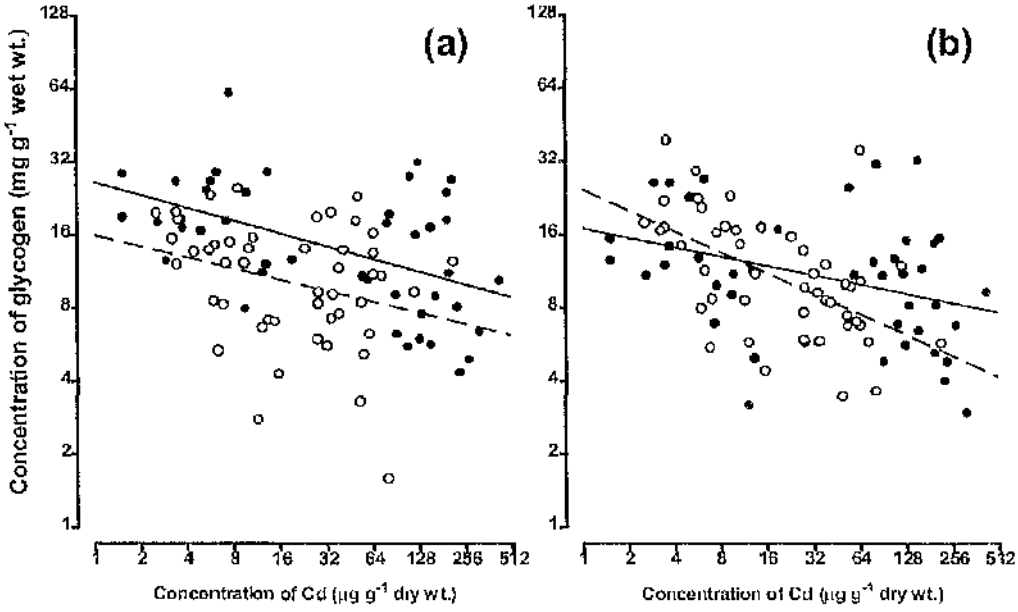

Figure 3.6. The relationship between the concentration of glycogen in the foot muscle (a) or digestive gland (b), and concentration of Cd in the remaining tissues of *Nucella lapillus* at 5°C (opened circle) and 10°C (solid circle). Significant regression lines ($P < 0.05$) for 5°C (dashed line) and 10°C (solid line) are shown.

Relationship between the Parameters

Stepwise multiple regression analyses were conducted in order to investigate the relationships between (1) MO_2, temperature, size and accumulation of Cd in the remaining tissues; and (2) concentrations of Cd, glycogen and MT in the various tissues, and temperature. First, Model I explained 76% of the variation of the data and indicated that MO_2 increased with increasing temperature but decreased with increasing body weight and Cd concentration in the remaining tissues (Table 3.6).

Secondly, Model II explained 83% of variance of the data and suggested that the Cd accumulation increased in relation to the ambient waterborne Cd levels and temperature; and correlated positively with MT in the kidney but negatively with the glycogen concentration of the foot muscles (Table 3.6). Without considering the ambient Cd concentration in seawater, Model III described Cd accumulation as elevated by increasing temperature; increasing Cd accumulation caused a reduction in the glycogen content of the foot muscle/ digestive gland, and an increase of MT in the Leiblein gland, although this model could only explain 36% of variation in the data (Table 3.6).

Table 3.6. Results of multiple regression analyses on the relationships between various parameters.

Model		B	Standard error of B	t	*P*	
I	Overall $MO_2 = 0.79 \cdot C^{-0.10} \cdot W^{-0.63} \cdot \exp^{(0.14t)}$ ($r^2 = 0.758$; $F_{3,58} = 60.632$, $P < 0.001$)					
	Constant	-0.242	0.147	-1.646	0.105	
	[Cd] in the tissue	-0.095	0.027	-3.523	0.001	**
	Soft-body wt	-0.630	0.062	-10.087	<0.001	***
	Temperature	0.137	0.016	8.547	<0.001	***
II	[Cd] in the tissue $= 8.66 \cdot C_w^{0.22} \cdot M_k^{0.26} \cdot G_m^{-0.35} \cdot \exp^{(0.08t)}$ ($r^2 = 0.831$; $F_{4,94} = 107.011$, $P < 0.001$)					
	Constant	2.159	0.390	5.533	<0.001	***
	Cd in water	0.220	0.013	17.544	<0.001	***
	MT in kidney	0.259	0.084	3.068	0.003	**
	Glycogen in f. m.	-0.354	0.116	-3.057	0.019	*
	Temperature	0.080	0.029	2.732	0.008	**
III	[Cd] in the tissue $= 36.23 \cdot G_d^{-0.73} \cdot G_m^{-0.85} \cdot M_l^{0.40} \cdot \exp^{(0.12t)}$ ($r^2 = 0.361$; $F_{4,94} = 12.310$, $P < 0.001$)					
	Constant	3.590	0.968	3.709	<0.001	***
	Glycogen in d. g.	-0.733	0.227	-3.225	0.002	**
	Glycogen in f. m.	-0.848	0.229	-3.701	<0.001	***
	MT in L. g.	0.404	0.133	3.041	0.003	**
	Temperature	0.116	0.055	2.118	0.037	*

Note: MO_2: oxygen consumption rate (mg O_2 g^{-1} d^{-1});
W: soft-body weight (g wet wt.);
t: temperature (°C);
C: Cd concentration in the remaining tissues (μg g^{-1} wet wt.);
C_w: Cd concentration in the water (μg l^{-1});
M_k and M_l are MT in kidney and Leiblein gland respectively (μg g^{-1} wet wt);
G_d and G_m are glycogen in digestive gland and foot muscles (mg g^{-1} wet wt.), respectively.

Results of Further Investigations

Recovery time of dogwhelks from upside-down posture was affected significantly by Cd exposure (Fig. 3.7; $\chi^2 = 8.67$, DF = 2, $P < 0.05$). Median recovery time for control and Cd-exposed groups were 2.42 and 5.55 min, respectively. Hence, Cd exposure generally caused a delay in recovery.

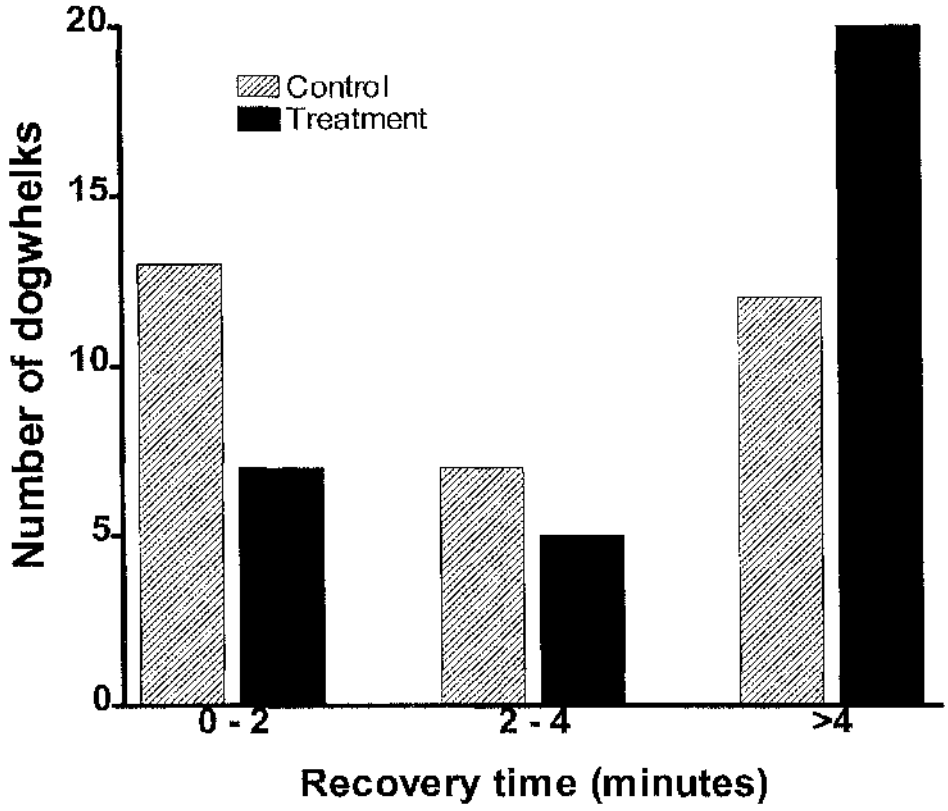

Figure 3.7. A comparison between the time required by control (shaded bar) and Cd-exposed (filled bar) *Nucella lapillus* to recover from the up-side-down position.

At 10°C, the oxygen carrying capacity of the haemolymph was significantly reduced in Cd-exposed dogwhelks (Student's t test: t = 2.93, DF = 8, $P < 0.01$; Fig. 3.8). However, the concentrations of copper in the haemolymph of both groups were not significantly different ($P > 0.05$; Fig. 3.8). *In vitro* biochemical reaction between Cd and haemolymph resulted in a significant reduction of oxygen carrying capacity (ANOVA: $F_{2,6} = 7.49$, $P < 0.05$; Fig. 3.9).

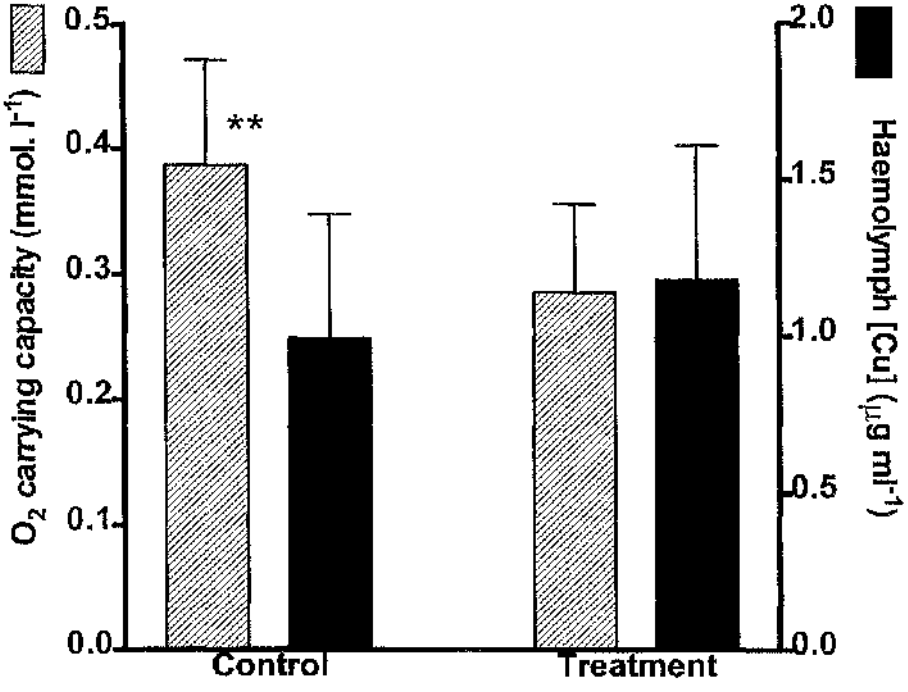

Figure 3.8. Values for the oxygen carrying capacity (shaded bar) and of the copper concentration (filled bar) of the haemolymph obtained from control and Cd-exposed *Nucella lapillus*. Values are mean ± SD. Significantly different means are indicated by asterisks (**$P < 0.01$).

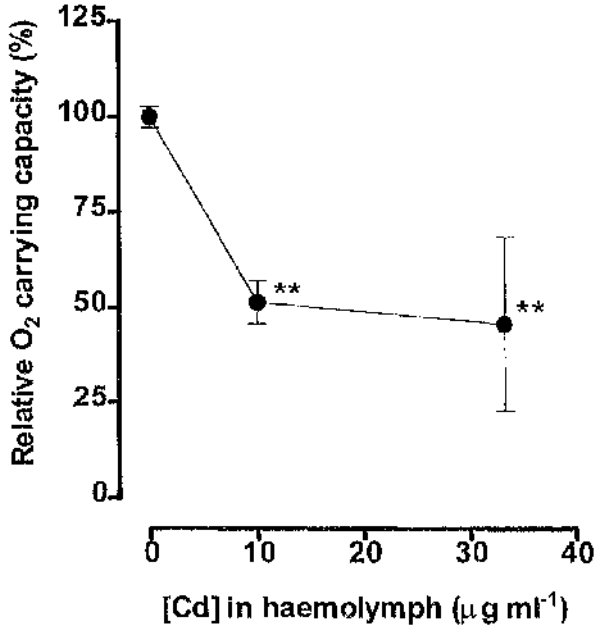

Figure 3.9. Results of an *in vitro* study of the effect of Cd on oxygen carry capacity of the haemolymph obtained from control *Nucella lapillus*. Values are mean ± SD. **Statistically significant from control ($P < 0.01$, Dunnett multiple comparison test).

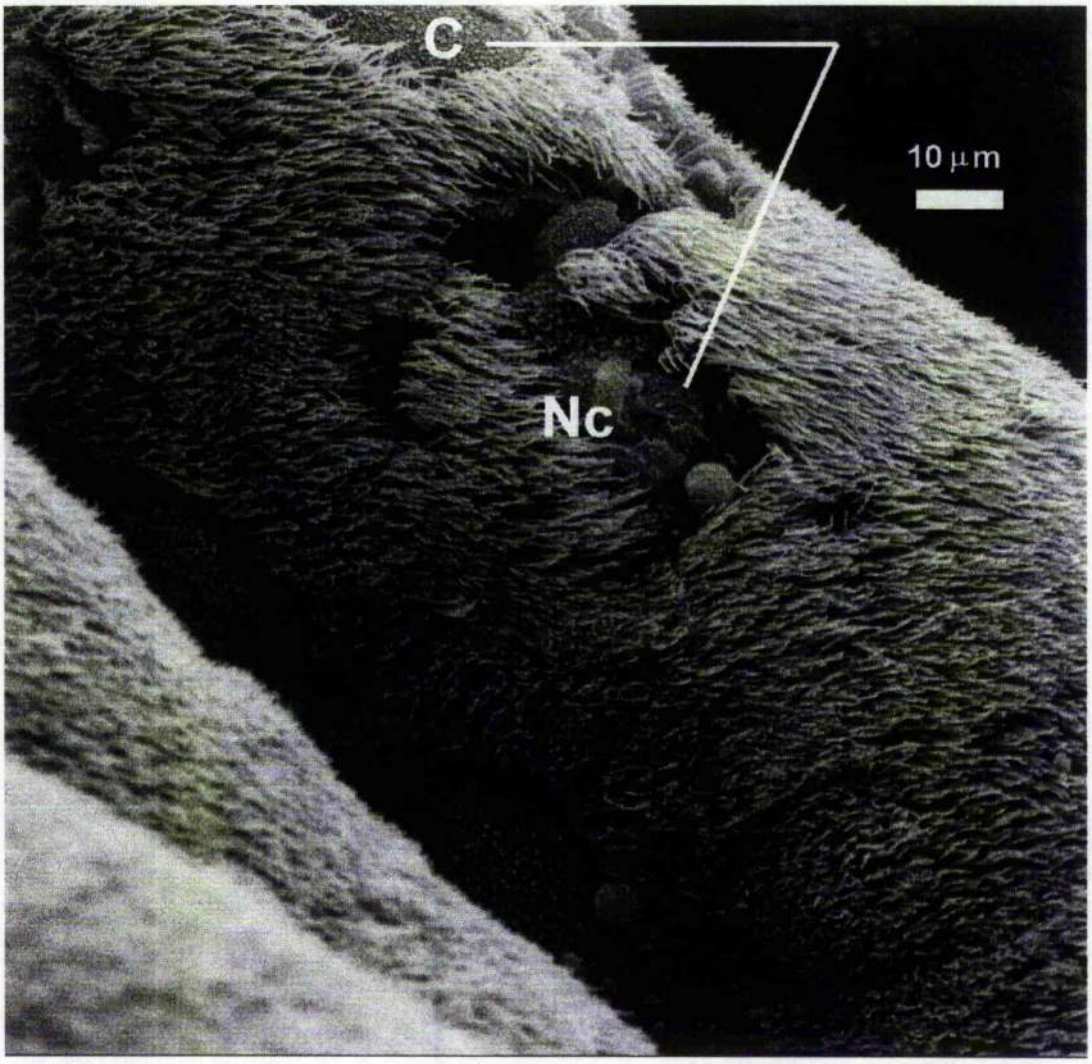

Fig. 3.10. SEM: Gill filament of Cd-exposed *Nucella lapillus*, showing a reduction of cilia (C) and necrotic cells (Nc).

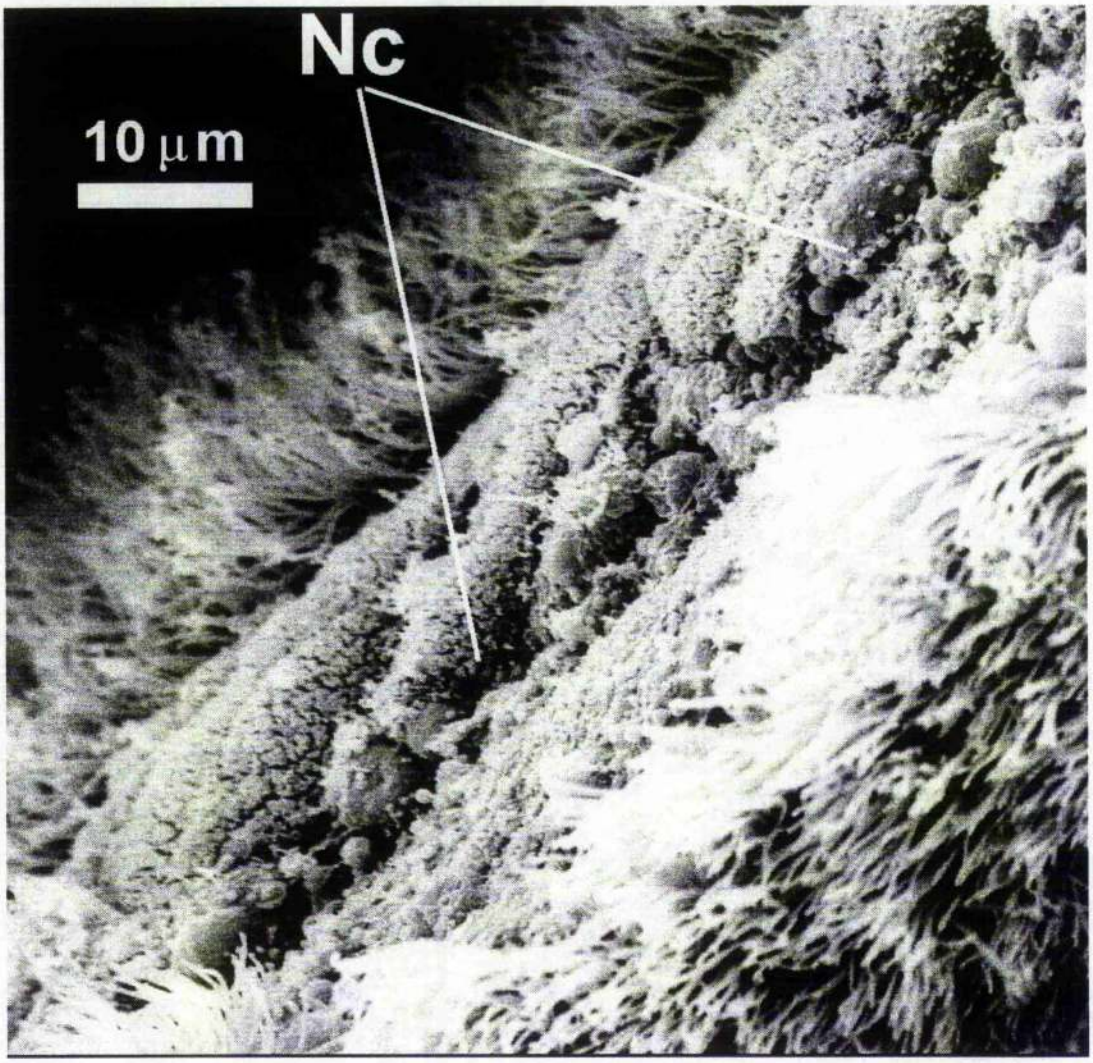

Fig. 3.11. SEM: Gill filament of Cd-exposed *Nucella lapillus*, showing necrotic cells (Nc), their internal organelles spilling into the extracellular space.

During sample preparation for histology, it was noted that the gills of dogwhelks exposed to Cd were covered with a thicker layer of mucus than found in the control specimens. Before fixation, the mucus layer was removed with a view to providing a better observation for the ultrastructure of gill filaments. There were no observable gross differences in the ultrastructure of gill filaments derived from control animals. In the Cd-exposed specimens, however, occasional areas on gill filaments were denuded of cilia (Fig. 3.10). In some cases, some abnormal, perhaps necrotic cells (their internal organelles spilling into the extracellular space) were also observed around these areas (Fig. 3.11).

DISCUSSION

Effects of Temperature on Cd Accumulation and Toxicity

Elevated temperature may not only increase thermodynamically the rate of physiochemical reaction in the aquatic medium (including the rate of binding of a free metal ion to a membrane transport ligand, leading to a consequent increase in metal uptake), but will also increase the rate of physiological processes such as metabolism, ventilation, and heart beat and these may enhance the uptake of trace elements (Phillips and Rainbow, 1993). In the present study, increased temperature resulted in an elevation of the metabolic rate (reflected by oxygen consumption) in *N. lapillus* and enhanced accumulation of Cd. An increased oxygen consumption resulting from an increase in temperature implies higher ventilation and metabolic activities, leading to higher Cd uptake through gill and body surfaces of the dogwhelks. *N. lapillus* exposed at 500 μg Cd l^{-1} for 60 days at 10°C, showed no reduction of Cd in their body tissues after 110 days of depuration, suggesting that the dogwhelks cannot regulate Cd (Leung and Furness, 1999a). Although increased temperatures may also increase the excretion rate in *N. lapillus*, any effect on the excretion of Cd might be minimal.

Zafarullah et al. (1989) carried out an *in vitro* study using cell lines of rainbow trout, *O. mykiss*, and showed that increased MT concentrations due to induction by Zn could be detected after 6 h at 20°C but required 192 h at 6°C. A recent *in vivo* study showed that liver MT levels of *O. mykiss* exposed to 100 μg Cd l^{-1} did not increase above control levels as the temperature dropped from 15°C to 2°C after 6 months, however, after a further 2 months of stabilisation at 2°C, the liver MT levels were significantly higher in

Cd-exposed fish (Olsson et al., 1996). Similarly, in the present study, a significant elevation of MT in the Leiblein gland of *N. lapillus* exposed to Cd was only observed at 10°C but not at 5°C, after 20 days of exposure. Based on these results, it is evident that MT induction is a temperature dependent process.

The magnitude and rate of MT induction in marine gastropods also varies in different tissues or organs (Bebianno and Langston, 1995, 1998; Leung and Furness, 1999a). Exposure of *N. lapillus* to 500 μg Cd l^{-1}, resulted in marked induction of MT in the Leiblein gland after 36 days of exposure but a similar increase of MT in the kidney was only observed after 60 days of exposure (Leung and Furness, 1999a). Transportation of Cd and Cd-MT from the different organs to the kidney for excretion might be a time-dependent process so that the renal MT induction was delayed. This might also explain why exposure of *N. lapillus* to Cd did not result in significantly higher MT in the kidney at both temperatures in the present experiment. Apart from the temperature effect, MT synthesis also appears to be a time-dependent process (e.g. Olsson et al., 1996). By prolonging the exposure period, MT in the kidney or in the Leiblein gland at 5°C might also increase significantly to above the control levels.

In wild populations, concentrations of MT or MT-like proteins in the Baltic clam, *Macoma balthica*, oyster, *Crassostrea gigas*, and periwinkle, *Littorina littorea*, increased with decreasing size (soft-body weight) (Bordin et al., 1997; Mouneyrac et al., 1998; Leung and Furness, 1999b). A similar MT-size dependent relationship was also observed in the kidney of dogwhelks in all groups (Fig. 3.4(c), (d)). However, the concentration of MT in the Leiblein gland of both control and Cd-exposed *N. lapillus* only increased with decreasing size at 10°C but not at 5°C (Fig. 3.4(a), (b)). Furthermore, the concentration of Cd in the remaining tissues increased with decreasing size of dogwhelks in treatment at both temperatures while no significant correlation occurred between the Cd concentration with size in the control groups (Fig. 3.3). Leung and Furness (1999b) suggested that the MT-size relationship could be explained by the significant relationship of MT with Cd, and Cd with size, in the periwinkle *L. littorea*. Nevertheless, the present results indicate that temperature is likely to be another factor that affects the MT-size relationship.

As MT induction can be influenced by temperature changes, levels of MT in biomonitors such as *N. lapillus* may be subject to seasonal (e.g. summer vs. winter) and geographical (e.g. temperate vs. tropics) variations. If MT increased in relation to temperature, MT levels in a biomonitor would be expected to reach a maximum during

summer and a minimum during winter. However, unlike laboratory experiments, there are multiple biological and environmental factors in the natural environment, affecting the physiology and biochemistry of individual organisms. In *M. balthica*, MT concentrations peaked in winter-spring but declined sharply and reached a minimum in summer (Bordin et al., 1997). These seasonal patterns can be partially explained by the changes in body weight throughout the season; weight loss occurring during the winter and weight gain during the summer. Although increases in temperature during the summer may increase accumulation of metals and rate of MT synthesis in the biomonitors, increased food consumption during the same period enhances their growth rate, leading to tissue dilution effects on both metal and MT concentrations (Phillips and Rainbow, 1993). Additionally, reproductive status may also be an important factor governing the seasonal variation of MT levels in biota (Langston and Spence, 1995). Nevertheless, our results suggests that environmental temperature should be considered while comparing MT or metal levels using mollusc populations from different latitudes with different temperature profiles.

Cd Mediated Metabolic Depression

The present results agree with a previous study that oxygen consumption by *N. lapillus* increased with increasing temperature (at salinity 35 ‰) and decreasing size, with a similar weight constant of 0.60 (Stickle and Bayne, 1982). The dogwhelks exposed to Cd showed significantly lower rates of oxygen consumption when compared to the control animals at 10°C but not at 5°C, indicating that respiration of *N. lapillus* was inhibited and/or depressed by Cd at 10°C. Coincidentally, significant increases in Cd accumulation and MT induction in *N. lapillus* were also noted at 10°C but not at 5°C. These results suggest that an elevated temperature increases Cd accumulation and toxicity in this gastropod species; and may explain why, in a previous study, inhibition of oxygen consumption by Cd was only observed in *N. lapillus* collected in summer (July) but not in those collected in winter (January) (Abdullah and Ireland, 1986).

Glycogen stores were greatly reduced in the dogwhelks by Cd-exposure. The glycogen stores decreased significantly with increasing Cd concentrations in the tissues of *N. lapillus*, strongly suggesting that Cd-exposure, -accumulation and -toxicity, directly cause the decline of glycogen stores. The reduction of glycogen in the foot muscle and digestive gland ranged from 17% to 45% of the control values regardless of

temperature changes. Such considerable amounts of energy expenditure have been attributed to the costs of combating the toxic effects of Cd (Gil et al., 1989; Calow, 1991; Forbes and Calow, 1996), although Cattani et al. (1996) have pointed out that the reduction of glycogen may also be the result of direct Cd-induced interference of mitochondrial function and activation of glycogen phosphorylase kinase. Obviously, there are metabolic costs for detoxification in the dogwhelks exposed to Cd such as production of mucus and MT (Leung and Furness, 1999a). The cost to gastropods of producing mucus is greater than the total cost of locomotion for mammals or reptiles of similar weight (Denny, 1980). The estimated cost of the production of mucus in *Patella vulgata*, for example, is 23% of energy input from ingestion (Davies et al., 1990). Thus, the cost for extra mucus production in the dogwhelks exposed to Cd should be considerable.

Metabolic depression commonly occurs in marine and terrestrial snails, especially during aestivation and hypoxia (Marchall and Mcquaid, 1991; Brooks and Storey, 1997). The large reductions in metabolic rate during aestivation and anoxia can translate into considerable energy savings so that gastropods can survive through these stresses. In this way, Cd may have induced metabolic depression in *N. lapillus*. The decrease of oxygen consumption together with the utilisation of glycogen during Cd exposure, suggest that *N. lapillus* might shift to anaerobic metabolism in order to minimise the uptake and toxicity of Cd by decreasing the rate of ventilation or water exchange (Devi, 1996; Isani et al., 1997). In fact, reducing the rate of filtration or ventilation, increasing the frequency of complete valve closure and switching to anaerobic metabolism, have been thought to be a strategy commonly adopted by bivalve molluscs to minimise the uptake and toxicity of metals (Manley, 1983; Krishnakumar et al., 1990; Kumarasamy and Karthikeyan, 1999).

Further Reasons Explaining the Depression of Oxygen Consumption by Cd

In nature, *N. lapillus* may occasionally be detached from the rock surface by wave-action or removal by predatory actions of shore crabs such as *Carcinus maenas*. When *C. maenas* is testing the thickness of individual shells and looking for thinner ones to attack, they will drop those with a thick shell (Michael T. Burrow, Dunstaffnage Marine Laboratory, personal communication). In these instances, the dogwhelks must recover as soon as possible from an abnormal or upside-down posture in order to avoid being washed away and to protect themselves from predation. Therefore, the

present behavioural study, by measuring the recovery time of dogwhelks from the upside-down position, is ecologically relevant and easy to conduct. Cd-exposed dogwhelks recovered comparatively slowly from the upside-down posture, suggesting that the dogwhelks were less active and/or less responsive. This result might imply that Cd-exposed individuals could reduce their normal energy requirement by minimising their activities and then channel the energy resource for detoxification and maintenance purposes, although this might also be the result of Cd toxicity (e.g. inhibition of acetylcholinesterase activity and damage to nervous systems (Reddy and Venugopal, 1993)). When rainbow trout *O. mykiss* were exposed to Cu in the diet for 3 months, the fish reduced the time spent swimming to help meet their metabolic demand for detoxification costs (Handy et al., 1999). Although the subtidal gastropod *Babylonia lutosa* exposed to Cu (0.02 to 0.2 ppm) exhibited significant reductions in normal locomotor activities (including burrowing), there was no alteration in feeding rate (Cheung and Wong, 1999), indicating that *B. lutosa* may save energy by reducing activity to combat Cu toxicity. A similar depression in activity caused by Cd was also observed in the mudsnail *N. obsoletus* (MacInnes and Thurberg, 1973). Apparently, the major metabolic trade-off in *N. lapillus* exposed to Cd, appears to be that the metabolic effort associated with detoxification is met by a reduction in locomotor activity. In addition to energy compensation for detoxification, reducing activity eventually reduces the overall oxygen consumption and indirectly minimises the uptake and toxicity of Cd.

Brouwer et al. (1982) demonstrated that Cd ions could bind to haemocyanin of the blue crab, *Callinectes sapidus*, increase the oxygen affinity and thus reduce the degree of their cooperative oxygen binding. Recently, Martin and Rainbow (1998) have also reported a similar observation while studying the binding of Cd with haemocyanin from *C. maenas*. In the present study, a consistent copper concentration between the control and treatment groups suggests that the levels of haemocyanin were similar in both groups. Thus, the decline of oxygen carrying capacity of the haemolymph, obtained from Cd-exposed dogwhelks, might be explained by binding of Cd to the haemocyanin, affecting structure and normal function. The results of our *in vitro* study further support the view that Cd may have been bound to the haemocyanin and hence reduce the oxygen carrying capacity.

Adaptive mechanisms used to reduce tissue metal burdens include the release of extracellular compounds that chelate and reduce the bioavailability of the metal in the

surrounding media, exclusion by the organism (lowered permeability), and increased elimination (Langston and Spence, 1995). A previous acute toxicity study noted that dogwhelks exposed to Cd increased the secretion of mucus, egestion and closing of the operculum (Leung and Furness, 1999a). We observed a thicker layer of mucus on the gill surface of dogwhelks exposed to Cd, indicating an enhanced mucus production. Increased mucus production may arise by a stimulation of secretion rates and by the increasing numbers of mucus-producing cells in the epithelium of exposed tissues such as skin, gills and intestine (Langston and Spence, 1995). Mussels, *Septifer virgatus* and *Perna viridis*, exposed to 50 Cu at μg l^{-1} for three months showed higher mucus production rates (1.85 and 2.65 times that of the control) and elevated concentrations of Cu in their mucus (Sze and Lee, 1995). Continuous production and shedding of mucus layers can, therefore, significantly limit absorption kinetics, both at body surfaces and in the gut (Langston and Spence, 1995). At the same time, mucus secretion may also reduce the efficiency of gaseous exchange through the gills and body surfaces. Inhibition of respiration by Cd in the mussel *Lampsilis ventricosa* has been attributed to mucus production (Naimo et al., 1992).

Damage of cellular structures and gill function may result from exposure to heavy metals. For example, *O. mykiss* exposed to Cu in the diet for 3 months showed a 9% increase in gill secondary lamellae length which may be an adaptation to Cu toxicity (Handy et al., 1999). Gill tissue of *M. edulis* exposed to Cu showed a depression in ciliary activity (Brown and Newell, 1972), proposed to be neuronally mediated by Cu ions (Micallef and Tyler, 1990). A time-course study on the histology of mussels *Perna perna* exposed to 50 μg Hg l^{-1} for 1 to 24 days, showed a gradual increase in the diameters of microvilli, a depletion of abfrontal cilia, an increase in abnormal necrotic cells and the number of cilia on the lateral surfaces (Gregory et al., 1999). We also observed a reduction in the number of cilia and an increase in necrotic cells on the gill surfaces of *N. lapillus* exposed to Cd. These might decrease the efficiency of gas exchange through the gill tissues and reduce oxygen uptake. Moreover, it has been suggested that metal ions, such as Cd and Zn may induce alterations in enzyme activity in the gills of marine invertebrates, leading to an osmoregulatory imblance (e.g. Bianchini and Carvalho de Castilho, 1999). Nevertheless, effects of Cd on enzyme activity have yet to be established in marine gastropods.

Other Possible Reasons Explaining the Depression of Oxygen Consumption by Cd

Either Cd, or Cd-MT, may directly interfere with the normal function of mitochondria, leading to a depression of oxygen consumption. For example, Cd causes a decrease in NADH-oxidase activity of the gill mitochondria in a freshwater clam *Anodonta cygnea* (Hemelraad et al., 1990). A recent *in vitro* study showed that the ADP-initiated oxygen consumption of mitochondria, isolated from rat liver, was inhibited by physiological concentrations of MT or MT and calcium synergistically (Simpkins et al., 1994, 1998). This may reduce the quantity of injurious oxygen free radicals produced within the cells (Simpkins et al., 1998). In the dogwhelk, a marked elevation of MT in the Leiblein gland at 10°C might also inhibit cellular oxygen consumption and thus significantly reduced the overall MO_2 at the same temperature. However, this hypothesis must be tested again in marine invertebrates because their cellular toxicity responses to MT may be very different from those observed in mammals.

The Norway lobster, *Nephrops norvegicus* infected with parasites *Hematodinium* sp showed an elevated oxygen consumption rate compared to healthy ones (Taylor et al., 1996). *Parorchis acanthus* and *Nucellicola holmanae* are the common parasites found in dogwhelks (Lamb et al., 1996). These parasites may contribute to the total oxygen consumption rate of a dogwhelk. The parasites could be killed or inhibited by Cd and thus reduce the total oxygen consumption by a dogwhelk. Further investigation is required.

Model Proposed to Describe the Physiological Responses of N. lapillus to Cd

Here, a model is proposed, based on the multiple regression models (Table 4.6), to describe the modes of Cd toxicity and the corresponding physiological responses of a fasted dogwhelk *N. lapillus* at 10°C (Fig. 4.12). The dogwhelk exposed to Cd will increase the accumulation of Cd in tissues. In order to minimise the uptake and toxicity of Cd, the animal produces extra mucus, reduces oxygen consumption (and gill ventilation rate), and shifts to anaerobic respiration. A reduction in oxygen consumption may be partially explained by the Cd-induced mucus production, structural damage to gills and a reduction in oxygen carrying capacity of haemocyanin. At the same time, both metabolic rate and normal activity are depressed. Such metabolic depression may cut down energy expenditure so that the extra energy demand for detoxification (e.g. an increase in MT synthesis and mucus secretion) and maintenance (e.g. repair of cellular damage caused by Cd) could be met. The major

physiological trade-off in combating Cd toxicity is the substantial depletion of energy reserves, i.e. glycogen. Furthermore, other direct inhibition of cellular respiration may be possible. We hope that this proposed model may set a foundation for further investigation of physiological and biochemical responses of marine gastropods to metal exposure.

Potential and Problems of Using Glycogen and MT as Biomarkers

In starved *N. lapillus*, Cd toxicity caused a decline in glycogen and an increase of MT, suggesting that measurement of glycogen and MT levels may be useful biomarkers for metal toxicity. However, in natural environments, the dogwhelks can increase energy stores by food consumption. With a good supply of food, dogwhelks might be able to repair damage and maintain normal health status. Field studies are needed to test whether continuous energy input could protect against metal toxicity in *N. lapillus* and keep glycogen stores constant. Moreover, populations in different areas may have different food availability and food types (e.g. mussels, barnacles and detritus), leading to different body conditions and glycogen reserves. Glycogen and MT concentrations may also vary according to season and reproductive status. For example, the oyster *Crassostrea virginica* depleted glycogen energy stores in the winter and spawned in summer, leading to two annual minima in soft-body weight (April and August) and thus affecting metal concentrations (Boyden and Phillips, 1981). Abdullah and Ireland (1986) also demonstrated that Cd concentrations in *N. lapillus* collected at Aberystwyth, Wales varied according to seasons, being high in January to April and low in July to September. Dogwhelks can breed throughout the year, although two major spawning periods, April-May and July-August have been reported (Feare, 1970). Therefore, measurement of glycogen and MT in biomonitors such as the dogwhelks may not be correctly interpreted unless other ecological factors are also taken into account.

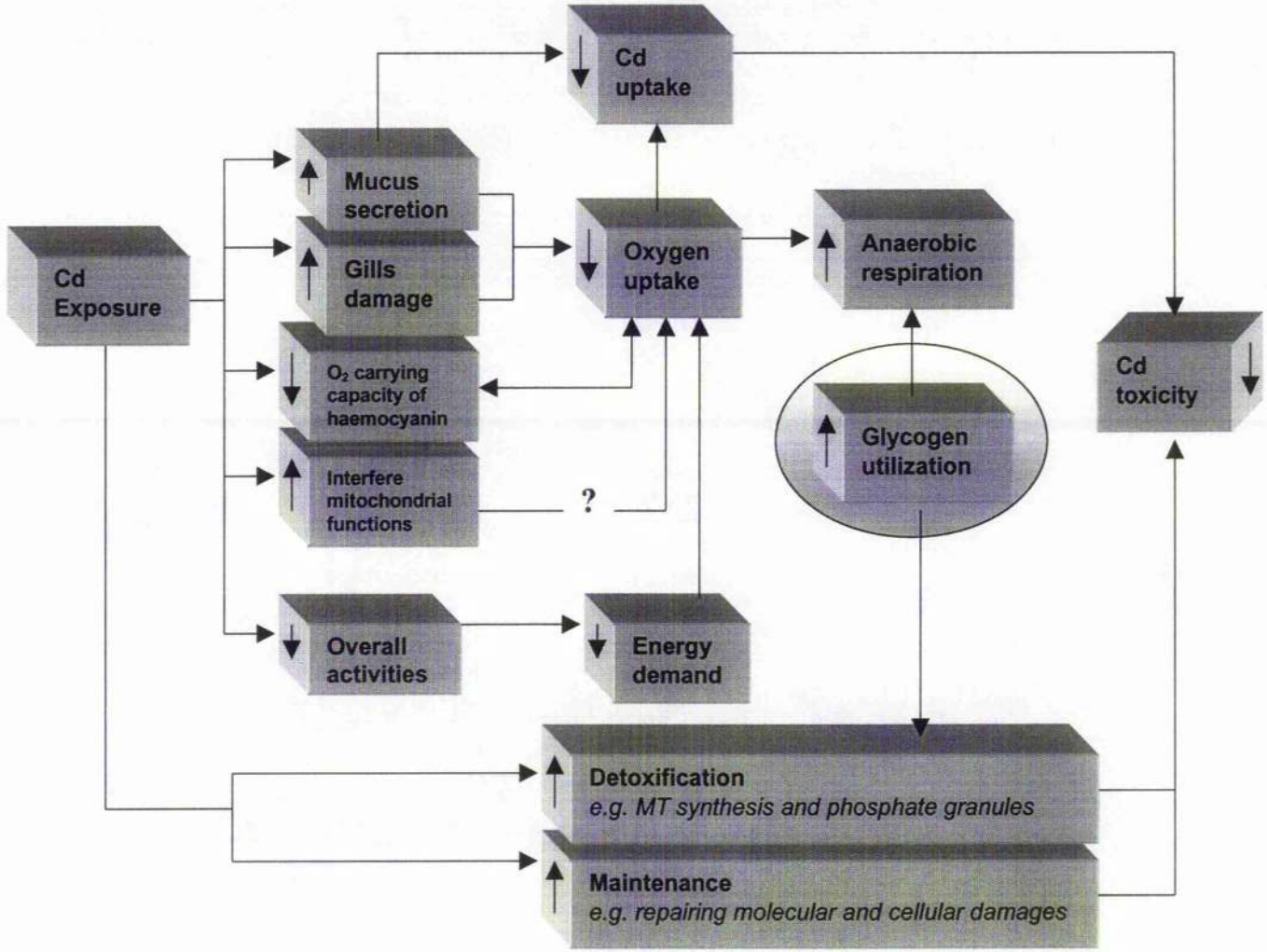

Figure 3.12.. A schematic diagram illustrating the modes of Cd toxicity and corresponding physiological responses of *Nucella lapillus*.

Another problem that will be encountered, if using MT and glycogen as biomarkers, is their high inherent variability within a population. For MT in the Leiblein gland of control dogwhelks at 5°C, the coefficient of variation (CV) was 66%. For glycogen in foot muscle, CVs of control and treatment were 45% and 51% respectively at 5°C, 54% and 91% at 10°C. Furthermore, CV of Cd concentration in the remaining tissue of control *N. lapillus* was 20% at 5°C and increased to 70% at 10°C. These variations suggest that there is high inherent variability in glycogen or MT contents and tolerances to Cd within the same population of dogwhelks. Such large variation might be explained by differences in pre-experimental food consumption rate, food type, activity, and reproductive status. Secondly, these variations might be due to inherent variability in tissue and body metal burden. Thirdly, environmental factors such as temperature might also affect the variations in responses to metal uptake, MT induction, glycogen distribution and utilisation within the population. If measuring glycogen and MT in dogwhelks or other biomonitors collected from field environments, such inherent variations would be expected within each population. For example, no significant difference was found in MT levels in *L. littorea* between polluted and clean areas because of large inherent variability of MT within an individual population (Leung and Furness, 1999b). In order to detect the differences between populations, the variation within each population should be minimal. The inherent variability may be reduced by improving sampling strategy (e.g. optimum sample size, time-bulking and space-bulking sample approach) and sensitivity of analysis (e.g. selection of tissue with most sensitive response, and improvement of accuracy and precision of measurement) (Phillips and Rainbow, 1993; Langston and Spence, 1995).

CONCLUSIONS

Our results suggest that temperature is an important factor affecting the uptake and toxicity of Cd in *N. lapillus* and it should be considered in ecotoxicity tests. Glycogen stores, oxygen consumption and activity were significantly reduced, indicating metabolic depression, in *N. lapillus* as a result of Cd-exposure at 10°C. Reduction in oxygen consumption by Cd may be associated with the extra-secretion of mucus, structural damage to gills, a shift to anaerobic respiration and a reduction in the

oxygen carrying capacity of haemocyanin. However, metabolic depression in Cd-exposed *N. lapillus*, may be a strategy to minimise the uptake and toxicity of Cd while meeting the extra energy demand for detoxification (e.g. mucus and MT synthesis) and maintenance (e.g. repair of cellular damage). The measurements of metallothionein and glycogen in this biomonitor species may be promising tools for monitoring of contamination and toxicity of metals in the coastal environment, but require the effects of other environmental factors to be considered.

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CHAPTER 4

Survival, Growth, Metallothionein and Glycogen Levels of *Nucella lapillus* (L.) Exposed to Chronic Cadmium Stress: the Influence of Nutritional State and Prey Type*

ABSTRACT

Dogwhelks *Nucella lapillus* feed mainly on mussels and barnacles, and may experience periods of starvation. We report effects of nutritional state and prey type on the survival, growth, cadmium (Cd) accumulation, metallothionein (MT) induction and glycogen stores in *N. lapillus* exposed to Cd in water. Adult dogwhelks, with similar shell length (30.0 ± 1.5 mm shell length), were either fasted or fed to satiation with barnacles *Semibalanus balanoides*, mussels *Mytilus edulis* or Cd-dosed *M. edulis*, and kept in filtered natural seawater (<0.01 μg Cd l^{-1}) or Cd-contaminated (400 μg Cd l^{-1}) seawater for 80 days. Mortality and individual growth rate were determined. Cd, MT and glycogen were measured in different tissues. Prolonged starvation and exposure to Cd synergistically reduced the survivorship of *N. lapillus*, but feeding could help dogwhelks to combat Cd toxicity and minimise mortality. Extended fasting also caused tissue wastage, leading to higher concentrations of Cd and MT in tissues, whereas fed animals increased in weight and had lower Cd and MT concentrations because of the tissue dilution effect. Prey type significantly affected growth rate of dogwhelks and indirectly influenced Cd accumulation, MT induction and glycogen stores. Eating mussels promoted better growth and higher glycogen reserves than eating barnacles. Individual growth rate decreased with increasing Cd accumulation. Cd-exposed survivors grew faster and consumed more than control animals, implying that these survivors may have better fitness and greater tolerance to Cd toxicity.

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There was no observable effect of sex on any parameters except significantly higher glycogen in foot muscles of male dogwhelks. This may be a sexual dimorphism in energy allocation in this gastropod species. The use of growth, condition index, MT and glycogen as biomarkers of environmental pollution are discussed. These results indicate a need to incorporate biological data including growth (or condition index) and prey type into biomonitoring programmes to allow sound interpretation.

INTRODUCTION

The dogwhelk, *Nucella lapillus* (L.), is a predatory prosobranch mollusc that lives on the rocky intertidal shores of Europe and eastern North America (Bayne and Scullard, 1978), and has been widely used as a biomonitor for trace metal contamination, especially for tributyltin. The species feeds principally on mussels (e.g. *Mytilus edulis*) and barnacles (e.g. *Semibalanus balanoides*), although they occasionally prey on other gastropods including *Patella vulgata, Gibbula cinerarea* and *G. umbilicalis, Littorina littorea* and *L. obtusata* (Moore, 1938a). However, in the presence of a variety of potential prey species, including barnacles, *Mytilus* is the preferred food (Morgan, 1972). A number of studies suggest that prey type and nutritional state can substantially affect growth rate of *N. lapillus*. They showed most shell growth on a diet of large barnacles, *S. balanoides*, and the highest rates of consumption on a diet of large mussels, *M. edulis* (Burrows and Hughes, 1990). Etter (1996) observed that juvenile *N. lapillus* grew best on a diet of mussels, either singly or in combination with barnacles, but grew less on barnacles alone. Davenport et al. (1998) showed that condition index of *N. lapillus*, fed on *M. edulis* to satiation, rose from 19.7 to 27.4 in 3 months, while that of starved dogwhelks fell from 19.7 to 16.3 (In nature, *N. lapillus* may experience periods of starvation, especially during winter).

Changes in food consumption or growth rate, such as those described above, may affect the accumulation of trace metals and toxicity (Langston and Spence, 1995). In theory, daily uptake rate of a particular metal (U_m, µg individual^{-1} d^{-1}) in a biomonitor can be simplified and described as a function of metal concentration in water (C_w) and time of exposure to water (T_w) (if metal uptake from diet is assumed to be minimal). If C_w and T_w are kept constant, then the U_m will be unchanging. In this instance,

concentration of the metal as microgram per gram soft-body weight in the biomonitor will be dependent upon its biomass, which can be directly affected by food type and consumption rate. Increases in biomass (i.e. growth) may dilute the concentration of the metal whereas poor growth or starvation causing tissue wastage may increase the metal concentration. For example, *L. littorea* sampled from a clean area had a lower body condition index (indicating poor growth) but showed high concentrations of Cd, Cu and Zn in their tissues that were similar to the metal levels in fast growing periwinkles obtained from polluted areas (Leung and Furness, 1999a). With good food availability and favourable conditions (e.g. protected habitat and low predation), the animals should grow well leading to the tissue-dilution of metals in their tissues, provided that the major route of metal uptake is solely from water and not from the diet. However, mussels and barnacles are themselves regarded as moderate and good accumulators (or non-regulators) of trace metals, respectively (Phillips and Rainbow, 1989) and thus may transfer significant amount of metals to their predators, such as *N. lapillus*. If the rate of metal uptake can be enhanced by food consumption itself, this may balance out or counteract the effect of tissue-dilution.

Measuring metallothionein (MT) concentration in biomonitors is an indirect method to assess bioavailability and toxicity of trace metals in marine invertebrates (Roesijadi, 1992, 1996). MT has also been quantified in various tissues of *N. lapillus*, especially in the gland of Leiblein which is the most sensitive tissue and may provide a more consistent measurement of MT (Leung and Furness, 1999b). However, there are several abiotic and biotic factors such as temperature, salinity, animal size, nutritional state, food consumption, prey type and growth, which may affect the metal accumulation in the biomonitor, and may influence the levels of MT (Langston and Spence, 1995; Mouneyrac et al., 1998). For example, the levels of MT in the Leiblein gland of dogwhelks (fasted and exposed to waterborne Cd for 20 days) increased with increasing temperature and decreasing animal size (Leung et al., in press). Further, in the same study, it was also noted that glycogen stores were significantly decreased in fasted *N. lapillus* exposed to Cd while MT increased in relation to the Cd concentration in the tissues, suggesting that there is a metabolic cost to combat Cd toxicity. Nevertheless, continuous food supply might affect MT induction, and sustain the glycogen stores while minimising Cd toxicity. Chandini (1989) showed that the toxicity of Cd (measured by survivorship, growth and reproduction) on the cladoceran *Daphnia carinata* was greatly reduced by feeding with high food levels. In the present study, we

investigate for the first time the effects of nutritional state (starvation and feeding) and prey type (barnacles, mussels, and mussels pre-exposed to Cd) on growth, mortality, MT induction and glycogen stores in adult *N. lapillus*, exposed to a sublethal level of Cd in water under controlled laboratory conditions. We will demonstrate how these factors affect the concentrations of Cd, and MT in this species. The results of this study will shed light on other similar studies using predatory prosobranch molluscs as biomonitors, or as test animals for toxicity studies.

MATERIALS AND METHODS

Experimental Animals

Adult *N. lapillus*, with similar shell length (30.0 ± 1.5 mm shell length; mean ± SD) were collected from Gourock, Clyde Sea, Scotland where *N. lapillus* feed upon *M. edulis*, and *S. balanoides* (prey cover 40% barnacles, and 60% mussels, approximately). Rocks with barnacles, dead-shells with barnacles, and mussels were collected from the same area for the feeding experiments. All animals were acclimatised to circulating seawater under controlled laboratory conditions (10 ± 0.5°C and 35 ± 1‰; fasted) for 1 wk. During this period, the dogwhelks were sexed by checking for the presence of a well-developed penis (as male characteristic) and then separated into seven groups (n = 36). Each group was assigned with 18 males and 18 females (sex of each individual was reconfirmed after the entire exposure period through dissection and examination for the presence of sperm receiving gland [as female characteristic]). Epifauna were removed from the shells of *N. lapillus*. Each individual was then labelled with a waterproof label using super-glue (151 Super Glue, SB limited, UK). Measurements were taken of shell length in the dogwhelks, and mussels, or opercular length in barnacles, using vernier callipers (± 0.1 mm).

Estimation of Initial Shell and Soft-body Mass

Shell and live body mass were estimated at the beginning of the experiment using the methods of Palmer (1982). Animals were maintained immersed for 48 h (9 days before Cd exposure) in order to allow air bubbles in the mantle cavity to dissolve completely. For estimation of shell mass, each dogwhelk was then transferred to a weighted-cradle, suspended from the arm of a balance (± 1.0 mg) by a stainless steel

rod and immersed in a small rectangular tank of seawater (Length × width × height: 16.3 × 6.3 × 10.0 cm^3; volume of seawater at 35‰ = 860 ml). The measured weight is largely due to the mass of the shell, as the density of body tissues is close to that of seawater (Burrows and Hughes, 1990). After weighing in water, each snail was dried and extra-visceral water removed by pressing an absorbent tissue firmly against the withdrawn foot, until no further fluid penetrated the tissue. The animal was left to dry for about 1 h before weighing in air. The true mass of shell was estimated using a regression of dry shell mass on both immersed total mass under water and the total mass in air (Table 4.1: regression [3]). The soft-body weight was obtained by subtraction of the estimated shell mass from the total mass in air.

Table 4.1. Morphometric relations of predators and prey used in growth and consumption calculation. Equations were calculated using least squares linear regression.

Regression number	N	Regression equation	R^2
Shell mass (g) estimates of destructively sampled *Nucella lapillus*			
Shell dry mass (Y) from immersed whole weight in gram (X)			
[1]	29	$Y = 1.7119X + 0.317$	0.9539
Shell dry mass (Y) from whole weight in air in gram (X)			
[2]	29	$Y = 2.1981X + 1.036$	0.8805
Shell dry mass (Y) from immersed whole weight in gram (X) and weight in air in gram (Z)			
[3]	29	$Y = 0.9427X + 0.3356Z - 0.3052$	0.9779
		($F_{2,26} = 575.25$, $p < 0.0001$)	
Dry flesh weight (g) as a function of prey size (g shell weight)			
S. balanoides: Body dry weight (Y) from dry shell weight immersed whole weight in gram (X)			
[4]	20	$Y = 0.0067X^{1.6414}$	0.8234
M. edulis: Body dry weight (Y) from dry shell weight immersed whole weight in gram (X)			
[5]	30	$Y = 0.0872X^{1.1116}$	0.9269

Experimental Set-up

Each experimental group of *N. lapillus* were held in a plastic enclosure (length × width × height: 20 cm × 15 cm × 10 cm) and placed in a closed-circulating system (40 litre) with a tidal controlling device (Fig. 4.1). Low tide was adjusted at 1100 to 1700 (i.e. 6 h) daily. The dogwhelks were fasted and acclimated in these experimental tanks

for another 7 days before the experiment commenced. After this period, the dogwhelks in Group 1 and Group 2 were continuously fasted; Group 3 and 4 were fed with barnacle *S. balanoides*; Group 5 and Group 6 were fed with mussels *M. edulis* while Group 7 were fed with *M. edulis* pre-exposed to Cd. All foods were provided *ad libitum*. Prey items consisted of rocks or dead-shells with live barnacles (3-5 mm opercular diameter) and live mussels (27-47 mm shell length; 36.5 ± 4.9 mm: mean ± SD). For group 7, live mussels exposed to 5 ppm Cd in filtered seawater for 48 h were used. These dosed mussels contained 61.4 ± 18.4 μg Cd g^{-1} dry soft-body weight (Table 4.5). Group 1, 3, 5 (as controls) and 7 were exposed to clean filtered seawater containing < 0.01 μg Cd l^{-1} while Group 2, 4 and 6 were exposed to filtered seawater containing 400 μg Cd l^{-1} (nominal concentration, equivalent to 1.7% of 96 h LC_{50} (Leung and Furness, 1999a)). The entire exposure period was 80 d. Water was renewed once every 4 days.

Mortality, Growth, Production and Condition Index

Mortality was checked regularly and defined (where movement was not immediately evident) as a failure to respond to probing with forceps. Any dead *N. lapillus* found was removed from the tanks immediately in order to avoid cannibalism. At the end of the experiment, live *N. lapillus* were re-measured for shell length and the soft-body was removed from the shells using a vice. All broken fragments of a shell were collected and dried at room temperature for 1 week before weighing. The wet weight of soft-body was measured after being blot-dried using an absorbent tissue. Growth rates in term of shell length, shell and soft-body mass were calculated based on the measured initial shell length, estimated initial shell and soft-body mass, respectively. Mortality, production and net production of each treatment group (g $cage^{-1}$ or kJ $cage^{-1}$) over the experimental period were estimated using the following equations:

$$\text{Mortality} = (N_i - N_f)(W_f + W_i)/2 \quad (1)$$

$$\text{Production} = [(N_i + N_f)/2](W_f - W_i) \quad (2)$$

$$\text{Net production} = (2) - (1) \quad (3)$$

Where N_i and N_f are the number of survivors at initial and final point, respectively; W_f and W_i are the mean soft-body weights of *N. lapillus* at start and the end of the experiment. The estimated production were also converted to energy units using

values of 22 J mg^{-1} for *N. lapillus* flesh (Hughes, 1972). Condition Index (CI) was calculated using the following equation:

$$CI = [\text{wet soft-body weight}/(\text{wet soft-body weight} + \text{dry shell weight})] \times 100 \quad (4)$$

The life expectancy of adult *N. lapillus* used in the present experiment was estimated for each group using the following equation (modified from Feare, 1969):

$$\text{Expected age} = 3 + [2 - (M + 0.27)]/[2 \times (M + 0.27)] \quad (5)$$

where, M is an observed mortality of a given age, expressed from 0 to 1. Assuming that the test animals were 3 years of age (based on their shell length (Moore, 1938a)) with 27 % natural mortality rate (Feare, 1969). If $M = 0$, the expected age is 6.2 years.

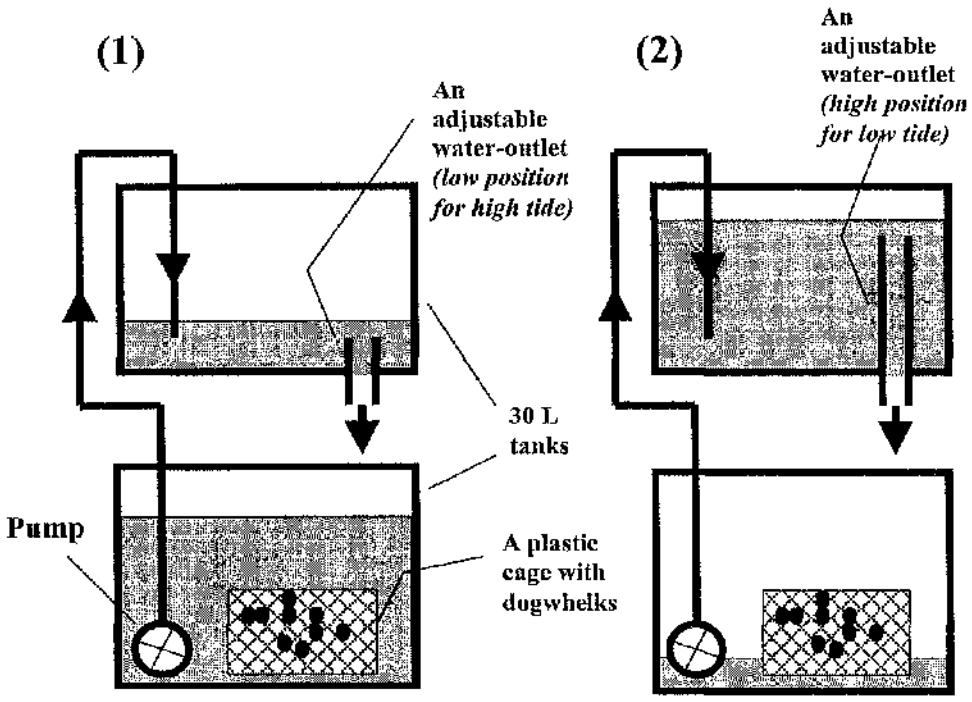

Figure 4.1. Experimental set-up with a tidal-control system: (1), high tide and (2), low tide.

Estimation of Food Consumption

The empty shells of barnacles and mussels were collected when new foods were provided. The dry flesh consumed from each prey item was estimated from regressions [4] and [5] (Table 4.1). Consumption of each prey type in each treatment group was obtained by converting the estimated total dry mass of ingested prey to energy units using values of 19.5 J mg^{-1} for mussels flesh (Elner and Hughes, 1978) and 23 J mg^{-1} for barnacle flesh (Wu and Levings, 1978). Consumption rate for individuals (J ind^{-1} d^{-1}) was estimated based on the estimated total consumption in each group (C, kJ $cage^{-1}$) over 80 d:

$$\text{Consumption} = \{C/[(N_i + N_f)/2]\}/80 \quad (6)$$

Concentrations of Cd, Glycogen and MT

At the end of the experiment, the soft-body was dissected into foot muscle, upper and lower parts of digestive gland/gonad complex, and gland of Leiblein. The lower half of digestive gland/gonad complex of *N. lapillus* and flesh of prey items were dried at 60°C for at least 96 h until constant mass was achieved. They were then digested in conc. HNO_3 for 24 h at room temperature followed by boiling for at least 2 h until a clear solution was obtained. Cd concentrations were determined using a Philips PU9200 Atomic Absorption Spectrophotometer (AAS) with deuterium background correction and expressed as microgram per gram dry tissue weight. Accuracy was regularly checked by including standard reference materials within batches.

The weighed whole Leiblein gland was homogenised with 0.4 ml of 0.25M sucrose using an Ultraturax (T25 Janke & Kunkel, IKA Labortechnik) at 4°C. The homogenate was centrifuged at 20,000*g* for 20 min at 4°C. Aliquots of 300 µl supernatant were analysed for MT content using the silver saturation method described by Leung and Furness (1999a). For glycogen analysis, 50-100 mg of the digestive gland/gonad complex (upper half) and foot muscle tissues were dissolved in 0.4 ml 30% KOH 90°C for 30 min. After cooling in ice, 1 ml of absolute alcohol was added to the tissue solution, mixed and kept at 4°C for 2 h. It was then centrifuged at 3,000*g* for 10 min. After removal of the supernatant, the pellet was re-dissolved in 1 ml distilled water. Subsequently, glycogen concentrations in these solutions were determined in triplicate by utilising the anthrone reagent (Seifter et al., 1950), with comparison against multiple glycogen standards. The results of MT and glycogen were expressed as µg or mg per g of wet tissue weight.

Data Analysis

Normality and homogeneity of variances of the data were checked using the Kolmogorov-Smirnov test and Bartlett's test, respectively. Since there was no replicate group for the mortality data, a Chi square test was employed to compare actual data and expected data based on 1:1 sex ratio. General linear models (GLM) were used in order to test significant effects of Cd exposure, sex, nutritional state and prey type on the data of growth, CI, Cd, MT and glycogen amongst the control and Cd-exposed dogwhelks (i.e. groups 1-6). For animals fed with mussels (i.e. groups 5-7), GLM were also utilised to test the differences in the mean values of the data among waterborne Cd-exposed, dietary Cd-exposed and control animals. The mean values of the data were subsequently compared using a Student Newman Keul's (SNK) multiple comparisons test. Apart from the data of glycogen in the foot muscles, all statistical analysis was based on combined data from both sexes, since no significant effect of sex was observed (GLM, $p > 0.05$). A partial correlation between growth and Cd data was conducted with a correction for size. Statistical significance was defined as $p < 0.05$. All statistics were run on standard software packages (SPSS for Windows, Release 7.5.1, 1996 and Graph Pad Prism™, version 2.0, 1995).

RESULTS

Mortality and Survivorship

Within the control groups (i.e. groups 1, 3 and 5), fasted dogwhelks showed higher mortality (19.4%), while a minimal mortality rate (2.8-5.6%) was observed in individuals fed with barnacles or mussels (Fig. 4.2). Amongst the treatment groups (i.e. groups 2, 4, 6 and 7), the order of mortality was ranked as followings: fasted > fed mussels > fed barnacles > fed Cd-dosed mussels (Fig. 4.2). The results indicate that prolonged fasting can reduce fitness and survivorship. In addition, *N. lapillus* exposed to waterborne Cd exhibited higher mortality when compared with the corresponding control, suggesting that their survivorship was significantly reduced by Cd toxicity. However, *N. lapillus* fed with Cd-dosed mussels had a similar mortality rate (5.6%) as the control animals fed with normal mussels. There was no significant difference in mortality between sexes ($\chi^2 = 3.521$, DF = 6, $p = 0.741$). Forced fasting and/or Cd exposure greatly reduced the estimated life expectancy of *N. lapillus* (Table 4.2).

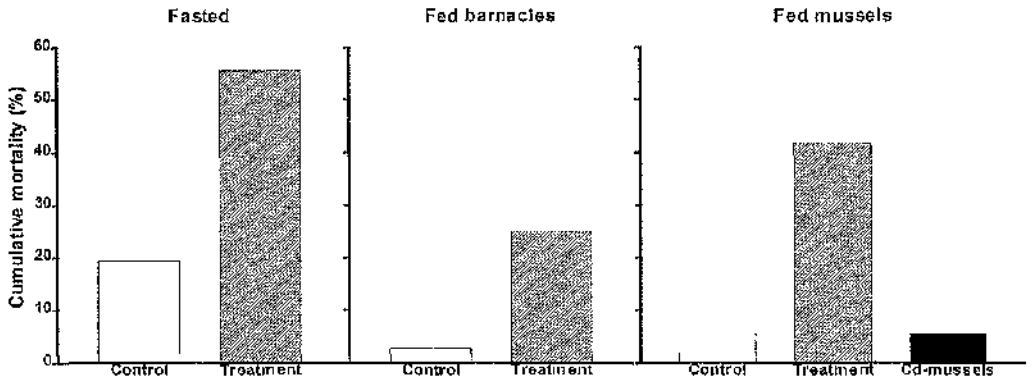

Figure 4.2. Cumulative mortality of *Nucella lapillus* in the controls (open bars); exposed to waterborne Cd (shaded bar); and fed with Cd-dosed mussels (solid bar) over the 80 days of experimental period.

Table 4.2. Estimated life expectancy of *Nucella lapillus* in nature population and in the present experimental groups.

Description	Expected age (year)	Sources
Population in natural environments		
Prey on mussels and barnacles	7 or more	Moore, 1938
Prey on mussels and barnacles	5 – 6	Feare, 1969
Prey on barnacles mainly	7 – 9	Wright, 1976
Population in laboratory (control)		This study
Fasting group	4.7	
Fed with barnacles	5.9	
Fed with mussels	5.6	
Population in laboratory (exposed to Cd in water)		This study
Fasting group	3.7	
Fed with barnacles	4.4	
Fed with mussels	4.0	

Growth, Condition Index and Production

After 80 days of exposure, daily growth rate was estimated individually for all survivors. There were significant differences in growth rate (mg g^{-1} d^{-1}) among groups 1-6 (Fig. 4.3; Table 4.3 [groups 1-6]). Fasted *N. lapillus* lost weight whereas all fed individuals gained weight. The results of GLM indicated that Cd-exposed *N. lapillus* surviving after 80 days, had a higher growth rate than the control animals (Fig. 4.3; Table 4.3). Within the control groups, *N. lapillus* fed with mussels grew better than those fed with barnacles alone (SNK, $p < 0.05$), but there was no significant difference in the growth rate of the Cd-exposed *N. lapillus* between these two diets. The growth rate of *N. lapillus* fed with mussels or Cd-dosed mussels were not significantly different (Table 4.3 [groups 5-7]). Furthermore, there was no observable difference in the growth of shell length among all groups ($F_{6, 189} = 0.849$, $p = 0.534$). Across all data, the growth of shell length ranged from 0 to 2.15 mm (0.08 ± 0.05 mm, mean ± SD).

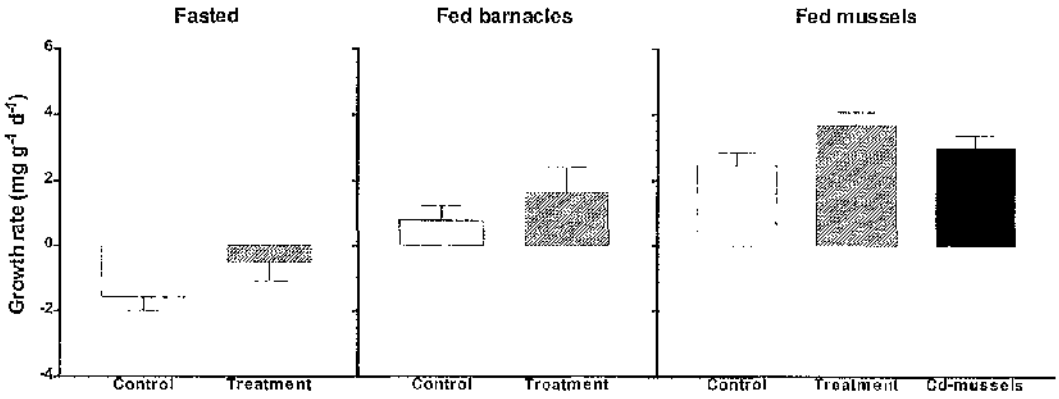

Figure 4.3. Estimated growth rate of *Nucella lapillus* in the controls (open bars); exposed to waterborne Cd (shaded bar); and fed with Cd-dosed mussels (solid bar). Mean and SEM are presented.

For groups 1-6, there was no significant effect of Cd on the CI values when compared between control and Cd-exposed *N. lapillus* (Fig. 4.4; Table 4.3). However, CI values were significantly affected by nutritional state and prey type (Table 4.3). In general, the CI of fasted *N. lapillus* was significantly lower than that of the fed animals while animals fed on mussels showed greater CI than those fed on barnacles (Fig. 4.4; SNK, $p < 0.05$). The CIs of *N. lapillus* fed with mussels or Cd-dosed mussels were not significantly different (Table 4.3 [groups 5-7]).

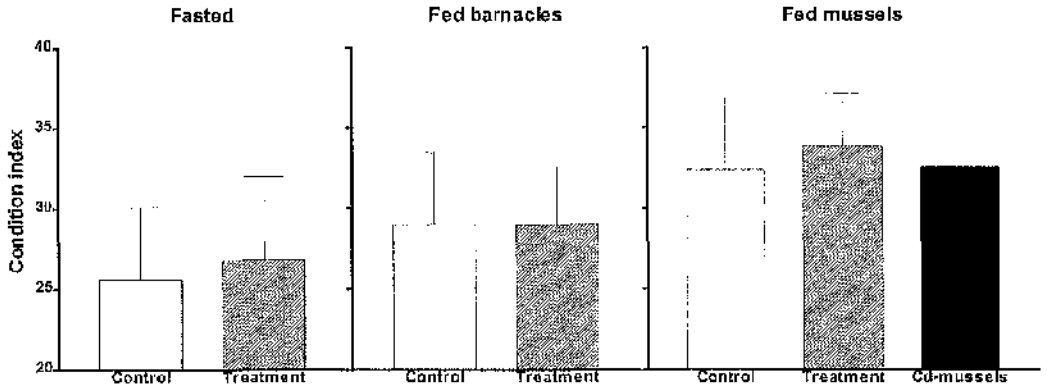

Figure 4.4. Condition index of *Nucella lapillus* in the controls (open bars); exposed to waterborne Cd (shaded bar); and fed with Cd-dosed mussels (solid bar) measured after 80 days of experimental period. Mean and SD are given.

Table 4.3. Results of general linear model for growth and condition index data.

Parameter	df1, df2	F value	Significance of F	
Growth rate				
[groups 1-6]				
Effect of Cd-exposure	1, 156	5.728	0.018	*
Effect of diet [a]	2, 156	27.864	< 0.001	***
[groups 5-7]				
Effect of treatment [b]	2, 86	2.099	0.129	
Condition index				
[groups 1-6]				
Effect of Cd-exposure	1, 156	1.630	0.204	
Effect of diet	2, 156	41.402	< 0.001	***
[groups 5-7]				
Effect of treatment	2, 86	1.020	0.365	

Note. [a] Diet includes no food, barnacles, mussels and Cd-exposed mussels.
[b] Treatment includes fed mussels, fed mussels and exposed to waterborne Cd, and fed Cd-exposed mussels. Asterisks denote $*p < 0.05$ and $***p<0.001$.

Although Cd-exposed survivors grew better than the control animals and had higher production, their net production was lower than that of the control groups (Table 4.4). This was due to high mortality caused by Cd toxicity. In fasting groups, the difference between the net production of control and Cd-exposed *N. lapillus* was 258.2 kJ. Between feeding groups, the differences were 184.3 kJ and 311.1 kJ for *N. lapillus* fed with barnacle and mussels, respectively. Nevertheless, dogwhelks fed with Cd-dosed mussels had higher net production than controls. Within control groups, animals fed on mussels had a better food conversion ratio (FCR: 22.9%), four time of those fed on barnacles (Table 4.4).

Table 4.4. Production, mortality, net production and food conversion ratio (FCR) of *Nucella lapillus* in each experimental group.

No.	Treatment	Production		Mortality		Net production		FCR*
		g	KJ	g	kJ	g	kJ	%
1	Fasting	-3.25	-71.5	7.92	174.3	-11.17	-245.8	NA
2	Fasting; exposed to Cd	-1.08	-23.7	21.84	480.4	-22.91	-504.1	NA
3	Fed barnacle	2.29	50.4	1.14	25.0	1.15	25.3	5.7
4	Fed barnacle, exposed to Cd	3.64	80.0	10.86	239.0	-7.22	-158.9	-34.6
5	Fed mussels	6.40	140.8	2.63	57.8	3.77	83.0	22.9
6	Fed mussels; exposed to Cd	9.88	217.3	20.25	445.4	-10.37	-228.1	-70.4
7	Fed Cd-exposed mussels	9.38	206.4	2.63	57.8	6.75	148.6	31.1

*FRC = 100 × (Net Production/Consumption); Consumption data are presented in Table 4.5; NA = not applicable.

Food Consumption and Dietary Cd Uptake

Food consumption was similar between the control and Cd-exposed *N. lapillus* fed with barnacles (Table 4.5) but in terms of dry biomass or energy intake consumption rates in these groups were lower than those fed with mussels. Animals exposed to Cd and fed on mussels showed a slightly higher individual consumption rate than those just fed on mussels or Cd-dosed mussels (Table 4.5). Mean concentrations of Cd in barnacles and mussels were 6.29 and 4.38 μg g^{-1} dry wt, respectively, while in Cd-

dosed mussels it was 61.43 μg g^{-1}, 14 times higher than the natural mussels (Table 4.5). Based on consumption and dietary Cd levels, individual dietary Cd uptake over the 80 days of exposure was calculated (Table 4.5). Uptake of Cd from diet was broadly similar in *N. lapillus* fed with barnacles and control animals fed with mussels (2.79-2.84 μg $individual^{-1}$). Cd-exposed *N. lapillus* fed on mussels had a higher dietary Cd uptake (3.62 μg $individual^{-1}$). However, the highest dietary Cd uptake rate was found in *N. lapillus* fed with Cd-dosed mussels (43.05 μg $individual^{-1}$).

Table 4.5. Concentration of Cd (mean ± sd, n = 15) in the prey items, estimated food consumption, and dietary Cd uptake by *Nucella lapillus* over 80 days of experimental period.

Group no.	Prey type	Estimated food consumption				[Cd] in prey	Cd uptake from prey (μg ind^{-1}) over 80 d
		g $cage^{-1}$	kJ $cage^{-1}$	mg ind^{-1} d^{-1}	J ind^{-1} d^{-1}	μg g^{-1} dry wt	
3	*S. balanoides*	15.7	361.8	5.54	127.4	6.29 ± 2.76	2.79
4	*S. balanoides*	14.1	324.0	5.59	128.6	6.29 ± 2.76	2.81
5	*M. edulis*	22.7	442.9	8.11	158.2	4.38 ± 1.30	2.84
6	*M. edulis*	23.6	459.2	10.32	201.4	4.38 ± 1.30	3.62
7	Cd-exposed *M. edulis*	24.5	478.3	8.76	170.8	61.43 ± 18.38	43.05

Cd, MT and Glycogen in Various Tissues

Among the control groups, the mean Cd concentration in the digestive gland/gonad complex of fed dogwhelks ranged from 12.2 to14.8 μg g^{-1} dry wt while in fasted individuals this value was 21.33 μg g^{-1} (Fig. 4.5). However, there was no significant difference in Cd concentration of these tissues among all control groups (SNK, $p > 0.05$). Exposure to Cd led to significantly higher tissue Cd levels in all treatment groups compared to the controls (Fig. 4.5; Table 4.6), with fasted individuals accumulating the highest concentrations (Table 4.6). For animals fed with mussels, the order of the Cd concentration in these tissues was ranked: waterborne Cd-exposed > dietary Cd-exposed > control animals (SNK, $p < 0.05$). In addition, partial correlation analysis (corrected for body weight) indicated that growth rate declined with increasing concentration of Cd in the tissues of fed *N. lapillus* in the control ($r = -0.3873$, $n = 66$, $p = 0.001$) or Cd-exposure groups ($r = -0.3655$, $n = 45$, $p = 0.012$) (Fig. 4.6).

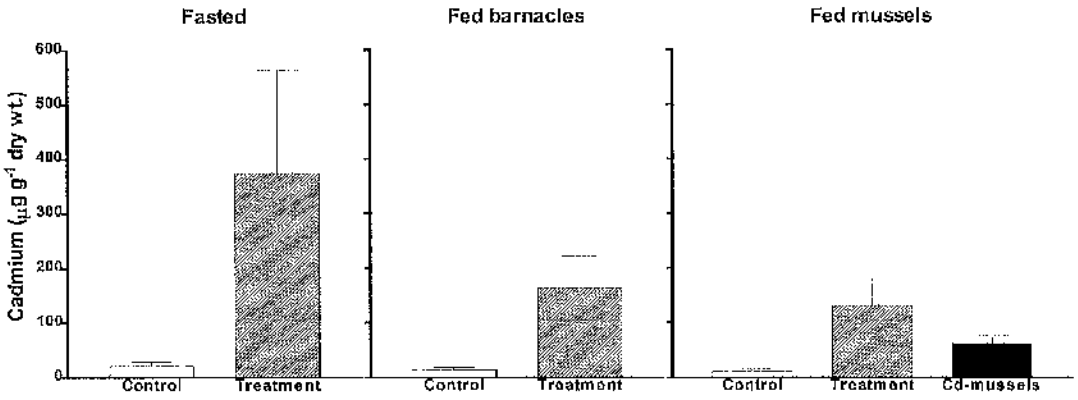

Figure 4.5. Cadmium concentration in the digestive gland/gonad complex of *Nucella lapillus* in the controls (open bars); exposed to waterborne Cd (shaded bar); and fed with Cd-dosed mussels (solid bar). Mean and SD are presented.

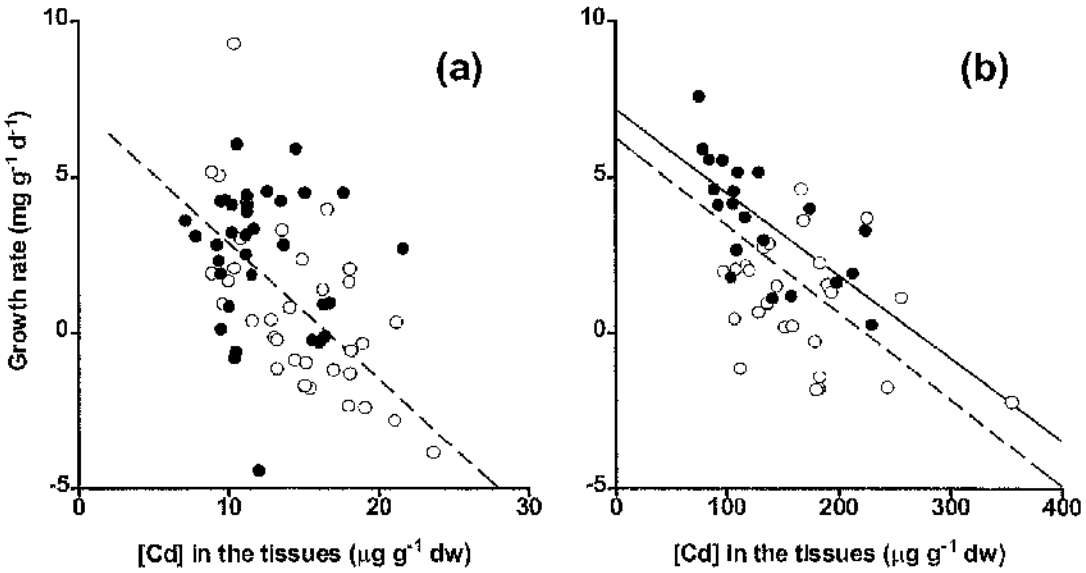

Figure 4.6. Relationship between the estimated growth rate and cadmium concentration in the digestive gland/gonad complex of (a) control and (b) Cd-exposed *Nucella lapillus* which were fed with barnacles (- - o - -) or mussels (—•—).

Table 4.6. Results of general linear model outputs for Cd, MT and glycogen data.

Parameter	df1, df2	F value	Significance of F	
[Cd] in digestive gland/gonad complex				
[groups 1-6]				
Effect of Cd-exposure	1, 156	370.308	< 0.001	***
Effect of diet	2, 156	47.596	< 0.001	***
Interaction	2, 156	41.343	< 0.001	***
[groups 5-7]				
Effect of treatment	2, 86	147.072	< 0.001	***
[MT] in the Leiblein gland				
[groups 1-6]				
Effect of Cd-exposure	1, 156	278.742	< 0.001	***
Effect of diet	2, 156	8.16	< 0.001	***
Interaction	2, 156	11.20	< 0.001	***
[groups 5-7]				
Effect of treatment	2, 86	36.004	< 0.001	***
MT content in the Leiblein gland				
[groups 1-6]				
Effect of Cd-exposure	1, 156	1094.505	< 0.001	***
Effect of diet	2, 156	22.248	< 0.001	***
[groups 5-7]				
Effect of treatment	2, 86	171.236	< 0.001	***
Glycogen in foot muscles				
[groups 1-6]				
Effect of Cd-exposure	1, 150	11.527	0.219	
Effect of diet	2, 150	25.737	< 0.001	***
Effect of sex	1, 150	6.355	0.013	*
[groups 5-7]				
Effect of treatment	2, 83	2.093	0.130	
Effect of sex	1, 83	4.831	0.031	*
Glycogen in digestive gland/gonad complex				
[groups 1-6]				
Effect of Cd-exposure	1, 156	0.016	0.901	
Effect of diet	2, 156	4.475	0.013	*
[groups 5-7]				
Effect of treatment	2, 86	2.161	0.121	

Asterisks denote *$p < 0.05$ and ***$p<0.001$.

The concentrations of MT in the Leiblein gland of *N. lapillus* were similar across all control groups, ranging from 264 to 326 μg g^{-1} wet wt. (Fig. 4.7a). Cd exposure through water or diet resulted in a significant elevation of MT in this tissue (Fig. 4.7a; Table 4.6). Within the treatment groups, the highest MT levels were noted in the fasted and barnacle fed *N. lapillus*, followed by those fed with normal or Cd-dosed mussels. Since we noted that the Leiblein glands of *N. lapillus* fed with mussels were generally bigger and heavier than the other groups, MT content per whole Leiblein gland was

calculated (Fig. 4.7b). There were significant differences in the MT content in this gland among all control groups (Fig. 4.7b; Table 4.6); the order was ranked as: fed mussels > fed barnacles > fasted (SNK, $p < 0.01$). Among the Cd-exposed groups, the MT content in the Leiblein gland of *N. lapillus* fed on mussels and barnacles was similar and significantly greater than that of fasted ones (SNK, $p < 0.05$).

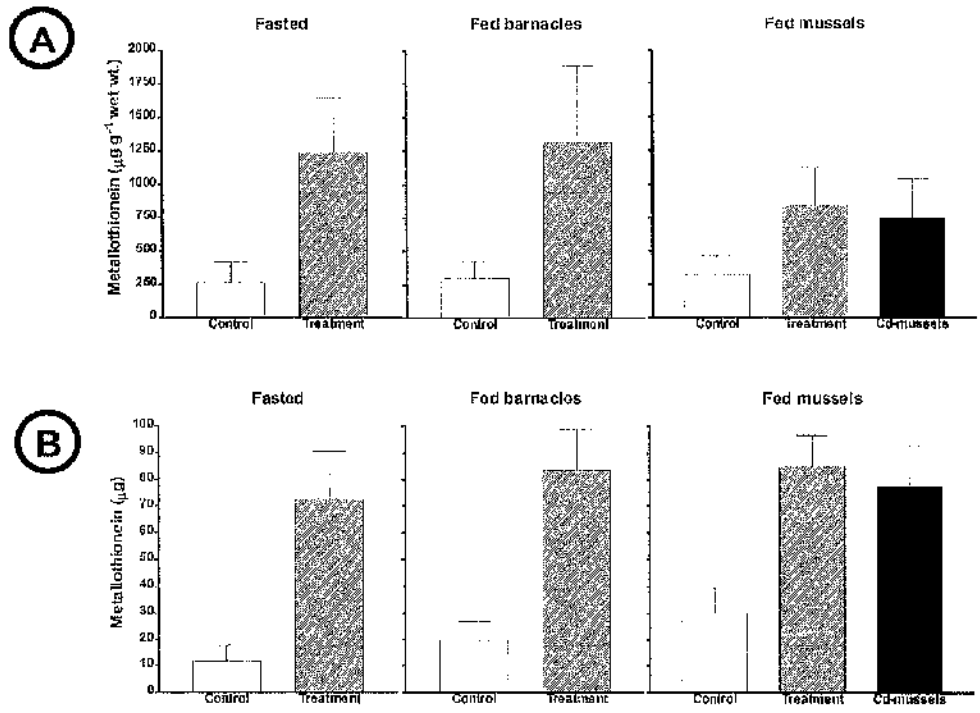

Figure 4.7. Concentration (A), and content (B) of metallothionein in the Leiblein gland of *Nucella lapillus* in the controls (open bars); exposed to waterborne Cd (shaded bar); and fed with Cd-dosed mussels (solid bar). Mean and SD are presented.

No significant effect of Cd-exposure on glycogen concentration in the foot muscles was observed (Fig. 4.8a; Table 4.6). Both fasted and barnacle-fed *N. lapillus* showed a similar foot muscle glycogen concentrations; values ranged from 9.6 to 12.8 µg g^{-1} wet weight. The foot muscles of *N. lapillus* fed on mussels had significantly higher glycogen levels (21.5-26.3 µg g^{-1}) than that of those fasted *N. lapillus* or those fed on barnacles (SNK, $p < 0.01$). Among mussel fed groups (groups 5-7), there was no significant difference in the concentration of glycogen in the foot muscles. The results of GLM revealed that sex had a significant effect on the glycogen concentration in foot muscles (Fig. 4.8b; $p < 0.05$). In general, male *N. lapillus* had a higher glycogen in their foot muscles, except in fasted or mussels fed control animals.

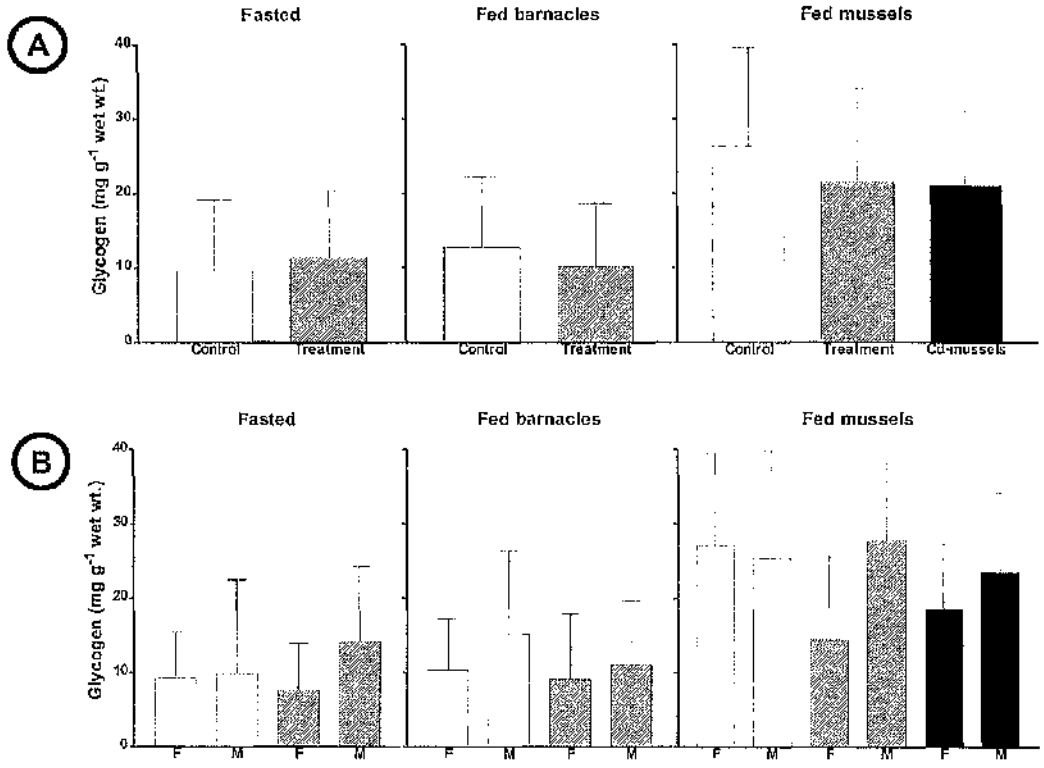

Figure 4.8. Concentration of glycogen in the foot muscles of *Nucella lapillus* in the controls (open bars); exposed to waterborne Cd (shaded bar); and fed with Cd-dosed mussels (solid bar): (a) pooled data from both sex and (b) data for each sex (F: female, M: male). Mean and SD are showed.

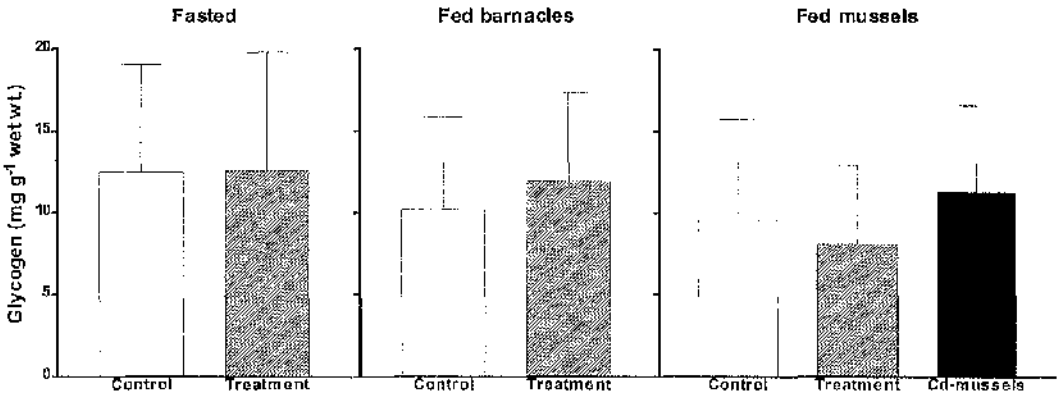

Figure 4.9. Concentration of glycogen in the digestive gland/gonad complex of *Nucella lapillus* in the controls (open bars); exposed to waterborne Cd (shaded bar); and fed with Cd-dosed mussels (solid bar). Mean and SD are showed.

As with foot muscles, there was no significant effect of Cd-exposure on glycogen concentration in the digestive gland/gonad complex (Fig. 4.9; Table 4.6). Both fasted and barnacle fed *N. lapillus* showed a similar glycogen concentration in these tissues; ranging from 10.2 to 12.6 μg g^{-1} wet weight. Concentrations of glycogen in these tissues of *N. lapillus* fed with mussels (8.1-9.6 μg g^{-1}) were slightly lower than in those fasted or fed with barnacles. Among mussel fed groups (groups 5-7), there was no significant difference in the concentration of glycogen in these tissues.

DISCUSSION

Effect of Nutritional State and Cd on Survivorship

It is evident that forced prolonged starvation significantly reduces the survival of *N. lapillus*. The dogwhelks fasted and exposed to Cd in water, showed the highest mortality rate. These results strongly suggest that starvation and Cd toxicity synergistically affect the dogwhelks. In order to survive, starved animals must minimise their energy expenditure as much as possible. As there is a metabolic cost to combat toxic effects caused by toxicants (Calow, 1991; Forbes and Calow, 1996), intoxification with Cd exerted extra energetic cost for starved individuals. Running out of energy reserves, the animals not only failed to protect themselves from Cd toxicity but also could not maintain normal function. Consequently, this synergistic effect caused mortality.

In general, longevity of a wild *N. lapillus* ranges from 5 to 7 years, with a maximum at ca. 9 years (Table 2; Moore, 1938b; Feare, 1969; Wright, 1976). Forced starvation and/or Cd exposure may greatly reduce the life expectancy of *N. lapillus* (Table 4.2). Long-term starvation during non-wintering seasons may decrease the expected life by 1 year while waterborne Cd exposure may reduce 1-2 years of life, depending on the nutritional state and prey type. Therefore, the results imply that both nutritional state and Cd toxicity play role in influencing life history of *N. lapillus*.

Another implication of the mortality results is that food consumption or energy intake is beneficial to the animals exposed to Cd. In an acute Cd-exposure study, glycogen stores of *N. lapillus* were found to be reduced in relation to the levels of Cd accumulated in the tissues, suggesting that there is a considerable cost for detoxification (Leung et al., in press). Food consumption thus helps to meet the extra

energy demand and provides the necessary nutrition, including vitamins, required to mediate poisoning effects of Cd. Chandini (1989) reached a similar conclusion. He demonstrated that *Daphnia carinata* with high food availability exhibited similar survivorship as the control animals, although exposed to a high Cd level (0.2 ppm). However, *D. carinata* fed at medium or low food levels significantly reduced in survivorship even at a low Cd level (0.05 ppm), despite that McGee et al. (1998) have recently shown that there is no significant difference in sensitivity to Cd between starved and fed juvenile amphipod *Leptocheirus plumulosus* through a comparison of 96-h LC_{50} values. However, we believe that the period over which this acute toxicity study was conducted was far too short to conclude whether there is a metabolic trade-off due to detoxification.

Growth Rate, Condition Index and Production

Growth is an important fitness component, and may have an overall impact on the success of natural populations (Burrows and Hughes, 1990; Wo et al., 1999). In addition, measurement of growth rate gives more sensitive and ecologically relevant responses of prosobranch molluscs to trace metals than the conventional lethal-concentration measurement such as 96h-LC_{50} (Thain, 1984; Gomot, 1997). Therefore, growth has been used as an indicator of pollution stress in marine invertebrates, such as the mussel *M. edulis* (Widdows, 1985), and amphipod *L. plumulosus* (McGee et al., 1998). In general, waterborne Cd exposure inhibits growth of intertidal gastropods. For example, growth of *Nassarius festivus* was significantly reduced after exposed to 160 μg Cd l^{-1} for 8 days indicated by scope for growth (Wo et al., 1999). The reduction of growth was 186% (relative to the control) for *Hydrobia ulvae* exposed to 160 μg Cd l^{-1} for 3 weeks at salinity 23‰ (Forbes and Depledge, 1992), while the reduction in percentage growth of *H. ventrosa* resulting from 3 weeks of exposure to Cd was only 24.2 at 100 μg Cd l^{-1} and 38.7 at 200 μg Cd l^{-1} at 23‰ (Forbes, 1991). Nonetheless, in our study, *N. lapillus* that survived exposure to 400 μg Cd l^{-1} for 80 days actually grew better than the control animals. Similarly, Walthall and Stark (1997) showed that surviving arthropod *Acyrthosiphon pisum*, were able to sustain higher rates of reproduction following acute exposure to an insecticide (imidacloprid).

Here, we propose a fitness hypothesis which may fairly explain why the survivors, *N. lapillus*, exposed to Cd grew faster. Individual *N. lapillus*, within each group, differ greatly in sensitivity and tolerance to Cd. For example, dogwhelks fasted and exposed

to Cd did not all die simultaneously, and only 55.6% mortality was recorded throughout the entire experimental period. If Cd exposure is a selection pressure, those individuals able to detoxify Cd and maintain normal function, are the ones that survive. Therefore, the survivors must have a better fitness in terms of genotypes or phenotypes (or both). Burrows and Hughes (1990) suggested that food consumption rate of *N. lapillus* can be used as an indicator for Darwinian fitness. Although there is no difference in estimated food consumption between control and Cd-exposed *N. lapillus* fed with barnacles, animals exposed to Cd and fed on mussels showed a higher consumption rate (127% of the control). Additionally, the consumption rate of *N. lapillus* fed with Cd-dosed mussels was 8% higher than the control. These results imply that these fast growers had a better fitness as reflected by their consumption. Indeed, it is also possible that these survivors might originally have better fitness (higher consumption and growth) in the natural habitat.

In terms of growth rate, our results are in agreement with the results of an early study on juvenile *N. lapillus* (Etter, 1996), in that prey type has a significant effect with mussels producing the fastest rate. The difference in growth rate is mainly explained by the difference in consumption rate and FCR. For example, an adult fed on barnacles to satiation would have an average consumption of 5.54 mg dry flesh d^{-1} (equivalent to 127.4 J; FCR: 5.7%) compared to a mussel fed individuals that would consume 8.11 mg d^{-1} (equivalent to 158.2 J; FCR: 22.9%). Less prey handling time and energetic cost of foraging might be attributed to the higher consumption rate when feeding with mussels.

Moore (1938b) observed that the rate of shell growth in *N. lapillus* significantly reduced towards ceasing at maturity (ca. 29.5 mm in shell length). As the mean shell length of dogwhelks used in this experiment was 30 mm, suggesting that they are mature, shell growth might have already ceased. We only observed a very small increment of shell length in the experimental animals and no significant difference in mean shell length between fasted and fed individuals.

No significant effect of Cd exposure was detected when using CI values as an indicator. These results suggest that CI is less sensitive to indicate stress responses when compared with growth measurement. Although CI can integrate stress responses in somatic growth, it can also be affected by nutritional and reproductive state (Phillips and Rainbow, 1993; Nicholson, 1999). The present results reveal that nutritional state and prey type, are rather more important factors affecting the CI values of *N. lapillus*

than Cd exposure. Nevertheless, CIs can indicate the significant effect of prey type and nutritional state on the growth of dogwhelks. In some cases, if growth measurement is not possible, CI could be at least useful to reflect the nutritional state and general growth performance of individual biomonitor populations that is very useful to interpret data of metal accumulation (Leung and Furness, 1999a; Nicholson, 1999).

In addition to study toxic effect on individual organisms, ecotoxicology also considers the impact of pollutants on populations, communities and ecosystems (Truhaut, 1977). In this way, measurements of net production used in the present study (integrating both production and mortality pointing to the impact of Cd at population levels), are more ecotoxicologically founded. Furthermore, population growth rate is a better predictor of toxic effects of toxicants than the mortality measurement alone (Walthall and Stark, 1997). In this study, Cd toxicity caused a great reduction in the net production, represented by negative values (Table 4.4). The reduction of net production in fasted *N. lapillus* by Cd was 2.0 times of the control value while the equivalent reductions in dogwhelks fed with barnacles and mussels were 7.3 and 3.8 times, respectively. These results are in opposition to those obtained by individual growth measurements on survivors but indicate the overall effect of Cd toxicity on population growth.

The present results also provide evidence that reduction of growth can be associated with Cd accumulation. Individual growth rate of *N. lapillus* reduced with increasing Cd in the digestive gland/gonad complex of control or Cd-exposed fed animals. The digestive glands and gonad are two of the major sites for storage of Cd in *N. lapillus*, the others being the Leiblein gland and kidney (Leung and Furness, 1999b). These results also indicate that there are individual differences in metal burden, indicated by the variability of Cd concentrations in the digestive gland/gonad complex of the control *N. lapillus*. As the excretion rate of Cd is extremely slow in this species (Leung and Furness, 1999b), the amount of Cd in the tissue might be related to the uptake rates of other trace metals and xenobiotics. Therefore, the higher the Cd concentration in the tissues, the higher the accumulation of contaminants. As a result, poor growth rate and fitness were associated with high levels of Cd in the tissues. Undoubtedly, Cd can inhibit growth of *N. lapillus* when its concentration reached a toxic level as suggested by the inverse correlation between growth and Cd concentration in the tissues. However, the tissue-dilution effect may also explain why the fast growers have less Cd in the tissues.

Waterborne Cd exposure has been shown to reduce food consumption rate of prosobranch molluscs (e.g. Lam, 1996; Wo et al., 1999), though feeding rates of mudsnails *H. ulvae* were unaffected by Cd exposure even at high levels (Forbes and Depledge, 1992). In our study, Cd exposure did not change the estimated consumption rates of *N. lapillus* fed with barnacles whereas Cd-exposed individuals exhibited a slightly higher consumption of mussels. However, due to lack of data on individual feeding rates (as it might vary individually), statistical analyses could not be performed. Further study on the effects of Cd on feeding in this species is needed to confirm the present results.

Cd Accumulation and MT Induction

Cd accumulation in the dogwhelks was significantly enhanced by Cd-exposure in water. Concentrations of Cd in the digestive gland/gonad complex of fasted *N. lapillus* were 2.3-2.8 times higher than that of fed animals following chronic exposure to Cd, indicating the effects of (1), tissue wastage in fasted individuals and (2), tissue dilution in fed animals, on the Cd accumulation. Similar effects of tissue dilution or wastage have been widely reported in various bivalve molluscs (Phillips and Rainbow, 1993; Langston and Spence, 1995). Nevertheless, the present results provide further evidence that accumulation of trace metals, especially Cd, in gastropod molluscs is also dependent on nutritional state and growth. Although it has been suggested that biomonitors from different populations should have identical growth rate and size (Langston and Spence, 1995), this idea is virtually impractical. Our results point to an urgent need for putting biological data such as growth rate, CI and prey type into biomonitoring programmes which would greatly help interpretation of pollutant data. Otherwise, misinterpretation of data might occur if the metal concentrations in biomonitors were strongly associated with nutritional state and growth rather than the level of contamination in the marine environment, especially when comparing different populations.

Young (1977) suggested that food consumption is the major route for accumulation of Zn and Fe by *N. lapillus* while seawater provides no more than 1% of the dogwhelk's uptake of these metals. In his study, the dogwhelks were starved for 50 days in seawater containing ^{65}Zn (14 $\mu g\ l^{-1}$) and ^{59}Fe (3.3 $\mu g\ l^{-1}$), or fed on barnacles, *Balanus balanoides*, rendered radioactive by feeding them on labelled *Artemia salina* nauplii (551 $\mu g\ ^{65}Zn\ g^{-1}$ and 84 $\mu g\ ^{59}Fe\ g^{-1}$). In contrast, in the present study, we used a relatively

high Cd concentration in seawater (400 μg l^{-1}) and a moderate Cd concentration in dosed mussels (61.4 μg g^{-1}); and observed higher Cd uptake through water than via food. The disparity between the results is probably due to the differences in the concentration of metal applied in seawater and diet. For example, Borchardt (1983) reported that ^{109}Cd uptake in *M. edulis* via algae was more efficient at low food levels while accumulation from seawater was linearly correlated with food quantities. He also suggested that the digestive route could only play a significant role if the diet was highly contaminated. It appears that *N. lapillus* can accumulate Cd from both seawater and food intake but the rate of uptake is dependent on the concentration in these mediums. Concentration of Cd in seawater in coastal or estuarine areas, varies from 0.01 to 85.0 μg l^{-1} (Philips, 1980) while Cd in mussels and barnacles are 1.8-34.6 μg g^{-1} dw and 0.15-6.29 μg g^{-1} dw, respectively (Philips, 1980; this study). One approach to showing the problem of whether the food pathway or seawater is the major uptake route of Cd in *N. lapillus* could be to use the concentrations of ^{109}Cd that represent the levels occurring in the natural marine environment (e.g. 5-50 μg l^{-1} in seawater and 5-30 μg g^{-1} dw in mussels) in an experimental set-up similar to this one.

Despite the fact that MT research began in the 1950s (Nordberg, 1998), study and quantification of MT in biomonitors have only developed since the mid 1980s. However, most laboratory studies on MT induction in marine invertebrates were conducted using fasted animals, and the effect of feeding on MT induction is virtually unknown. An attempt, therefore, has been made in this study to investigate whether fasting or feeding affect the MT concentration in *N. lapillus*, since any change in growth rate by nutritional state, can influence the concentrations of MT-inducing metals (e.g. Ag, Cd, Cu, Hg and Zn), and may indirectly affect the MT induction. Waterborne Cd exposure consequentially induced MT synthesis in the Leiblein gland of *N. lapillus*. Similar to Cd accumulation, tissue dilution effect on the concentration of MT was also noted in dogwhelks fed with normal or Cd-dosed mussels (which grew faster). Among the control groups, the highest MT content was found in dogwhelks fed with mussels, followed by those fed with barnacles while fasted animals presented the lowest MT content. Within the Cd-exposure groups, the MT contents in this tissue of fed *N. lapillus* were higher than that of fasted animals. The pattern of the MT content is similar to the one observed for growth rate (Fig. 4.3 and 4.7b). Synthesis of MT requires amino acids (especially cysteine), enzymes and energy, which can be obtained directly or indirectly from the diet. In fasted *N. lapillus*, the induction of MT might have drawn on body

reserves which could be one of the limiting factors for MT induction. On the contrary, fed animals have more efficient anabolism, as there is a continuous supply of resources from food (Schmidt-Nielsen, 1990). Our results highlight that growth rate, which is affected by nutritional state and prey type, has a significant effect on synthesis and concentration of MT in *N. lapillus*. Indeed, a recent study on baltic clam *Macoma balthica* also indicated that concentrations of MT-like protein in this bivalve species are greatly influenced by body weight which varies in different populations and seasons (Mouneyrac et al., 2000).

The dogwhelks fed with Cd-dosed mussels had very low mortality (similar to the control) and comparatively lower Cd concentrations in the tissues (only five times greater than control level, and half that of animals fed on mussels and exposed to Cd in water (Fig. 4.6)). However, MT levels in the Leiblein gland of these two mussel-fed treatment groups (dietary or waterborne Cd exposed) were similar. The low mortality in animals fed with Cd-dosed mussels may be explained by their relatively lower Cd accumulation, implying lower Cd toxicity compared with those exposed to waterborne Cd. In barnacles *Semibalanus balanoides*, the majority of detoxified zinc is stored as phosphate granules, which are excreted directly by *N. lapillus* in faeces, and are therefore biologically unavailable (Nott and Nicolaidou, 1990). Thus, Cd uptake from Cd-dosed mussels by *N. lapillus* could be in a less bioavailable form, resulting in lower toxicity. Nonetheless, this would not explain why animals fed with Cd-dosed mussels had a similar MT induction as those with higher Cd accumulation after being exposed to waterborne Cd. It may be that the form and route of Cd uptake could affect the MT induction in addition to the effect of Cd concentration. In seawater, Cd exists mainly as chloro-complexes ($CdCl^+$, $CdCl_2^0$, $CdCl_3^-$) with only ca. 2.5% present as free (hydrated) ions Cd^{2+} (Rainbow, 1997). Such forms of Cd can be transported through body surfaces such as gills (e.g. via calcium channel, organic ligand-mediated transportation and active transport pump). On the other hand, Cd in dosed mussels is mostly bound to proteins or stored in granules. The uptake of dietary Cd mainly occurs in the whole digestive system. Thus, differences in Cd species and transportation routes within the organism might lead to differences in biochemical responses and thus MT induction in the Leiblein gland, but these effects have yet to be tested.

Glycogen Stores

In general, dogwhelks store more glycogen in the foot muscles than in the digestive gland/gonad complex. This is possibly that there is a higher metabolic demand for locomotion activities and mucus secretion in the foot muscles (Voltzow, 1994). In accord with a similar pattern to the one observed for growth, feeding with mussels lead to higher glycogen reserves in their foot muscles. This suggests that glycogen stores are directly contributed to by food consumption. Moreover, fasted individuals had slightly higher level of glycogen in the digestive gland/gonad complex, suggesting a different energy allocation strategy although this may be a result of tissue wastage.

Glycogen represents the readily mobilisable storage form of glucose for most organisms. Lagadic et al. (1994) suggested that glycogen could be a useful biomarker since changes in glycogen concentrations are not as transient or sensitive to non-toxicant stress. In general, waterborne Cd exposure results in reduction of glycogen in marine organisms (e.g. Abdullah and Ireland, 1986; Gil et al., 1989; Cattani et al., 1996). It came as a surprise that there was no significant effect of Cd on glycogen concentrations in the foot muscles and digestive gland/gonad complex of fasted or fed *N. lapillus* in this study. These results are in disagreement with previous acute studies in which glycogen stores in fasted dogwhelks were significantly reduced by Cd exposure in water (Abdullah and Ireland, 1986; Leung et al., in press). However, our chronic study lasted 80 days (compared with < 20 days in the acute studies), and therefore metabolic reserves of fasted animals could have been depleted to threshold levels since mortality began on Day 40 of the experiment. Though it might be argued that there is no difference in glycogen concentrations of the foot muscles and digestive gland/gonad complex between fasted dogwhelks and those fed with barnacles. Growth data indicate that feeding with barnacles increased biomass in *N. lapillus* but fasted individuals lost weight. Therefore, in terms of glycogen content per individual (glycogen concentration × biomass), higher glycogen content would be expected in animals fed with barnacles. In another chronic study, adult *N. lapillus*, which were exposed to 500 μg Cd l^{-1} for 60 days and then to depuration for 110 days, showed a significant decline in CI values during the beginning of the exposure period (Leung and Furness, 1999b). However, the CI values were similar as that of the control animals after further starvation of 110 days. This might imply that the metabolic reserves have

already been expended during prolonged starvation and reaching a threshold limit, regardless of the extra energetic cost for detoxification.

A continuous input of energy (via food consumption) may help meet the basal and extra energy demands for combating Cd toxicity without depleting the glycogen reserves. Therefore, there was no observable difference in glycogen concentrations in fed animals between control and Cd-exposed groups. Clearly, glycogen concentration in this species can be strongly influenced by growth, which is affected by nutritional state and prey type. As these physiological factors can complicate interpretation of responses, there is some doubt as to whether glycogen could be used as an indicator of stress caused by pollutants.

Effect of Sex

Sex and reproductive state may affect metal burden and glycogen utilisation in marine invertebrates (Langston and Spence, 1995). Nevertheless, there were no significant effects of sex on mortality, growth, Cd accumulation and MT induction in *N. lapillus* throughout all treatment groups. Moore (1938b) also observed that there were no between sex differences in shell growth of this species. Interestingly, male *N. lapillus* had higher glycogen concentrations in the foot muscle, suggesting that there might be some sexual dimorphism in glycogen utilisation in this species. This could be explained by possible differences in behaviour and energy allocation (males are likely be more active while females possibly store more glycogen in other tissues such as gametes for reproductive purposes). This proposed hypothesis of sexual dimorphism in glycogen utilisation is an obvious choice for further investigation.

CONCLUSIONS

One of the major purposes of ecotoxicological research is to establish relevant knowledge in order to aid in the prediction of actual effects of pollutants on wildlife as they occur in the real world. The present study for the first time has revealed that nutritional state and prey type not only have substantial effects on survival, growth, but also affect Cd accumulation, MT induction and glycogen store in control *N. lapillus* and those exposed to Cd. Prolonged starvation and Cd-exposure synergistically reduced the survivorship of *N. lapillus*, whereas feeding could minimise mortality. Thus, results of conventional acute toxicity tests could be confounded by the nutritional state of test animals. Extended fasting also caused weight loss, leading to

higher concentrations of Cd and MT in their tissues, whereas fed animals increased in weight and had relatively lower Cd and MT concentrations because of the tissue dilution effect. Prey type significantly affects the growth rate of dogwhelks and indirectly influences Cd accumulation, MT induction and glycogen stores. Feeding on mussels promoted better growth and higher glycogen reserves than feeding on barnacles. It is also evident that individual growth rate decreased with increasing Cd in the tissues. In conclusion, these results point to the need to incorporate biological data including growth (or at least CI) and prey type into biomonitoring programmes in order to permit data interpretation.

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CHAPTER 5

Metallothionein Induction and Condition Index of Dogwhelk *Nucella lapillus* (L.) Exposed to Cadmium and Hydrogen Peroxide*

ABSTRACT

It has been suggested that metallothionein (MT) not only can regulate essential metals and detoxify toxic metals, but that MT can also play a significant role as an antioxidant and can be induced by oxidative stresses other than metals. This study aimed at investigating the effect of hydrogen peroxide (H_2O_2), and the combined effect of H_2O_2 and cadmium (Cd) on MT induction and condition index (CI) in dogwhelks *Nucella lapillus*. Adult male dogwhelks (27 ± 1 mm in shell length) were exposed for 20 days to (1), control (filtered natural seawater only); (2), 0.50 ppm Cd; (3), 2.0 ppm H_2O_2 + 0.50 ppm Cd; (4), 1.0 ppm H_2O_2 + 0.25 ppm Cd; (5), 2.0 ppm H_2O_2; (6), 1000 ppm H_2O_2 or (7), 1000 ppm H_2O_2 + 0.50 ppm Cd. The concentration of MT in the Leiblein gland of *N. lapillus* was quantified using the silver saturation method. MT or MT-like proteins in the animals were induced by Cd (0.5 ppm), H_2O_2 (2.0 ppm) or Cd + H_2O_2, indicating that MT in this gastropod species can be induced by either metal or oxidative stresses. Exposure to high H_2O_2 (1000 ppm) alone or combined with Cd, and exposure to Cd (0.50 ppm) or H_2O_2 (2.0 ppm), resulted in significant weight loss, indicated by a reduction of CI. However, CIs of groups (3) and (4) were similar to that of the control suggesting that Cd antagonistically reduces toxicity caused by H_2O_2 since Cd-induced MT may have a protective function against hydroxyl radicals.

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INTRODUCTION

Metallothioneins (MTs) are low molecular weight (ca. 6-10 kDa), cysteine-rich (25-33%) proteins which are induced by, and bind Cu, Zn, Cd, Hg, Ag and Au (e.g. seven equivalents of Zn and/or Cd or Cu per mole MT) (Carpenè, 1993; Aspholm and Hylland, 1998). The major functions of MTs in invertebrates include regulation of essential metals (e.g. Zn and Cu) and detoxification of toxic metals (e.g. Cd and Hg) (Roesijadi 1992, 1996). Therefore, it has been proposed that MTs can be used as biomarkers for metal pollution (Bebianno and Machado, 1997).

Research on mammals has demonstrated that MTs also play a significant role as antioxidants (like glutathione) and can be induced by other oxidative stresses. For example, rabbit liver (Cd, Zn)-, Zn-, and Cd-MTs can defend against hydroxyl and superoxide radicals (Thornalley and Vasak, 1985). Metallothionein induction by pre-treatment of mice with zinc protected against nickel-induced mortality and hepatic lipid peroxidation (Srivastava et al., 1993). Although MT induction by metals, especially Cd, has been widely studied in marine molluscs, very few *in vivo* study in molluscs has been conducted to investigate whether MT can be induced by oxidative stresses, and to study its function as an antioxidant (Viarengo et al., 1999).

The dogwhelk *Nucella lapillus* is a predatory prosobranch mollusc that lives on the rocky intertidal shores of Europe and eastern North America, and has been commonly used as a biomonitor for trace metal contamination, especially for tributyltin. A previous study demonstrated that Cd can induce Cd-MT in various tissues of *N. lapillus* (Leung and Furness, 1999). The present study was designed to investigate the effect of hydrogen peroxide (H_2O_2), and combined effects of Cd and H_2O_2 on MT induction by *N. lapillus*. Toxic effects caused by these chemicals are also quantified using a condition index.

MATERIALS AND METHODS

Experimental Animals

The dogwhelks *Nucella lapillus* were collected from Gourock, Clyde Sea, Scotland, at low tide mark during summer 1999. They were then acclimated in laboratory conditions with running seawater (34‰ and 10°C) and fed *ad libitum* with mussels

Mytilus edulis. They were fasted for 7 days before experimentation. During this acclimatisation period, the dogwhelks with similar shell length (27 ± 1 mm) were preliminarily sexed by checking for the presence of a well-developed penis (as male) and then divided into 7 groups (Table 5.1). Sex of each individual was reconfirmed after the entire exposure period through dissection and examination for the absence of sperm receiving gland. For each group, 15 male dogwhelks were caged, submerged in a 750 ml glass tank and exposed to: (1), control (filtered natural seawater only); (2), 0.50 ppm Cd (as $CdCl_2$); (3), 2.0 ppm H_2O_2 and 0.50 ppm Cd; (4), 1.0 ppm H_2O_2 and 0.25 ppm Cd; (5), 2.0 ppm H_2O_2; (6), 1000 ppm H_2O_2 or (7), 1000 ppm H_2O_2 and 0.50 ppm Cd. Seawater was renewed daily in order to maintain constant levels of H_2O_2 and Cd. Conditions of seawater were identical to those in acclimation. After 20 days of exposure, the animals were frozen at -25°C to await further analysis.

TABLE 5.1
Concentrations of Cadmium and Hydrogen Peroxide Applied for Each Experimental Group in This Study.

Group no.	Cd (ppm)	H_2O_2 (ppm)
1 (Control)	0.00	0.00
2	0.50	0.00
3	0.50	2.00
4	0.25	1.00
5	0.00	2.00
6	0.00	1000
7	0.50	1000

Condition Index

No mortality was observed throughout the experimental period. At the end of the experiment, the soft-body of each live dogwhelk was removed from the shells using a vice. All broken fragments of a shell were collected and dried at room temperature for 1 week before weighing. The wet weight of soft-body was measured after being blot-dried using an absorbent tissue. Condition Index (CI) of each snail was calculated using the following equation:

$$CI = [\text{wet soft-body weight}/(\text{wet soft-body weight} + \text{dry shell weight})] \times 100 \quad (1)$$

Metallothionein in the Leiblein gland

The Leiblein gland is one of the most important tissues for storing Cd and synthesising MT (Leung and Furness, 1999). The weighed whole Leiblein gland was homogenised with 0.4 ml of 0.25M sucrose using an Ultraturax (T25 Janke & Kunkel, IKA Labortechnik) at 4°C. The homogenate was centrifuged at 20,000*g* for 20 min at 4°C. Aliquots of 300 µl supernatant were analysed for MT content using the silver saturation method described by Leung and Furness (1999). Samples were incubated with 0.5 ml of 20 mg/litre silver solution for 20 min at 20°C to saturate the metal binding sites of MT. Excess silver ions were removed by the addition of 100 µl bovine red blood cell hemolysate to the assay tubes followed by heat treatment in a water bath (100°C for at least 10 min). The heat treatment caused precipitation of silver-bound haemoglobin and other proteins, except for MT, which is heat stable. The denatured proteins were removed by centrifugation at 1,200*g* for 10 min. The hemolysate addition, heat treatment and centrifugation were repeated three times in each sample. Finally, the supernatant was centrifuged at 20,000*g* for 10 min. The amount of silver metal in the final supernatant fraction which was proportional to the amount of MT present was determined by atomic absorption spectrophotometry using a Philips PU9200 A.A.S with deuterium background correction. Assay tubes containing purified horse kidney MT obtained from Sigma Chemicals in a range of concentrations from 2 to 20 µg underwent the same process in order to establish a calibration curve (µg Ag ml^{-1} vs. µg MT ml^{-1}) for MT quantification. The results of MT concentration and content were expressed as µg per g of wet tissue weight, and µg per Leiblein gland, respectively.

Data Analysis

Normality and homogeneity of variances of the data were checked using the Kolmogorov-Smirnov test and Bartlett's test, respectively. Differences in CI and MT between control and treatments were compared using one-way analysis of variance (ANOVA), with a subsequent comparison of all individual means against the control values using Dunnett multiple comparison tests. Student t test was also used to test the difference in mean values between two selected treatment groups.

RESULTS

Effects of Hydrogen Peroxide and Cadmium on Condition Index

No mortality was observed throughout the experimental period. There were significant differences in CIs between the control dogwhelks and animals exposed to Cd and/or H_2O_2 (ANOVA: $F_{6,98} = 9.883$, $p < 0.0001$) (Fig. 5.1). All treatments exhibited lower CI values than the control, except the groups (3) and (4), exposed to 2.0 ppm H_2O_2 + 0.50 ppm Cd, and 1.0 ppm H_2O_2 + 0.25 ppm Cd having a similar CI compared to the control (Dunnett's test: $p > 0.05$). Exposure to high H_2O_2 (1000 ppm) alone or combined with Cd, and exposure to Cd (0.50 ppm) or H_2O_2 (2.0 ppm) alone, caused a significant weight loss in *N. lapillus*, indicated by decreasing CIs. We also observed that animals exposed to 1000 ppm of H_2O_2 alone or combined with 0.5 ppm of Cd, suffered from a swollen foot and head as a result of increasing water ventilation rate.

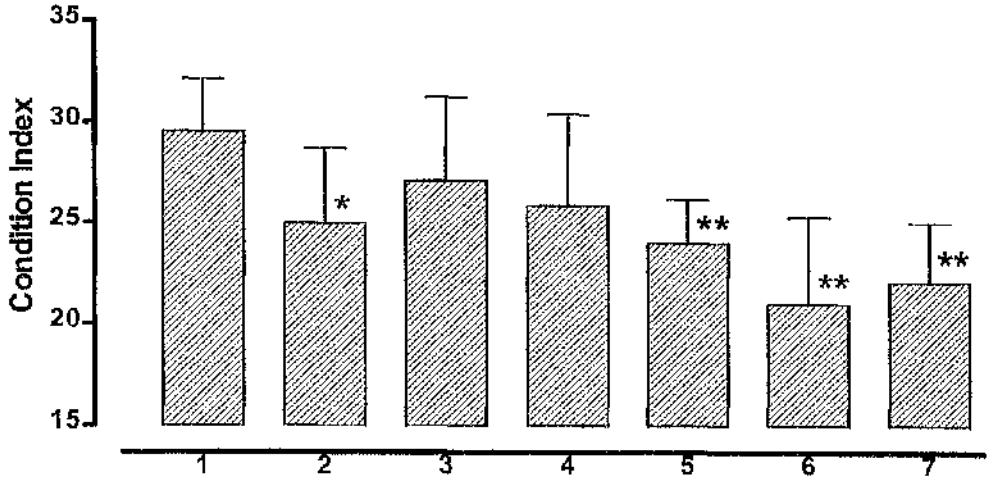

Figure 5.1. Condition index of *Nucella lapillus* exposed for 20 days to (1), control; (2), 0.50 ppm Cd; (3), 2.0 ppm H_2O_2 and 0.50 ppm Cd; (4), 1.0 ppm H_2O_2 and 0.25 ppm Cd; (5), 2.0 ppm H_2O_2; (6), 1000 ppm H_2O_2 or (7), 1000 ppm H_2O_2 and 0.50 ppm Cd. Mean and SD are presented. Asterisks indicate significant difference from control based on Dunnett's tests: * $p < 0.05$ and ** $p < 0.01$.

In order to investigate the combined toxic effect of Cd and H_2O_2, we compared the CI values between groups 2 and 3, groups 3 and 5, and groups 6 and 7, respectively using student t tests. The CI values were similar between groups 2 and 3, and groups 6 and 7 ($p > 0.05$). Nevertheless, the CI of dogwhelks exposed to 2.0 ppm H_2O_2 + 0.50 ppm Cd was significantly higher than that of those exposed to 2.0 ppm H_2O_2 alone ($t = 2.55$, DF = 28, $p = 0.0165$), suggesting that Cd might antagonistically reduce the toxicity caused by H_2O_2.

Effects of Hydrogen Peroxide and Cadmium on Metallothionein Induction

In general, MT concentrations in the Leiblein gland of treatments were higher than that of control (ANOVA: $F_{6,98} = 6.973$, $p < 0.0001$) (Fig. 5.2a). Among the treatments, dogwhelks exposed to 0.5 ppm Cd alone or combined with H_2O_2 (i.e. groups 2, 3 and 7) showed a significantly higher MT concentration than the control (Dunnett's test: $p < 0.01$). Exposure to 2.0 ppm H_2O_2 also lead to a significantly higher MT concentration in *N. lapillus* ($p < 0.05$). However, there was no significant difference in MT concentration between control dogwhelks and the animals exposed to 0.25 ppm Cd + 1.0 ppm H_2O_2 or 1000 ppm H_2O_2 (i.e. groups 4 and 6). In addition, only the dogwhelks exposed to 2.0 ppm H_2O_2 and 0.50 ppm Cd, had a significantly greater MT content than the control (Fig. 5.2b).

To study the effects of Cd and H_2O_2 on MT induction, we compared the MT concentrations between groups 2 and 3, groups 3 and 4, groups 3 and 5, and groups 6 and 7, respectively using student t tests. The MT concentrations were similar between groups 2 and 3, and groups 6 and 7 ($p > 0.05$). The dogwhelks in group 3, exposed to 0.25 ppm Cd + 1.0 ppm H_2O_2, showed a significantly lower MT concentration than in group 4 (exposed to 0.5 ppm Cd + 2.0 ppm H_2O_2) ($t = 6.386$, DF = 28, $p < 0.0001$). Furthermore, the MT concentration of dogwhelks exposed to 2.0 ppm H_2O_2 + 0.50 ppm Cd (group 3) was significantly higher than that of those exposed to 2.0 ppm H_2O_2 (group 5) alone ($t = 2.748$, DF = 28, $p = 0.0104$). The significant higher MT induction in groups 2 and 3, indicated that Cd or Cd + H_2O_2 promoted a higher induction of MT than H_2O_2 although exposure to H_2O_2 alone also induced MT synthesis.

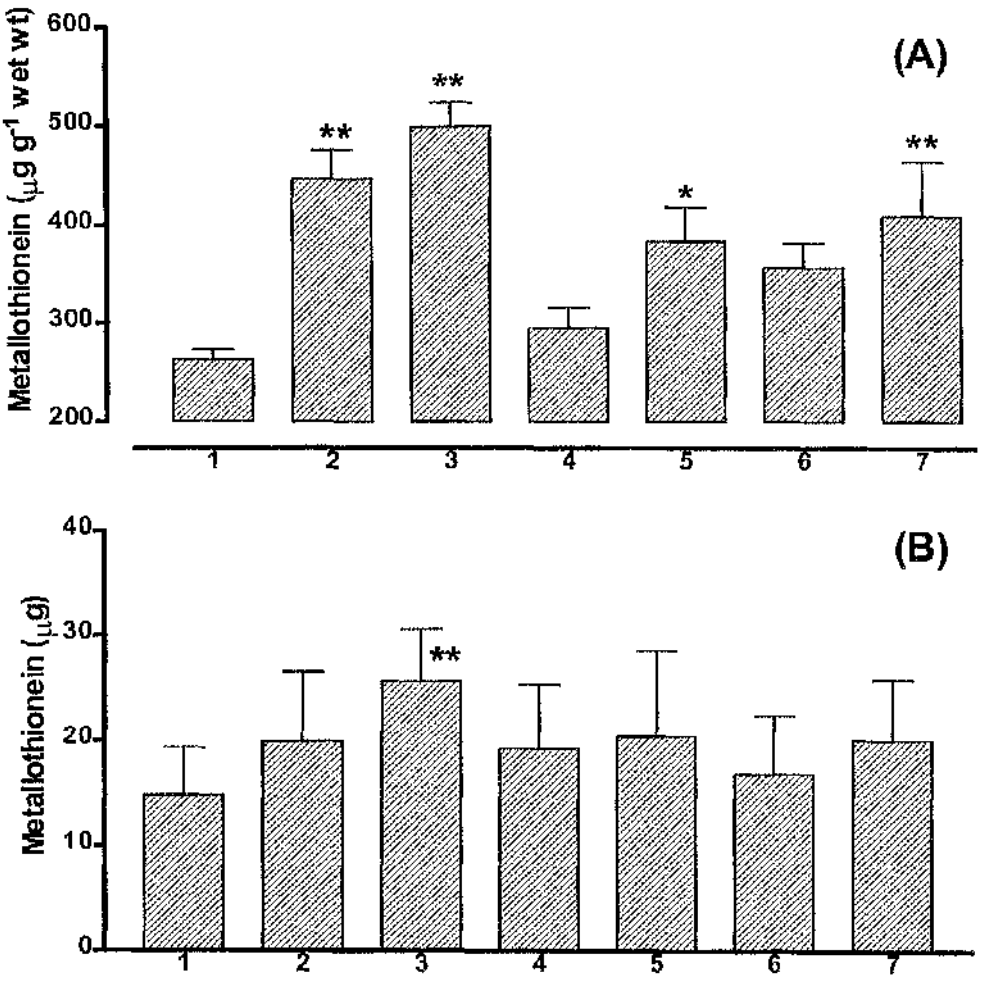

Figure 5.2. Metallothionein concentration (A), and content (B) in the Leiblein gland of *Nucella lapillus* exposed for 20 days to (1), control; (2), 0.50 ppm Cd; (3), 2.0 ppm H_2O_2 and 0.50 ppm Cd; (4), 1.0 ppm H_2O_2 and 0.25 ppm Cd; (5), 2.0 ppm H_2O_2; (6), 1000 ppm H_2O_2 or (7), 1000 ppm H_2O_2 and 0.50 ppm Cd. Mean and SD are presented. Asterisks indicate significant difference from control based on Dunnett's tests: $^*p < 0.05$ and $^{**}p < 0.01$.

DISCUSSION

Hydroxyl radicals ($HO^\cdot$) can be produced by the reduction of H_2O_2 by metal cations such as Fe(II) or Cu (I) (Fridovich, 1998). These radicals are extraordinarily powerful oxidants, which attack most organic compounds at diffusion-limited rates and cause oxidative damage to cell membranes, proteins and DNA; thus they are cytotoxic (Suzuki, 1996). The concentrations of H_2O_2 in rainwater collected in

Miami, ranged from 0.01 ppm to 1.31 ppm, with an average 0.35 ppm (Deng and Zuo, 1999). In coastal marine waters, the concentration of H_2O_2 increases in relation to the degree of anthropogenic pollution, ranging from 0.11 ppb to 0.56 ppb (Herut et al., 1998). In this study, H_2O_2 was therefore used as a model chemical to provide oxidative stresses to the dogwhelks. Intoxification of H_2O_2 (especially at 1000 ppm) increased the ventilation rate, indicated by a swollen body. This change in behaviour might be a strategy for detoxification. Dogwhelks exposed to H_2O_2 (2.0 or 1000 ppm) exhibited a significant lower CI (i.e. lost weight) implying that there is a metabolic cost for detoxification such as producing antioxidants (e.g. glutathione) and antioxidant enzymes; and toxicity of H_2O_2 may alter normal metabolism of this gastropod species.

It is evident that H_2O_2 (2.0 ppm) can induce MT-like proteins in *N. lapillus*. Nevertheless, the isoform(s) and mechanism of MT-like proteins induced by oxidative stress have yet to be established in this gastropod. The present results also raise a question whether total MTs can be used as a specific biomarker for metal contamination and toxicity. In coastal marine environments, oil pollution and sewage disposal may release a considerable quantity of oxidative substances, which might induce MT-like proteins in the biomonitors. To improve the present measurement using total MT, specific MT isoforms (e.g. Cd-MT or Zn-MT) should be quantified (Dallinger et al., 1997).

The dogwhelks exposed to 0.5 ppm Cd + 2.0 ppm H_2O_2 presented highest MT concentration in the Leiblein gland and CI value among all treatments, while animals exposed to 2.0 ppm H_2O_2 had comparatively low MT and CI. These results suggest that the present of Cd may protect *N. lapillus* from oxidative stresses by H_2O_2. *In vitro* studies using mammal and fish cell lines have revealed that MT can scavenge oxidative radicals, and thus reduce cellular damage caused by oxidative stresses (Wallace, 1996; Schlenk and Rice, 1998). Further, a recent *in vivo* study also demonstrated that Cd-preexposed mussels *Mytilus galloprovincialis* had a significantly higher tolerance and resistance to oxidative stresses, indicating the oxyradical scavenging role of Cd-induced MT (Viarengo et al., 1999). In this way, Cd-induced MT may function as an antioxidant in the dogwhelks.

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CHAPTER 6

Effects of Animal Size on Concentrations of Metallothionein and Metals in Periwinkles *Littorina littorea* Collected from the Firth of Clyde, Scotland*

ABSTRACT

Different sizes of *Littorina littorea* were collected from four areas in the Firth of Clyde, Scotland. Their metallothionein (MT) and heavy metal concentrations were analysed using the silver saturation method and atomic absorption spectrophotometry respectively. Concentrations of MT, Cd and Zn (as $\mu g\ g^{-1}$ dry soft-body weight) generally decreased with an increase in size of *L. littorea*. MT concentrations were better correlated with Cd than with Zn or Cu concentrations. Nevertheless, MT and the metals in periwinkles (as μg individual^{-1}) increased significantly with increasing size. Concentrations of MT and the metals among the sampling areas were compared at a standardised soft-body weight (10 mg). The results from discriminant analysis based on all metal concentrations indicate that Largs is different from the other areas and characterised by high Fe concentrations in *L. littorea*. The problems and differences in using either soft-body weight or shell length as independent variable for size-standardisation are discussed.

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INTRODUCTION

At present, pollution by metals in the environment is generally monitored by measuring the levels of selected metals in whole organism or organs. However, any detrimental effects of such exposure will initially manifest themselves at the cellular level where measurement should be made for the greatest sensitivity (Haux and Forlin, 1988). Subcellular or biochemical biomarkers have the advantage that they are specific to a given group of contaminants and that it is possible to relate the effect to essential cellular processes (Depledge and Fossi, 1994). It has been suggested that measurement of metal binding proteins, especially metallothionein (MT) in aquatic animals may be useful in evaluating metal exposure as well as predicting metal toxicity since cellular toxicity may result after the metal binding capacity of MT has been exceeded (Roseijadi, 1992; 1996). MT level increases following metal exposure and generally relates to the concentration of heavy metals in the animal (Benson *et al.*, 1990). However, bioaccumulation of heavy metals in marine invertebrates may be affected by factors such as environmental conditions and, the age, size and feeding rate (Phillips, 1990). These factors therefore may affect the level of MT in the biomonitors. If measuring MT in a monitoring programme, these factors should be carefully considered (Mouneyrac *et al.*, 1998).

The earliest monitoring studies have shown that animal size is an important independent variable influencing metal levels in marine gastropods such as dogwhelk *Nucella lapillus*, and limpet *Patella vulgata* (Nickless *et al.*, 1972; Peden *et al.*, 1973; Stenner and Nickless, 1974). Boyden (1974, 1977) showed that levels of metal in molluscs, including gastropods, were generally dependent on size. For example, concentrations of Fe, Mn, Pb and Zn in periwinkles *Littorina littorea* (as $\mu g\ g^{-1}$ dry soft-body weight) decrease with increasing soft-body weight while heavy metal contents in individual periwinkles (μg individual^{-1}) always increase significantly according to size (Boyden, 1977). Recently, Mouneyrac *et al.* (1998) have also observed an inverse relationship between weight of soft tissues and metals (Cd, Cu and Zn) or MT-like protein concentrations in the whole soft tissues of oysters *Crassostrea gigas*. Nevertheless, the effect of animal size on the MT levels in marine gastropods has not been studied.

MT-like proteins have been found in *L. littorea* (Langston & Zhou, 1986, 1987; Bebianno *et al.*, 1992; 1995). This gastropod species has been used for monitoring heavy metal pollution in the UK (Ireland & Wootton, 1977; Bryan *et al.*, 1983; Langston & Zhou, 1986). The present study aims to investigate the effect of animal size on MT concentrations and on the bioaccumulation of trace metals (including Cd, Cu, Fe and Zn) in *L. littorea* collected from four different sites in the Firth of Clyde, Scotland.

Comparisons of metal concentrations among sampling areas are commonly based on a comparison at a standardised size of biomonitor. Size of a periwinkle can be represented by its dry soft-body weight, shell length, or shell weight. Although relating metal content of soft-body tissues to weight of soft-body has been accepted and commonly used for expressing analytical results of 'Mussel Watch' programmes, the great variability of soft-body weight has been recognised as a limitation of this conventional approach (Fisher, 1983). A suggested alternative is to relate bioaccumulated metal content with a less variable parameter, e.g. with the protein fraction of the soft-body (Zaroogian, 1980); with the shell weight (Fischer, 1983; Marigómez *et al.* 1990); with shell length and age measured by *in situ* studies of shell growth (Cain & Luoma, 1990). In the present study, the effects on using different independent variables: (1) dry soft-body weight and (2) shell length, for size-standardisation procedure are also investigated.

MATERIALS AND METHODS

Sampling Areas

Different sizes of periwinkle *L. littorea* were collected randomly from four different areas, namely Gourock (G), Largs (L), Dunoon (D) and Loch Fyne (LF), in the Firth of Clyde, Scotland (Fig. 6.1). Water and sediment quality in the Gourock, Largs and Dunoon areas are influenced by sewage, heavy transportation, historically polluted sediments and surface run off from the River Clyde (Balls *et al.*, 1997; Edwards, 1997). Gourock is situated at the Clyde Estuary and regarded as a polluted area, Dunoon and Largs lie in the Clyde Sea so are likely to be of intermediate pollution status, while Loch Fyne is a relatively remote area, mainly serving for aquaculture and recreation purposes.

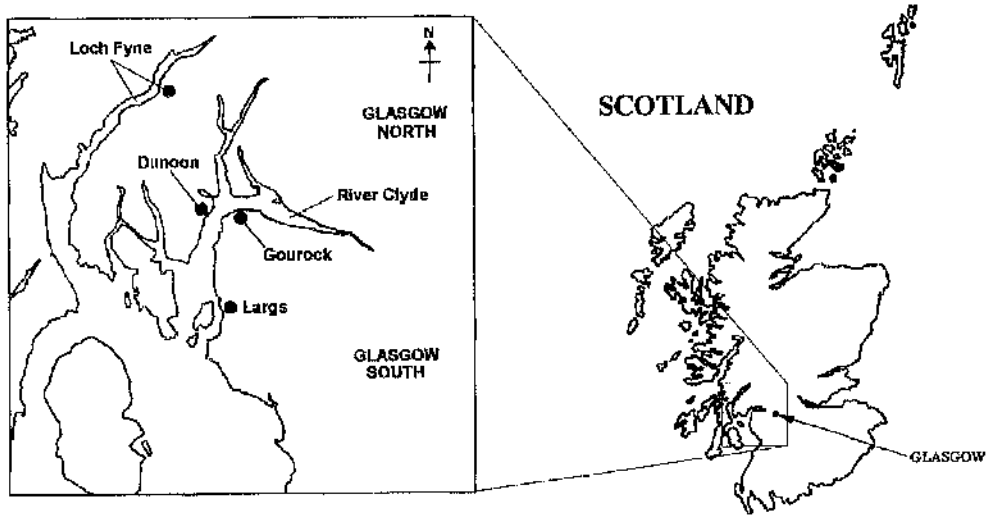

Figure 6.1. A map, showing the sampling locations in the present study.

Sample Preparation

The periwinkles were collected from the above areas near low tide mark in July 1997. They were acclimated in the laboratory with running seawater at 10 ± 1°C and 34 ‰ salinity for 48 hours to allow clearance of their gut contents. Afterward they were stored at -70°C for further analysis. Shell length of each individual gastropod had been measured before they were ranked, grouped and pooled for MT and metals analysis according to their shell length (Table 6.1). Mean shell length was then calculated for each pooled sample and used as one of the independent variables.

Metallothionein Analysis

For determination of MT contents, tissue samples were weighed and placed in a homogenising tube with a solution of 0.25 M sucrose in 1:4 w/v ratio. The mixture was homogenised using an Ultraturax (T25 Janke & Kunkel, IKA Labortechnik) at 4°C. The homogenate was centrifuged at 20,000*g* for 20 min at 4°C. Aliquots of 400 µl supernatant were analysed for MT content by the silver saturation method of Scheuhammer and Cherian (1991) with small modifications. First, bovine red blood cell hemolysate was used instead of using human blood cell hemolysate. Secondly, standard addition was applied for each sample using a freshly prepared solution of 20 µg l^{-1} purified horse kidney MT obtained from Sigma Chemicals in order to minimise

any sample matrix effects. The amount of silver metal bound to MT present was determined by atomic absorption spectrophotometry using a Philips PU9200 AAS. The MT contents were expressed in μg g^{-1} dry soft-body weight.

Table 6.1. Shell length (mean ± sd) and number of animals (*n*) pooled for MT and metals analysis.

Gourock		Largs		Dunoon		Loch Fyne	
mm	*n*	mm	*n*	mm	*n*	mm	*n*
10.1 + 0.8	10	4.5 ± 0.6	16	11.0 ± 1.3	11	8.5 ± 1.0	10
11.1 ± 1.8	10	6.3 ± 0.5	15	11.1 ± 0.8	10	10.9 ± 0.7	10
14.1 ± 0.9	10	6.4 ± 0.8	15	13.7 ± 0.6	10	11.6 ± 1.0	10
16.8 ± 1.0	10	6.6 ± 1.2	15	16.0 ± 0.8	10	13.4 ± 0.9	10
19.1 ± 1.1	10	8.8 ± 1.1	10	16.7 ± 0.6	10	13.6 ± 0.6	10
19.5 ± 0.8	10	8.9 ± 1.0	10	16.7 ± 1.0	10	16.6 ± 0.7	10
21.2 ± 1.4	7	12.6 ± 1.1	10	18.7 ± 0.9	10	17.0 ± 1.0	10
21.6 ± 1.2	7	14.5 ± 0.6	10	19.0 ± 0.5	10	17.6 ± 0.9	10
21.7 ± 2.0	7	15.0 ± 0.7	10	19.0 ± 0.7	10	22.8 ± 1.0	10
		16.3 ± 0.8	10	20.5 ± 0.9	8	22.9 ± 1.1	10
		17.0 ± 1.0	10	20.8 ± 1.5	7	23.2 ± 1.3	10
		17.2 ± 1.0	10	20.9 ± 0.9	8	25.1 ± 0.4	4
		19.0 ± 0.7	10			25.8 ± 0.8	4
		20.2 ± 0.2	4			26.0 ± 1.1	4
		21.6 ± 0.2	3				

Dry Weight Determination

The water content of the homogenate of whole body tissue was obtained by weight difference after drying at 80°C for 48 h. Dry weight of the tissue was obtained by the difference between the total dry weight and the amount of sucrose added. Mean soft-body weight for each pooled sample was then obtained by dividing the dry tissue weight with the number of animals pooled in the sample.

Metal Analysis

Samples of 0.5–1.0 g dried whole body tissue, were digested in 10 ml concentrated nitric acid and then diluted to 25 ml with distilled water. Metal concentrations were analysed using the Philips PU9200 AAS. Detection limits in the digested sample were 0.014 μg/g for Cd, 0.01 μg/g for Zn, 0.035 μg/g for Cu, and 0.01 μg/g for Fe. All metal concentrations were expressed on a dry weight basis. The reproducibility and accuracy of this method were tested by analysing a reference material, Tuna Fish Flesh (IAEA-

350) from the Marine Environment Laboratory, International Atomic Energy Agency of Monaco (Table 6.2).

Table 6.2. Comparison of metal concentrations (μg g^{-1} dry weight) in standard reference material IAEA-350 tuna fish flesh certified by the Marine Environment Laboratory, International Atomic Energy Agency of Monaco, and analytical results from the current study. (Mean and 95% confidence interval are shown)

Metal	Certified values		Our values (n=8)	
	Mean	Confidence Interval	Mean	Confidence Interval
Cd	0.032	0.018 - 0.050	0.049	0.030 - 0.067
Cu	2.83	2.55 - 3.10	2.76	1.24 - 4.28
Fe	72.1	66.7 - 77.3	73.4	56.9 - 89.9
Zn	17.4	16.6 - 18.5	17.3	16.0 - 18.5

Tissue Distribution

In order to investigate whether the major tissues for MT production represented the same percentage of whole soft-body for all sizes of periwinkles, kidney (n = 25), gonad/digestive gland complex (n = 25), kidney/gonad/ digestive gland complex (n = 26) were dissected from different sizes of periwinkles collected from Largs. The sex of the specimen was also identified and recorded. Both dissected and remaining tissues were dried at 80°C for 48 h to obtain a constant weight. Relative size of the dissected tissue was expressed as a percentage of whole soft-body weight on a dry weight basis and plotted against size (as mg dry soft-body weight). Results of analysis of covariance (ANCOVA), using size as covariate, showed that there was no significant difference between sexes. The regression analysis, therefore, was based on data from both sexes. Any significant differences in tissue in relation to size was tested by regression analysis (i.e. deviation from slope = 0).

Data Analysis

All regression analyses were carried out by using Log-transformed data and weighted by Log-number of animals pooled in a sample. Partial correlation analysis was also performed on the concentrations of MT and MT-inducing metals (including

Cd, Cu and Zn) with size correction. Analysis of covariance (ANCOVA), using Log-size (mg or mm) as covariate, was utilised to test (1) differences among the slopes of the regression between Log-metal content (μg individual^{-1}) and Log-size; and (2) the interaction between size and area. Contents of MT and metals (μg individual^{-1}) at size of 10 mg dry soft-body weight or 10 mm shell length were calculated from the above regression. Analysis of variance (ANOVA) was applied to test the differences between the mean values of MT and metal contents with a subsequent comparison between individual means using Tukey-Kramer Multiple Comparisons Test (Zar, 1989). Concentrations of MT and metals (μg g^{-1}) were calculated from their contents in individual animals. Discriminant analysis was also utilised to describe the results by grouping the areas using the concentrations of all metals measured. All data analysis was carried out using SPSS for Window 5.0 version with significance level $p < 0.05$.

RESULTS AND DISCUSSION

Across all individual samples, concentration of MT generally decreased with increasing size of periwinkles ($n = 50$, $r = -0.684$, $p < 0.001$; Fig. 6.2(a) & Table 6.3) while their MT contents (as μg individual^{-1}) increased significantly according to size of the specimen ($n = 50$, $r = 0.750$, $p < 0.001$; Fig. 6.2(b)). Similar results have also been demonstrated in a marine bivalve *Crassostrea gigas* (Mouneyrac *et al.*, 1998), suggesting that animal size of marine invertebrates (i.e. biomonitors) should be considered while applying MT in a monitoring programme.

Kidney and gill are suggested to be the major sites for MT-induction in *L. littorea* (Babianno and Langston, 1995). In an early study, digestive glands of periwinkles also showed significantly higher levels of MT than in the remaining tissues (Babianno *et al.*, 1992). In addition, metals are commonly accumulated by the kidney and digestive gland of *L. littorea* (Mason & Simkiss, 1983). If the growth rate or relative size of the above organs or tissues were different among different size of animals, this might explain why the MT and metals were size dependent. In the present study, an attempt has been made to test this hypothesis. The size of the kidney and gonad/digestive gland complex, in terms of dry weight to whole soft-body weight, is independent of size of *L. littorea* (Fig. 6.3(a),(b)). When the periwinkles were dissected into two parts: (1) kidney/gonad/digestive gland complex and (2) the remaining tissues including the

gills, no significant correlation was found between the relative size (measured as mg dry soft-body) of these tissues and dry soft-body weight (Fig. 6.3(c)). These results indicate that the relative size of these tissues is similar for all sizes of periwinkles. Therefore, the size effects on bioaccumulation of metals and production of MT are not due to the differences in relative size of these organs.

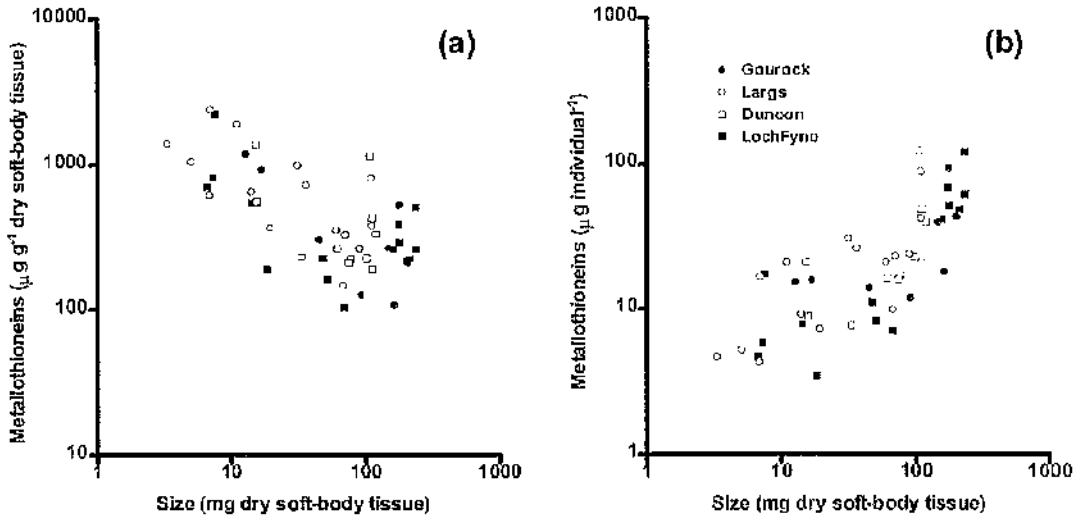

Figure 6.2. Scatter plots of size (as dry soft-body weight) against metallothioneins in *Littorina littorea* as (a) content (μg individual^{-1}) and (b) concentration (μg g^{-1} dry soft-body weight) on a double logarithmic basis.

Metallothioneins are low molecular weight cysteine-rich metal-binding proteins, which normally bind with d^{10} metal ions, such as Zn^{2+}, Cd^{2+}, Hg^{2+} and Cu^{+} (Wang *et al.*, 1996). In the present study, results of partial correlation analysis showed that MT was better correlated with Cd (n = 50, r = 0.4142, p = 0.003) after correcting for size but not with Zn (n = 50, r = 0.0343, p = 0.815) and Cu (n = 50, r = -0.0744, p = 0.611) in *L. littorea*. MT, therefore, is a good predictor of Cd in this biomonitor species. Similar to MT, Cd concentrations decreased significantly with increasing size in all areas except Dunoon (Table 6.3). Across all individuals, Cd is negatively correlated with size (n = 50, r = -0.652, p < 0.001; Table 6.3). Based on these findings, the size-dependent nature of MT may be associated directly with the Cd concentrations in *L. littorea*.

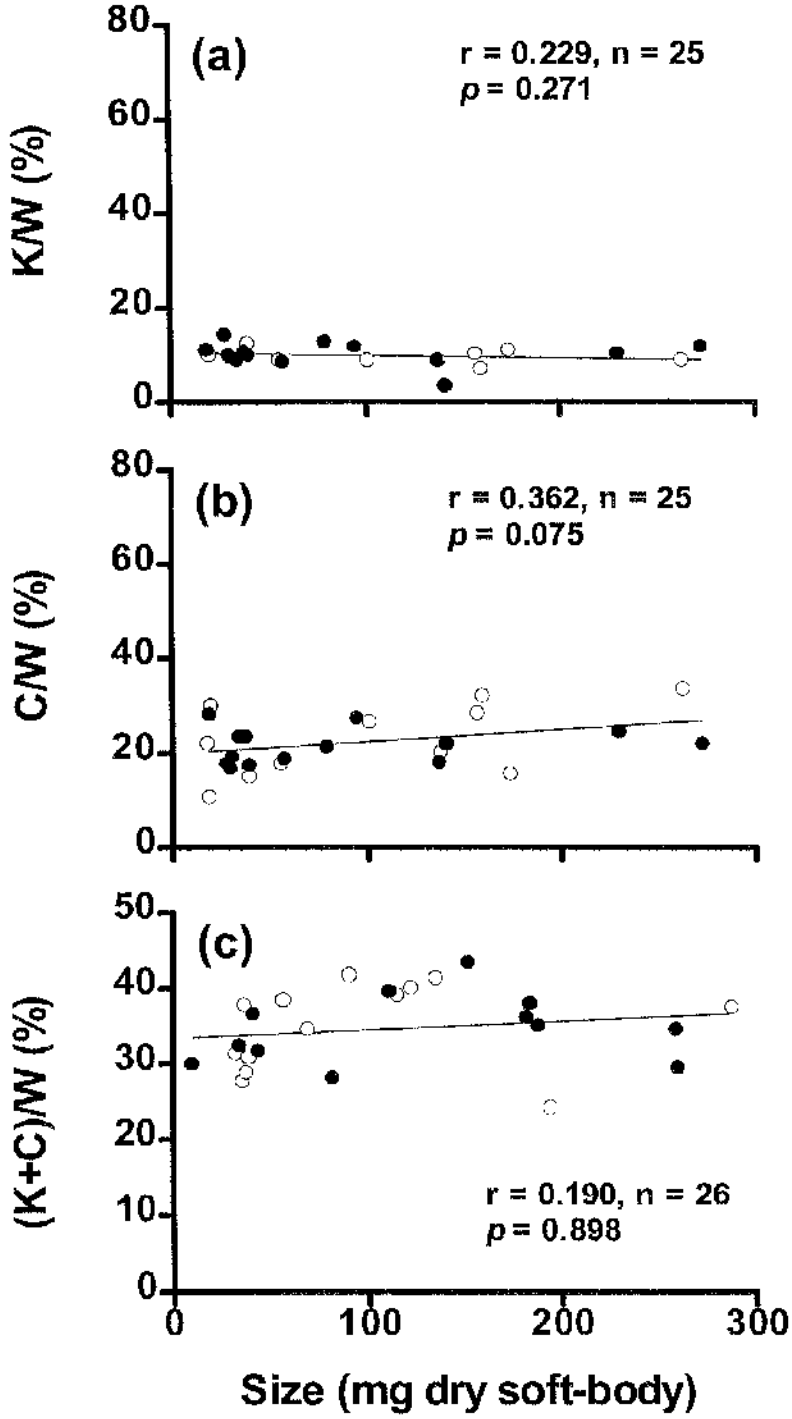

Figure 6.3. Relationship between whole soft-body dry weight and relative size, as percentage of whole soft-body dry weight (W) of separated tissues (a) kidney (K); (b) gonad/digestive gland complex (C); and (c) kidney/gonad/digestive gland complex (K+C). Solid and open circles denote male and female, respectively.

Table 6.3. Results of the weighted regression analysis. Log-concentration (μg g^{-1} dry soft-body) is regressed on log-dry soft-body weight (mg) and weighted by Log-number of individuals[a].

Area	*n*	log *a*	SE of *a*	*b*	SE of *b*	*r*	*p*
Metallothionein							
Gourock	9	3.623	0.366	-0.596	0.189	-0.767	0.0159*
Largs	15	3.460	0.178	-0.493	0.127	-0.734	0.0019*
Dunoon	12	3.234	0.451	-0.376	0.247	-0.434	0.1592
Loch Fyne	14	3.112	0.246	-0.340	0.141	-0.572	0.0326*
Overall	50	3.364	0.120	-0.456	0.070	-0.684	0.0000*
Cadmium							
Gourock	9	0.460	0.210	-0.362	0.108	-0.785	0.0122*
Largs	15	0.304	0.160	-0.323	0.113	-0.621	0.0135*
Dunoon	12	-0.108	0.354	-0.163	0.194	-0.257	0.4191
Loch Fyne	14	0.342	0.104	-0.302	0.060	-0.826	0.0003*
Overall	50	0.286	0.089	-0.310	0.052	-0.652	0.0000*
Copper							
Gourock	9	2.089	0.147	-0.102	0.076	-0.452	0.2214
Largs	15	1.467	0.100	+0.246	0.071	+0.694	0.0041*
Dunoon	12	2.143	0.115	-0.125	0.063	-0.532	0.0747
Loch Fyne	14	2.342	0.098	-0.200	0.056	-0.717	0.0039*
Overall	50	1.868	0.077	+0.020	0.045	0.065	0.6530
Iron							
Gourock	9	2.772	0.161	-0.286	0.083	-0.794	0.0106*
Largs	15	2.555	0.095	+0.293	0.068	+0.769	0.0008*
Dunoon	12	2.961	0.202	-0.185	0.111	-0.467	0.1258
Loch Fyne	14	2.581	0.116	-0.070	0.066	-0.294	0.3080
Overall	50	2.888	0.134	-0.174	0.078	-0.306	0.0309*
Zinc							
Gourock	9	2.286	0.095	-0.185	0.049	-0.819	0.0069*
Largs	15	1.915	0.093	+0.035	0.066	+0.145	0.6068
Dunoon	12	2.482	0.076	-0.280	0.042	-0.904	0.0001*
Loch Fyne	14	2.579	0.087	-0.302	0.050	-0.868	0.0001*
Overall	50	2.146	0.064	-0.094	0.038	-0.339	0.0161*

[a] *n* is sample size; Log *a* is the intercept; *b* is the regression coefficient and *r* is the correlation coefficient. Significant correlations are denoted by $p < 0.05$.

The present results support the idea of induction of MT in relation to Cd in *L. littorea* and agree with previous field and laboratory studies (Langston & Zhou 1986, 1987; Bebianno *et al.*, 1992). Roesijadi (1992, 1996) have reviewed and suggested that use of MT in assessing the status of metal exposed aquatic invertebrates may be most suitable with Cd due to the specific of the response, the nonessential role of Cd in biological processes, and the normally low concentrations of Cd in tissues. However, with Cu and Zn, high basal levels of MT synthesis and MT-bound metals, the natural

variability associated with nutritional requirements, and effects of external factor besides metals may serve to confound analysis of toxicologically-related responses in some cases (Roesijadi, 1992). Recently, Dallinger *et al.* (1997) have isolated and characterised specific MT isoforms for individual metal (Cu-MT isoform & Cd-MT isoform) in a terrestrial snail *Helix pomatia*. With such advanced molecular technologies, we may detect and quantify any specific MT isoform (e.g. Cd-MT isoform) in marine biomonitors in the near future.

The relationship between size and concentration of metals varied among the areas (Table 6.3). For example, concentrations of Zn in *L. littorea* were negatively correlated with size in all areas except Largs while concentrations of Cu and Fe in the periwinkles from Largs also showed significant positive correlations with size but negative correlations (some significant and some not) at the other sites (Table 6.3). Previous workers have also demonstrated that the relationship between metal concentrations and size in molluscs species varied from site to site and metal to metal (Boyden, 1977; Riget *et al.*, 1996). The negative correlations may be explained by (1) relatively higher growth and metabolic rates in small animals than in large ones; and (2) tissue-dilution effect due to increasing body weight (Rainbow, 1996). The positive correlations indicate that the metal accumulation rate is faster than the growth rate of the organisms (Riget *et al.*, 1996).

However, contents of MT or metal in individuals (μg individual^{-1}) always increased significantly with size in all areas (for MT: $p < 0.05$; for metals: $p < 0.001$). In fact, larger gastropods generally have higher net accumulation of metals (as μg individual^{-1}) in their body tissue than smaller ones (Boyden, 1974, 1977; Phillips, 1990). Thus large gastropods are able to produce more MT for regulation of essential metals (Zn and Cu) and detoxification of toxic metals like Cd. Boyden (1974, 1977) has suggested that the significant relationship between metal content and size allows comparison among different sampling areas. In general, a highly metal-contaminated area may show a steeper slope and/ or higher elevation compared to a 'clean' area. As it is important to monitor bioaccumulation of pollutants and their effects at the population level which have individuals of various sizes (Forbes & Depledge, 1996), data obtained from such a population will not only include the variation among individuals but also reflect the general degree of metal contamination in the population (Riget *et al.*, 1996).

Table 6.4. Results of ANCOVA on the relationship between Log-content of MT and metals (cd, cu, fe & zn) (μg individual^{-1}) and (1) Log-soft body dry weight (mg) or (2) Log-shell length (mm) among different areas[a].

Parameter	Log-dry soft-body tissue (mg) as covariate				Log-shell length (mm) as covariate			
	$F_{df1, df2}$ value	MS	Significance of F value		$F_{df1, df2}$ value	MS	Significance of F value	
MT								
Size Effect	$F_{1, 42}$ = 38.62	2.84	0.000	***	$F_{1, 42}$ = 44.19	3.04	0.000	***
Area Effect	$F_{3, 42}$ = 0.64	0.05	0.595		$F_{3, 42}$ = 2.82	0.19	0.051	
Interaction	$F_{3, 42}$ = 0.47	0.03	0.703		$F_{3, 42}$ = 2.24	0.15	0.098	
Cd								
Size Effect	$F_{1, 42}$ = 130.27	4.48	0.000	***	$F_{1, 42}$ = 138.57	4.45	0.000	***
Area Effect	$F_{3, 42}$ = 0.76	0.03	0.525		$F_{3, 42}$ = 3.63	0.12	0.020	*
Interaction	$F_{3, 42}$ = 0.31	0.01	0.816		$F_{3, 42}$ = 3.14	0.10	0.035	*
Cu								
Size Effect	$F_{1, 42}$ = 595.83	7.61	0.000	***	$F_{1, 42}$ = 943.88	6.93	0.000	***
Area Effect	$F_{3, 42}$ = 15.48	0.20	0.000	***	$F_{3, 42}$ = 1.20	0.01	0.323	
Interaction	$F_{3, 42}$ = 10.65	0.14	0.000	***	$F_{3, 42}$ = 1.39	0.01	0.258	
Fe								
Size Effect	$F_{1, 42}$ = 432.30	7.43	0.000	***	$F_{1, 42}$ = 495.86	6.79	0.000	***
Area Effect	$F_{3, 42}$ = 1.08	0.02	0.368		$F_{3, 42}$ = 8.16	0.11	0.000	***
Interaction	$F_{3, 42}$ = 8.94	0.15	0.000	***	$F_{3, 42}$ = 3.50	0.05	0.023	*
Zn								
Size Effect	$F_{1, 42}$ = 575.00	5.68	0.000	***	$F_{1, 42}$ = 1125.67	5.27	0.000	***
Area Effect	$F_{3, 42}$ = 11.84	0.12	0.000	***	$F_{3, 42}$ = 3.47	0.02	0.024	*
Interaction	$F_{3, 42}$ = 8.40	0.08	0.000	***	$F_{3, 42}$ = 3.72	0.02	0.019	*

[a] Levels of significance are indicated by asterisks (*$p < 0.05$ and ***$p < 0.001$).

Results of ANCOVA showed that there was no significant difference among the slopes of the relationship between MT content and dry soft-body weight or shell length (Table 6.4). Large variations in MT content may have caused this failure in detection of difference among the areas. First, this problem may be resolved by increasing the sample size. Secondly, it is advantageous in monitoring programmes to select a specific tissue which shows maximal proportional response to changes in the concentration of MT rather than analyse the whole organism. Babianno and Langston (1995) have implied that the kidney of *L. littorea*, which is the most sensitive tissue for measurements of MT, may provide a more consistent measure of MT. Therefore, use of the kidney for MT analysis might improve the results of present study.

Nevertheless, the slopes of the relationship between individual metal content and size generally varied from area to area (Fig. 6.4). The difference in Cd, Cu and Zn contents among areas decreased with increasing size (Fig. 6.4; Table 6.4), indicating that no significant differences could be detected in large animals (e.g. > 15 mm shell length). These results may be explained by the different slopes and elevations of the regression between dry soft-body weight and shell length among the areas (ANCOVA, $p < 0.001$; Fig. 6.5). The difference in body weight decreased with increasing shell length and vice versa. As the slope of the regression indicates the body conditions of the animals (i.e. slope = body weight/shell length), different slopes of the regression indicate the body conditions of *L. littorea* vary between areas. These differences may be caused by differences in food consumption, food types, growth rate, morphology and development of shell, and genotype of periwinkles among areas (Tresierra-Aguilar, 1985; Janson, 1987). Similar metal contents were observed in the large periwinkles among the areas suggesting that selection of small sized individuals (e.g. 10 mm shell length) for monitoring is preferable than large individuals (>15 mm) in order to detect differences in metal contamination between areas. However, there are several problems in using small individuals (< 5 mm) such as species identification (it is very difficult to distinguish among *Littorina* sp. at small size) and requirement of large sample size to obtain enough tissues (e.g. kidney) for analysis. Thus, following procedures recommended (e.g. Riget *et al.*, 1996), we compared the results using either 10 mg dry soft-body weight or 10 mm shell length in the present study.

Different standardisation variables provided different patterns of results in terms of metal content. For example, based on dry weight of 10 mg individuals, Cu content in *L. littorea* from different areas were compared and ranked as: LF > D = G > L while the order was D = G > L > LF based on individuals 10 mm in size (Fig. 6.6(a)). The difference is mainly due to the inconsistent relationship between soft-body weight and shell length of periwinkles among the areas (Fig. 6.5). At 10 mm shell length, the estimated corresponding dry soft-body weight varied among the areas (L = 17.5 mg; G = 12.5 mg; D = 12.3 mg and LF = 6.6 mg). Based on comparison of Cu content at 10 mg individuals between sites, it is suggested that periwinkles from Loch Fyne might take longer to reach that body weight compared to those in the other areas. They would thus have been exposed for a longer period and would have higher Cu content (Fig. 6.6(a)). Although high metal content in periwinkles from Loch Fyne may also be caused by relatively high ambient metal levels, metal concentrations in blue mussel

Mytilus edulis from Loch Fyne are significantly lower compared to those from areas adjacent to the Clyde Sea (unpublished data), indicating low bioavailability of heavy metal in Loch Fyne.

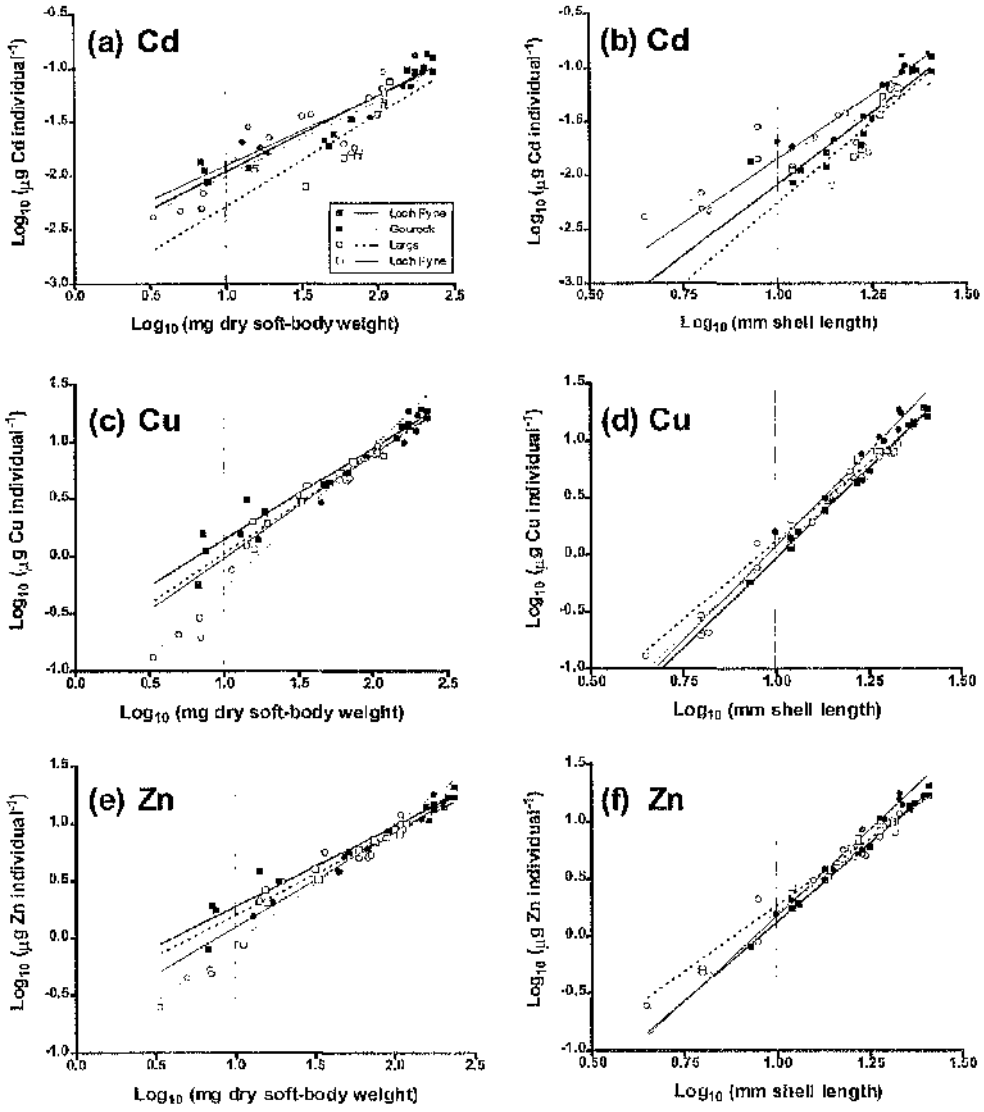

Figure 6.4. Examples showing differences in the relationship of size-metal contents in *Littorina littorea* among the areas on a double logarithmic basis. Cd, Cu and Zn contents are regressed with size as dry soft-body weight [(a), (c) and (e)] and shell length [(b), (d) and (f)], respectively. Comparison of metal concentrations among area is standardised at either 10 mg soft-body weight or 10 mm shell length, and represented by the inserted vertical line.

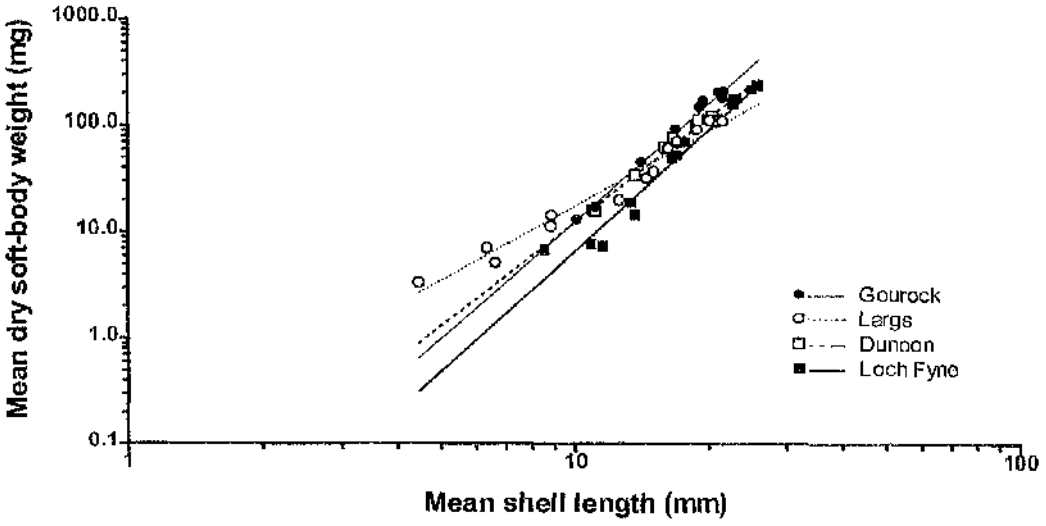

Figure 6.5. Relationship between mean dry soft-body weight and mean shell length of *Littorina littorea* collected from different areas.

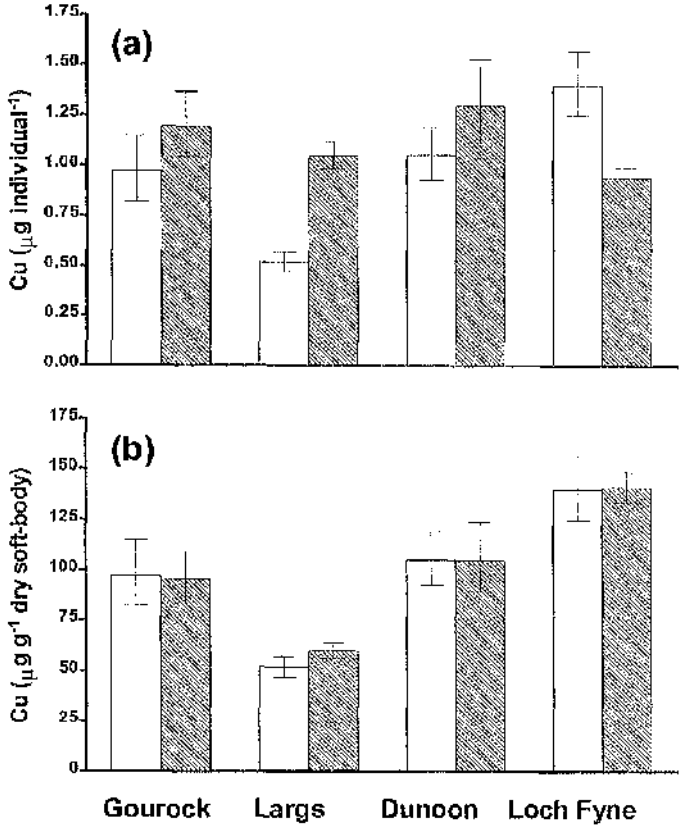

Figure 6.6. Comparison of (a) Cu content and (b) Cu concentrations in *Littorina littorea* collected from different areas at a standardised size: 10 mg dry soft-body weight (opened bar) and 10 mm shell length (shaded bar). Mean values and standard deviations are shown.

On the basis of concentrations (μg g^{-1}), however, the patterns of the results concur for both standardised biomass (10 mg dry body weight) and size (10 mm shell length) (Fig. 6.6(b)). There is an advantage in using dry weight as the independent variable since transformation of the content to concentration only requires multiplication by a single factor for all areas after normal-transforming the data. This also allows direct statistical comparison of mean values among areas based on the log-transformed data. When shell length is used as the independent variable, different conversion factors for dry weight are required for individual areas, respectively and direct comparison between mean values based on the log-transformed MT or metal content is impossible. Therefore, concentrations of MT and the metals were only calculated based on the regressions using dry weight as the independent variable (Fig. 6.7).

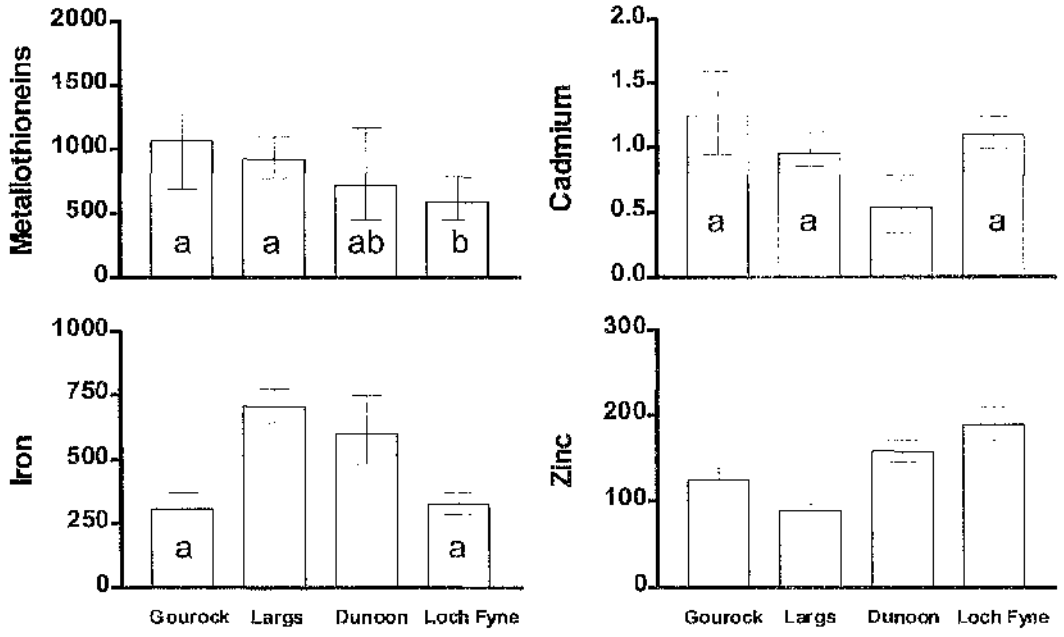

Figure 6.7. Concentrations of MT, Cd, Fe and Zn (μg g^{-1} dry soft-body weight) in *Littorina littorea* collected from different areas at a standardised size: 10 mg dry soft-body weight. Mean values and standard deviations are shown. Bars with same letter are not significantly different (p<0.05).

Bebianno & Machado (1997) have proposed that measurement of MT concentrations in biomonitors can provide an accurate indication of subtle environment increases in metal contamination. Moreover, MT may be useful in predicting metal toxicity in aquatic organisms because cellular toxicity may result after the metal binding capacity

of MT has been exceeded (Roesijadi, 1992), and the level of MT also can be linked and associated with the stress response in aquatic animals (Cattani *et al.*, 1996; Tort *et al.*, 1996; Leung & Furness, 1999 press). MT concentrations in periwinkles at 10 mg from Gourock and Largs were significantly higher than those from Loch Fyne (Fig. 6.7) suggesting that current levels of metal contamination and toxicity might be higher in these areas.

The metal concentrations in periwinkles were compared at 10 mg and ranked as follows: G = L = LF > D for Cd; LF > D = G > L for Cu; L > D > LF= G for Fe; and LF > D > G > L for Zn. Although the Clyde Estuary has been regarded as one of the areas most contaminated by trace metals in Scotland (Balls *et al.*, 1997), periwinkles (at 10 mg) from Gourock did not show significantly higher metal concentrations in their soft-body compared with the other sites. In addition, periwinkles from Loch Fyne, which is a 'clean' area, showed relatively high Cd, Cu and Zn concentrations. Ireland & Wootton (1977) also noted that contamination profiles generated from the analysis of *L. littorea* and *N. lapillus* at nine sites on the coast of Wales did not match the comparative levels of metals in the seawater. The different contamination profiles displayed by gastropod species may be explained by (1) an inconsistent relationship between the comparative levels of metals in solution and metals associated with particulates among all sampling areas (Phillips, 1990); (2) different concentrations of metals in the diet consumed by the animals, as well as different food types and food availabilities among the areas (Bryan *et al.*, 1983).

Furthermore, body condition of periwinkles can partially explain the different profiles obtained in using the periwinkle as a biomonitor in the present study. Higher concentrations of essential metals (Cu and Zn) in *L. littorea* from Loch Fyne may be associated with their poor body condition or slower growth rate compared to the other areas while lower concentrations of Cu and Zn in periwinkles from Largs may be due to their better body condition, faster growth and metabolic rates. Higher metabolic rate, implying greater metal removal rate (e.g. through excretion of metal-rich granules; Nott & Langston, 1989; Phillips & Rainbow, 1989), and tissue dilution effect of accumulated metals are normally observed in faster-growing animals (Rainbow, 1996). Therefore, caution has to be taken during interpretation of the results of metal concentrations. Finally, based on all metal concentrations measured in periwinkles, results of discriminant analysis showed that Largs was significantly different from the

other three areas because of elevated Fe concentrations (Fig. 6.8). These results suggest that Largs has a significantly higher bioavailable iron compared to the other areas.

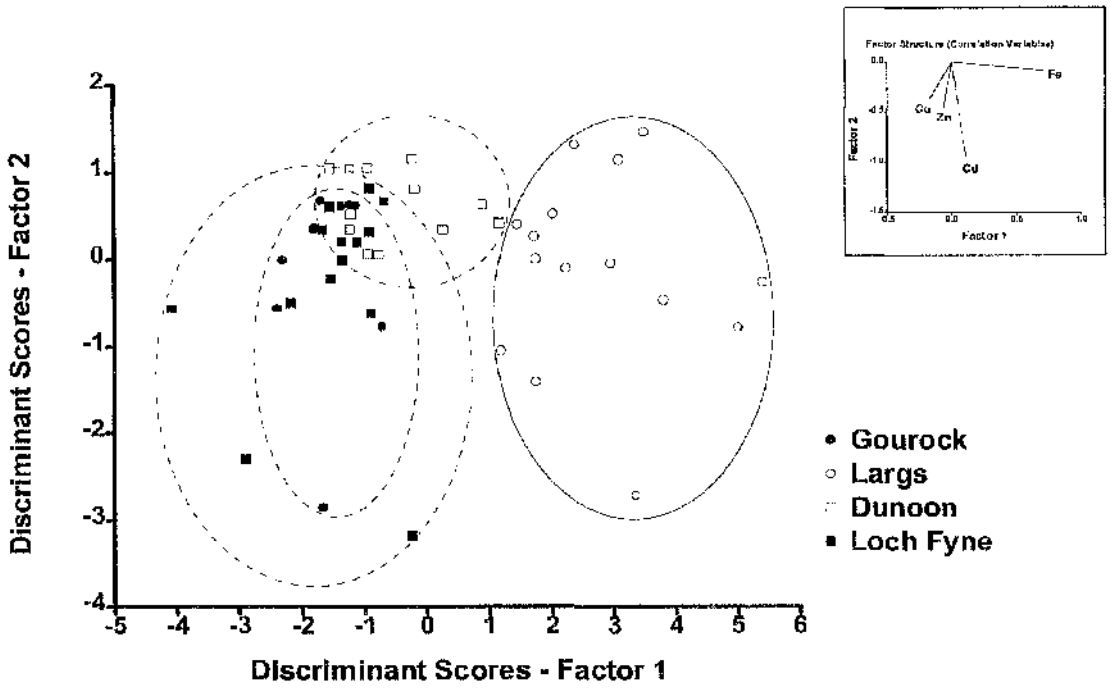

Figure 6.8. Results of discriminant analysis describing the different profiles of metal contamination among the sampling areas. Factor structure is illustrated as the inserted figure.

CONCLUSIONS

Concentrations of MT and metals like Cd and Zn in *L. littorea* generally decrease with increasing size while MT content (μg individual^{-1}) is a linear function of size. Knowledge of the regression coefficients and slopes of lines relating the individual MT content to size of gastropods is potentially a considerable aid in the monitoring of metal pollution. Also, MT has been found to be a good predictor for Cd in *L. littorea*. Dry soft-body weight is a better independent variable for size-standardisation procedure compared with shell length because it allows direct comparison on log-transformed data. The relationship of dry soft-body weight and shell length, which varies among areas, is essential to data interpretation. It is, therefore, very important to incorporate biological data such as size and growth rate in using molluscs as a monitoring tool.

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CHAPTER 7

Growth rate as a factor confounding the use of dogwhelks *Nucella lapillus* (L.) as biomonitors of heavy metal contamination*

ABSTRACT

Growth rate of individually tagged dogwhelks *Nucella lapillus* were measured in free-living animals at three sites of differing heavy metal contamination in the Firth of Clyde, west Scotland. After 6-10 weeks, dogwhelks were collected from these sites in order to determine *in situ* growth rate, condition index (CI), concentrations of metals (Cd, Cu, Pb and Zn), metallothionein (MT), RNA (the RNA/protein ratio) and glycogen in different tissues. In general, the marine environments of Gourock and Largs were contaminated with significantly higher tributyltin (TBTs), Pb and Zn than Loch Fyne, as indicated by the results of imposex indices, and metal concentrations in transplanted Chelex® 100 and *Mytilus edulis*. However, metal accumulation in the dogwhelks displayed a very different pattern. At a standard size (0.5 g wet soft-body weight), *N. lapillus* from Largs showed higher MT, Cd and Cu in the tissues than in animals from the other two populations. Levels of Pb and Zn were similar among the populations despite different concentrations in Chelex and mussels. Gourock's dogwhelks showed similar levels of Cu and MT but lower Cd when compared to those of Loch Fyne. These differences can be attributed primarily to differences in dogwhelk growth rate between sites. Largs dogwhelks grew slowly, whereas Gourock animals grew faster and had with higher CI, and RNA/protein ratio in the foot muscle than those in Loch Fyne, especially at small sizes. Among populations, differences in growth rate may be due to differences in prey availability, predation pressure, and/or genotype. In accord with a laboratory study, fast growing Gourock animals showed lower metal concentrations because of a tissue dilution effect. In contrast, higher levels

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of MT, Cd and Cu in dogwhelks from Largs can be attributed to their growth rate being slower than the rate of metal accumulation. Slow growing animals in Loch Fyne had relatively high MT, Cd, Pb and Zn although Loch Fyne has been regarded as a clean reference site. The present results demonstrate that inter-site differences in growth rate can confound the use of dogwhelks as biomonitors of metals.

INTRODUCTION

Marine intertidal gastropods such as dogwhelks *Nucella lapillus*, periwinkles *Littorina littorea*, and limpets *Patella vulgata*, meet most important requirements of the ideal biomonitor (Phillips 1980, 1990). These gastropod species are sedentary. They are abundant throughout northern America and Europe, easy to identify and sample at all times of year, and large enough to provide sufficient tissue for analysis. They can tolerate wide ranges of contaminant concentration and of physicochemical variables such as salinity, thereby permitting the design of transplant experiments and laboratory studies of contaminant kinetics. These gastropod species are also strong accumulators of certain metals such as Cd and Ag although they can regulate essential metals like Cu and Zn (Langston & Spence, 1995).

However, they may not fulfil the final criterion as an ideal biomonitor because the metal concentration in tissues is not always correlated with the average ambient bioavailable metal concentration (Phillips 1980, 1990). Ireland and Wootton (1977) noted that contamination profiles generated from the analysis of *L. littorea* and *N. lapillus* at nine sites on the coast of Wales did not match the comparative levels of metals in local seawater. Studies using *L. littorea* as biomonitors for trace metals reached a similar conclusion (Bryan et al. 1983, Leung & Furness 1999a). The different contamination profiles displayed by gastropod species might be explained by an inconsistent relationship between the comparative levels of metals in solution and metals associated with particulates among all sampling sites (Phillips, 1990). Alternatively, Bryan et al (1983) suggested that such discrepancies might be due to different concentrations of metals in the diet consumed by the snails, as well as different food types and food availabilities amongst the areas. Another possibility is that the disagreement in the metal concentration profiles could be due to differences in growth rate among populations, as suggested by differences in condition index of *L.*

littorea between study sites (Leung & Furness, 1999a). However, the last hypothesis has not been tested by field experiments.

It has been widely accepted that weight-specific metal concentration (e.g. $\mu g\ g^{-1}$ dry tissue weight) in a biomonitor is affected by growth rate (Phillips & Rainbow, 1993, Langston & Spence, 1995; Rainbow, 1996). In theory, the concentration of a metal will increase with age and weight provided that growth is slow relative to the rate of accumulation of that element. Alternatively, if growth is rapid compared to metal accumulation, the observed concentration of the element will decrease with age and weight, even though the overall metal content may be increasing (Phillips & Rainbow, 1993). The latter phenomenon is called the "tissue dilution effect". If this effect is substantial, any biomonitoring programme should incorporate growth measurement of individual biomonitor populations, as growth rate may vary amongst individuals and amongst populations. Nonetheless, reports on biomonitoring of trace metals seldom consider the possibility of interspecific differences in growth of biomonitors between study areas, *a priori* assuming that all populations have a similar growth rate. If growth rate is a predominant factor affecting the metal concentration in a biomonitor, the conventional approach without taking growth into account will lead to a potential pitfall in biomonitoring. For example, a population of biomonitors growing fast in a metal contaminated area may present similar or even lower metal concentrations than in animals with a slow growth rate in clean areas. Such an effect might be anticipated since metal concentration is often associated with organic enrichment.

Both food type and food availability have significant effects on growth rate of marine gastropods, and thus may affect the concentrations of metals and metallothionein (MT) in tissues. In dogwhelks, feeding mussels alone or combined with barnacles promotes better growth than feeding barnacles alone (Etter 1996). In the laboratory, adult dogwhelks fed with mussels or barnacles and exposed to Cd had a significantly lower Cd concentration in the tissues than that of fasted Cd-exposed dogwhelks, consistent with a tissue dilution effect occurring in fed individuals (Leung & Furness submitted). These laboratory results invite experiments to determine whether these results apply in wild populations.

Apart from trace metals, concentrations of biomarkers such as MT or MT-like proteins, and glycogen stores may also be influenced by growth. A key objective of the present study is to examine whether growth influences metal accumulation, MT synthesis and glycogen stores of *N. lapillus* under natural environmental conditions.

We investigated *in situ* growth rates, nutritional status and metal contamination of three different intertidal populations of dogwhelks *N. lapillus* from three areas of the Firth of Clyde, western Scotland. Based on this comprehensive biomarker approach, accumulation and toxicity of trace metals in these populations of *N. lapillus* were evaluated.

MATERIALS AND METHODS

Description of Study Sites

The Firth of Clyde is a semi-enclosed sea area on the west coast of Scotland which receives direct inputs of contaminants, via rivers, pipelines, sea dumping, farm and fish farm waste discharges, as well as from the adjoining North Channel and the atmosphere (Muller, 1998). The major inflow of freshwater to the Clyde Sea, via the inner Firth of Clyde, is from the Clyde Estuary into which drain the effluents of approximately half of Scotland's population and industry (Fig. 7.1; Thomason et al 1997). Population samples of *N. lapillus* were collected from Gourock, Largs and Loch Fyne, from enclosed and protected rocky intertidal shores (Fig. 7.1). Water and sediment quality in Gourock and Largs are greatly influenced by sewage, heavy transportation, historically polluted sediments and surface run off from the River Clyde (Balls et al 1997). Gourock is situated at the Clyde Estuary and regarded as a contaminated area, Largs lies in the Clyde Sea so is likely to be of intermediate pollution status while Loch Fyne is in a relatively remote area, mainly serving forestry, extensive grazing, aquaculture and recreation purposes.

Sampling

Different sizes of dogwhelks were collected at low tide from all these areas during 24-30 May 1998, returned to the laboratory and maintained in water tanks supplied with circulating seawater (35‰and 10°C). Amongst the areas, Gourock had the highest abundance of *Nucella*, followed by Largs, while Loch Fyne had the lowest abundance, indicated by the catch per unit effort (Table 7.1). At the time of collection a note was made of the presence or absence of the two barnacles *Semibalanus balanoides* and *Chthamalus* spp., and also of the abundance of the mussel, *Mytilus edulis* (Table 7.2), all of which are regular foods of dogwhelks (Moore 1936, Borrows & Hughes 1990).

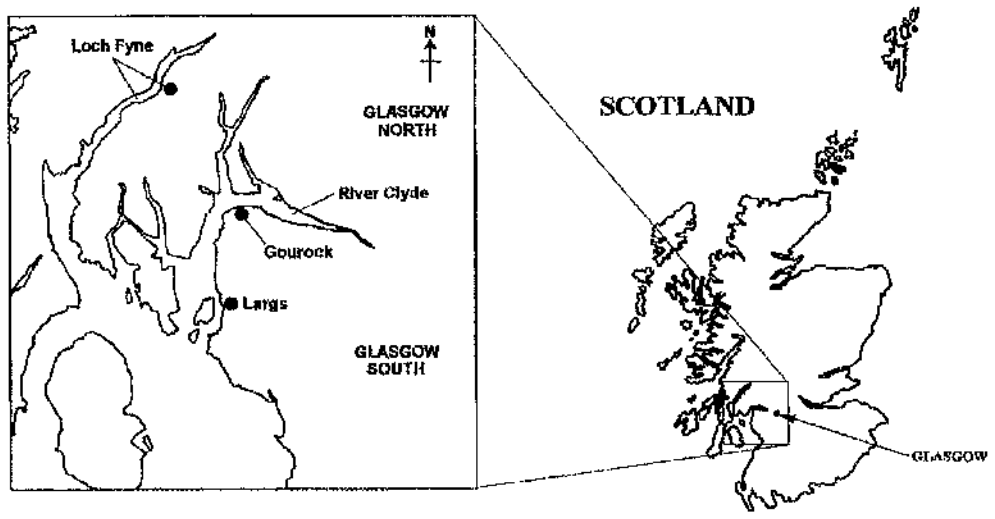

Figure 7.1. A map showing the study sites.

Table 7.1. Date of release and sampling of tagged *Nucella lapillus* from different study sites. Numbers and percentage recovery of labelled dogwhelks are given.

Area	Release Date	No. of tagged dogwhelks released	Catch per unit effort (no. h^{-1} $person^{-1}$)	Sampling date	Time of exposure (days)	No. of tagged dogwhelks collected	% Recovery
Gourock	04.06.98	355	142	20.7.98	47	30	8.4
Largs	03.06.98	288	115	15.8.98	73	17	5.9
Loch Fyne	30.05.98	89	30	21.7.98	53	12	13.5

Table 7.2. Observed characteristics of food availability in the study sites.

Area	Presence of *Chthamalus* spp.	Presence of *S. balanoides*	Abundance of *Mytilus**
Gourock	Absent	Present	++
Largs	Absent	Present	+
Loch Fyne	Present	Present	+++

**Note:* + Either doubtfully present, or else absent in the immediate locality, but present in the neighbourhood; ++ Present in small numbers; +++ Present in large numbers.

Estimation of In Situ Growth Rate

After acclimation in the aquarium for 1-2 days, each individual was tagged with a waterproof label (4 mm × 2 mm) using super-glue (151 Super Glue, SB limited, UK). Measurements were taken of shell length in the dogwhelks using vernier callipers (± 0.1 mm). Shell and live body mass were estimated at the beginning of the experiment using the methods of Palmer (1982). Animals were maintained immersed at least for 24 h in order to allow air bubbles in the mantle cavity to dissolve completely. Each dogwhelk was then transferred to a weighted-cradle, suspended from the arm of a balance (± 1.0 mg) by a stainless steel rod and immersed in a small rectangular tank of seawater (Length × width × height: 16.3 × 6.3 × 10.0 cm^3; volume of seawater at 35‰ = 860 ml). The measured weight is largely due to the mass of the shell, as the density of body tissues is close to that of seawater (Burrows and Hughes, 1990). After weighing in water, each snail was dried and extra-visceral water removed by pressing an absorbent tissue firmly against the withdrawn foot, until no further fluid penetrated the tissue. The animal was left to dry for about 1 h before weighing in air. The true mass of shell for each population was estimated using regressions of dry shell mass on both immersed total mass under water and the total mass in air (Table 7.3: regressions). The soft-body weight was obtained by subtraction of the estimated shell mass from the total mass in air. For each population, different sizes of *Nucella* (n = 23-44) were put through the same procedures and sacrificed to establish calibration regressions for each site (Table 7.3). Condition Index (CI) of each snail was also calculated using the following equation:

$$CI = [\text{wet soft-body weight}/(\text{wet soft-body weight} + \text{dry shell weight})] \times 100 \quad (1)$$

As an expression of the shape of the shell, the half-apical angle θ was measured directly using a protractor according to Moore (1936). The tagged and weighed animals were then released back to the original habitats within 5-7 days. After 6 to 10 weeks, the tagged dogwhelks were recollected (Table 7.1). The recapture rate of tagged individuals was 5.9-13.5%. Non-tagged animals were also collected and included for metal, and biochemical analysis (there was no statistical difference in all parameters between tagged and non-tagged animals in each area; Student t-tests or ANCOVA: $p > 0.05$). Shells of *Nucella* were removed using a vice. Wet weight of soft-body was measured using an electronic balance after being blot-dried with an absorbent tissue. The soft-body tissues were stored at -25°C to await further analysis. Growth rate for

individual dogwhelks was calculated by the difference between the final and initial estimated soft-body weight, and expressed as mg g^{-1} d^{-1}.

Table 7.3. Morphometric relations of *Nucella lapillus* used in growth estimation.

Regression number	N	Regression equation	R^2
Shell mass (g) estimates of destructively sampled *Nucella lapillus*			
Shell dry mass (Y) from immersed whole weight (X) and weight in air (Z) in gram			
Gourock [1]	44	$Y = 0.2330X + 0.5557Z - 0.0562$	0.9876
		($F_{2,41} = 1638.13$, $p < 0.001$)	
Largs [2]	39	$Y = 0.2715X + 0.6233Z - 0.0064$	0.9878
		($F_{2,36} = 1460.31$, $p < 0.001$)	
Loch Fyne [3]	23	$Y = 0.6877X + 0.4658Z - 0.0001$	0.9924
		($F_{2,26} = 575.25$, $p < 0.001$)	

Metal Analysis

The soft-body of each live dogwhelk was dissected into foot muscles, upper and lower parts of digestive gland/gonad complex, and gland of Leiblein (n = 30 for each area). The tissues of digestive gland/gonad complex (upper part) of dogwhelks were dried at 60°C for at least 96 h until constant mass was achieved. They were then digested in conc. HNO_3 for 24 h at room temperature followed by boiling for at least 2 h until a clear solution was obtained. The concentrations of Cd, Cu, Zn and Pb were determined using a Philips PU9200 Atomic Absorption Spectrophotometer (AAS) with deuterium background correction and expressed as μg per g dry tissue weight. Accuracy was regularly checked by including a standard reference material (dogfish muscle, DORM-1, from the National Research Council, Canada) within batches (Table 7.4).

Table 7.4. Comparison of metal concentrations (μg g^{-1} dry weight) in standard reference material DORM-1 certified by the National Research Council of Canada and analytical results from the current study. Mean and 95% confidence interval, are given.

Metal	Certified values	Current study values (n = 8)
Cd	0.086 ± 0.012	0.072 ± 0.010
Cu	5.22 ± 0.33	4.87 ± 0.19
Pb	0.40 ± 0.12	0.40 ± 0.07
Zn	21.3 ± 1.0	22.6 ± 2.9

Quantification of MT and Glycogen

The weighed whole Leiblein gland or kidney was homogenised with 0.4 ml of 0.25M sucrose using an Ultraturax (T25 Janke & Kunkel, IKA Labortechnik) at 4°C. The homogenate was centrifuged at 20,000*g* for 20 min at 4°C. Aliquots of 300 µl supernatant were analysed for MT content using the silver saturation method described by Leung and Furness (1999b).

For glycogen analysis, 50-100 mg of the digestive gland/gonad complex (lower part) or foot muscle tissues was dissolved in 0.4 ml 30% KOH 90°C for 30 min. After cooling in ice, 1 ml of absolute alcohol was added to the tissue solution, mixed and kept at 4°C for 2 h. It was then centrifuged at 3,000*g* for 10 min. After removal of the supernatant, the pellet was re-dissolved in 1 ml distilled water. Subsequently, glycogen concentrations in these solutions were determined in triplicate by utilising the anthrone reagent (Seifter et al., 1950), with comparison against multiple glycogen standards. The results of MT and glycogen were expressed as µg or mg per g of wet tissue weight.

RNA and the RNA/Protein Ratio

Approximately 100 mg of frozen foot muscle was homogenised in 3 ml of ice-cold 0.2 M PCA in a 5 ml test tube for 20s and transferred to a clean, 15 ml polypropylene centrifuge tube on ice. The test tube was washed twice for 20s with 1 ml PCA which was added to the centrifuge tube. The homogenate (4 ml) was centrifuged at 6000*g* for 10 min at 4°C and supernatant discarded. The pellet was re-suspended (by sonication) and washed in 3 ml 0.2M PCA twice and then re-suspended in 4.5 ml distilled water. The suspension was gently mixed with 0.5 ml of 3M NaOH and incubated at 37°C for 1 h, with regular shaking to ensure solubilisation (The alkaline condition re-dissolved proteins; separated rRNA from ribosomal protein and totally solubilised this RNA). Samples were allowed to cool and mix. Two 50 µl aliquots were stored in 15 ml polypropylene centrifuge tubes at -20°C for later Lowry protein assay (Lowry et al., 1951 as modified by Schacterle & Pollock, 1973). 0.9 ml of 20% PCA (3.4M) was added to the remaining 3.9 ml samples, mixed and centrifuged at 6000*g* for 10 min at 4°C. (This caused all the protein and DNA to precipitate but left protein-free RNA in solution). The supernatant was stored on ice in labelled 15 ml polypropylene centrifuge tubes for later RNA assay, using Orcinol reagent. A series of RNA (yeast RNA from Sigma) standards (0 to 50 µg RNA/ml) were prepared with 0.5M HCl. Orcinol reagent

was prepared by dissolving 120 mg Orcinol per 20 ml $FeCl_3$/HCl solution (20 mg $FeCl_3$ dissolved in 100 ml conc. HCl). 1 ml of sample or standard was pipetted into a glass test tube and mixed with 1 ml 0.5M HCl, and 2 ml Orcinol reagent in a fume cupboard. The assay tubes were heated at 100°C in a water bath for 35 min in a fume cupboard, and then allowed to cool to room temperature. Absorbance of each sample or standard was read at 665 nm. The results of RNA were expressed as μg RNA g^{-1} dry tissue weight and μg RNA mg^{-1} protein.

Imposex Determination

On the date of re-sampling, non-tagged adult dogwhelks (n = 40) were collected at the same areas for imposex determination. The relative penis size index (RPSI) is the mean bulk of the female penis (length^3) expressed as a percentage of the mean bulk of the male penis (length^3) in a sample (Davies et al. 1997). The vas deferens stage index (VDSI), which is an index for the individual stage of the development of a vas deferens in the female, was determined as described by Gibbs et al. (1987).

Trace Metals Monitored by Chelex® 100 and Mussels

The mussels *Mytilus edulis* (48 ± 1 mm in shell length) were collected from Loch Fyne and acclimated in an aquarium with circulating seawater at 35‰and 10°C for 5-10 days. During acclimation, 30 mussels and 15 Chelex tubes (Wu and Lau, 1996) were caged. Three such cages were prepared and deployed in the study areas by anchoring them with rocks. They were recovered after 6 weeks of exposure. Shell length and dry biomass of the mussels were measured in order to determine the condition factor (mg mm^{-1}). The concentrations of Cd, Cu, Pb and Zn in the Chelex and mussels were determined using ICPES and graphite furnace atomic spectrometry (Thermal Jarrell Ash Smith-Hieftie 12), respectively, after acid-digestion with concentrated nitric acid (Wu and Lau, 1996). Results were expressed as μg per g dry weight.

Statistical Analysis

All data were natural-log transformed, except growth rate (there were negative values, and thus normal data were utilised). Normality and homogeneity of variances of the data were checked using the Kolmogorov-Smirnov test and Bartlett's test, respectively. General linear models (GLM) were used to test the effects of animal size and sampling site on all parameters. There were no size effects on MT, glycogen and

RNA/protein data. Therefore, comparisons between these size-independent data were made based on mean or median values. Nevertheless, analyses of growth, metal, CI and shell weight-length data were made using analysis of covariance (ANCOVA in GLM) using wet soft-body weight as covariate. To test whether there was difference in each parameter between tagged and non-tagged animals, ANCOVA was used to test size-dependent data while Student t-tests were used to test size-independent data. There were no significant differences in any parameters between tagged and non-tagged animals ($p > 0.05$). Therefore, 18 and 13 non-tagged dogwhelks with different sizes were added to Largs and Loch Fyne tagged samples, respectively, and analysed together with the tagged animals (total $n = 30$). For parametric data with similar SDs, differences in size-independent parameters between areas were compared using one-way analysis of variance (ANOVA), with subsequent comparison between individual means using a Tukey-Kramer multiple comparison test. For non-parametric data or data with different SDs, differences in the size-independent parameters between sites were compared using Kruskal-Wallis tests, with subsequent comparison between individual means using a Dunn's multiple comparison test. Correlations between different parameters were examined using Pearson's correlation analysis. Partial correlation analysis was also performed on the concentrations of MT and MT-inducing metals (including Cd, Cu and Zn) or RNA/protein ratio with a correction for size. Concentrations of metals ($\mu g\ g^{-1}$ wet wt.) were standardised at size of 500 mg wet soft-body weight using the regression between the metal concentration and wet soft-body weight. ANOVA was applied to test the differences between the mean values of metal concentrations with a subsequent comparison between individual means using the Tukey-Kramer Multiple Comparison Test. Statistical significance was defined as $p < 0.05$. All statistics were run on standard software packages (SPSS for Windows, Release 7.5.1, 1996 and Graph Pad Prism™, version 2.0, 1995).

RESULTS

General Metal Contamination Profiles

The highest imposex index (PRSI or VDSI) was observed in *Nucella* from Gourock followed by Largs (Table 7.5). Among animals from Loch Fyne, very few of them presented with 1-2 stages of the vas deferens sequence so that RPSI approached zero. These results indicated that Gourock was highly contaminated with TBTs; Largs was in

intermediate pollution status while Loch Fyne showed very little contamination with these estrogenic chemicals.

Table 7.5. Imposex indices (RPSI and VDSI) in adult *Nucella lapillus* collected from study sites. Mean and SD are presented.

Area	Shell length (mm)	RPSI	VDSI
Loch Fyne	34.7 ± 3.1	0.00	0.50 ± 0.90
Largs	31.6 ± 4.0	0.03	1.86 ± 1.51
Gourock	32.2 ± 3.3	0.82	3.25 ± 1.14

To compare the average environmental trace metal levels in these three areas, Chelex tubes and mussels *M. edulis* were deployed. Chelex resins mainly accumulate the ionic fraction of metals, while *M. edulis* accumulates both particulate and ionic forms (statistical comparisons are in Table 7.6). There was no significant difference in the condition factor of *M. edulis* among all sites ($F_{2,37}$ = 2.39, p = 0.106), indicating that the growth rate of these mussels were similar. There was no significant difference in Cd concentration of Chelex or mussels among all areas. Copper concentration in Chelex from Largs was similar to Loch Fyne but significantly higher than Gourock, but there was no significant difference in Cu concentration in mussels among areas. The highest concentration of lead was observed in Largs Chelex samples, followed by Gourock and Loch Fyne, while the Chelex from Gourock presented the highest concentration of Zn. In mussels, the concentrations of Pb and Zn were similar between Gourock and Largs samples but lower in Loch Fyne. Therefore, Loch Fyne was less contaminated with Pb and Zn.

Characteristics of Nucella Populations

The size-frequency distribution of each population was constructed based on the shell length of specimens used for tagging (Fig. 7.2). All populations showed a continuous size structure. Gourock's population peaked at 18-22 mm and 26-28 mm while size structure of the Largs population was similar, with highest abundance at 26-28 mm. Dogwhelks from Loch Fyne showed a different size distribution, with a high abundance of large animals (30-36 mm), the size class 32-34 mm being the most abundant. Maximum size in this population was 40-42 mm, much greater than that of the populations from Gourock (32-34 mm) and Largs (36-38 mm).

Table 7.6. Concentration of various metals in transplanted mussels *Mytilus edulis* and Chelex® 100 tubes and at different study areas. Values of condition factor (CF) of mussels from each sites are also shown. Mean and SD are presented (n = sample size). For concentration of each metal in either Chelex or mussel, values with same letter are not significantly different (Tukey-Kramer Multiple Comparison test, $p > 0.05$).

		Concentration (μg g^{-1} dry weight)				CF
	n	Cd	Cu	Pb	Zn	mg mm^{-1}
Transplanted *M. edulis*						
Gourock	10	2.70 ± 3.39^{a}	8.99 ± 4.87^{a}	6.98 ± 2.48^{a}	110.50 ± 62.17^{a}	10.1 ± 3.2^{a}
Largs	15	1.56 ± 0.52^{a}	8.74 ± 6.56^{a}	5.12 ± 2.63^{ab}	85.36 ± 19.58^{ab}	8.2 ± 2.0^{a}
Loch Fyne	15	1.31 ± 0.39^{a}	6.53 ± 0.72^{a}	3.64 ± 3.84^{b}	64.94 ± 12.72^{b}	8.1 ± 2.2^{a}
Chelex® 100						
Gourock	12	2.69 ± 1.04^{a}	0.81 ± 0.97^{b}	2.49 ± 0.05^{b}	18.78 ± 9.75^{a}	
Largs	10	2.27 ± 0.64^{a}	1.92 ± 0.52^{a}	4.34 ± 1.66^{a}	6.42 ± 2.55^{b}	
Loch Fyne	9	3.19 ± 1.79^{a}	0.97 ± 1.40^{ab}	1.20 ± 0.53^{c}	9.64 ± 5.00^{b}	

The dogwhelks of Gourock had significantly higher CIs than those of Largs, while the lowest CIs were observed in Loch Fyne whelks (Fig. 7.3; Table 7.7; ANCOVA: $p < 0.001$). The lower CIs in Largs and Loch Fyne animals could be partially explained by a heavier shell weight, especially in large whelks (Fig. 7.4; Table 7.7; ANCOVA: $p < 0.001$). The apical angle θ, when plotted against shell-length (Fig. 7.5), decreased in both Gourock and Largs population. However, shell-length of *Nucella* from Loch Fyne increased with increasing θ. Results of the *in situ* growth study showed that *Nucella* from Gourock and Loch Fyne grew faster than those from Largs under natural environmental conditions (Fig. 7.6, Table 7.7). At small sizes (< 0.7 g wet soft-body weight), Gourock's population grew faster than the dogwhelks from Loch Fyne, but the latter population had a higher growth rate at large sizes (> 1 g) (Table 7.7: Interaction $p < 0.001$). During the study period, weight loss was observed in most dogwhelks from Largs, and in large individuals from Gourock.

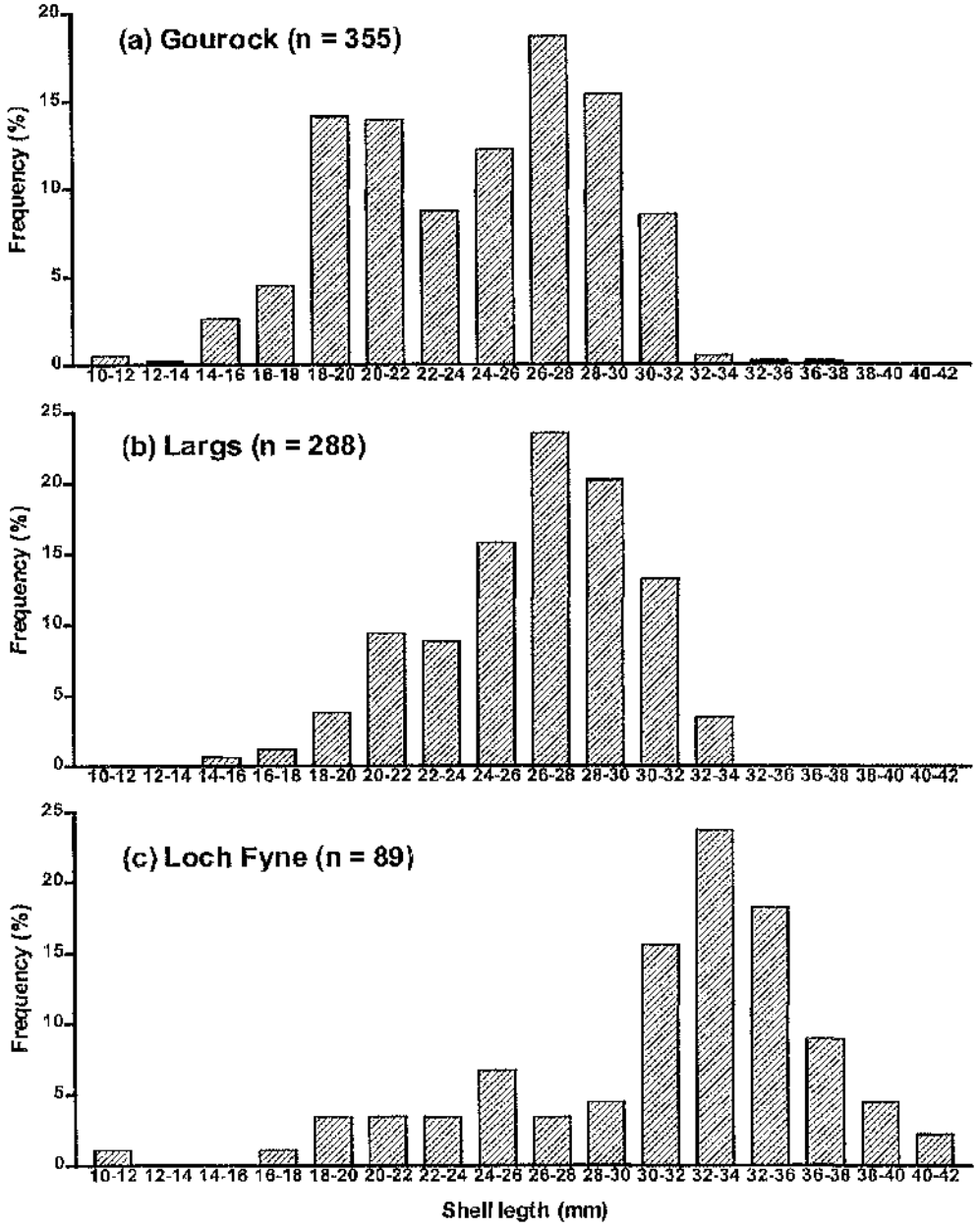

Figure 7.2.. The size-frequency distribution of the three *Nucella* populations.

Table 7.7. Analyses of covariance of condition index, shell weight, growth rate and the apical angle versus area and size (wet soft-body mass) where size is the covariate.

	Condition index			Shell weight		Apical angle			Growth rate		
	d.f.	M.S.	P	M.S.	P	d.f.	M.S.	P	d.f.	M.S.	P
Regression	3	2.181	<0.001***	12.561	<0.001***	3	0.0039	0.275	3	2558.1	<0.001***
Area	2	1.245	<0.001***	1.483	<0.001***	2	0.0040	0.264	2	1310.8	<0.001***
Size	1	3.385	<0.001***	30.988	<0.001***	1	0.0004	0.699	1	6605.2	<0.001***
Area x size	1	1.392	<0.001***	19.434	<0.001***	1	2.0220	<0.001***	1	7802.3	<0.001***
Residual	102	0.023		0.028		59	0.0029		55	142.1	

*p< 0.05, **p<0.01 and ***p<0.001.

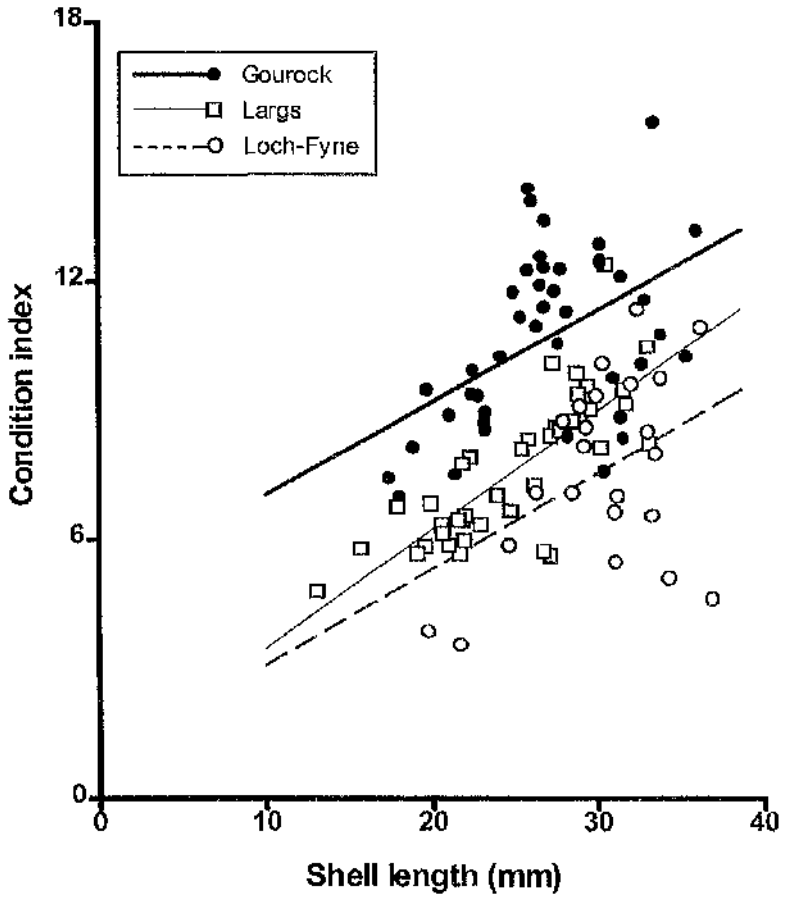

Figure 7.3. The relationship between shell length and condition index of *Nucella* samples from Gourock (solid circle, thick-solid line), Largs (open square, thin-solid line) and Loch Fyne (open circle, dashed line).

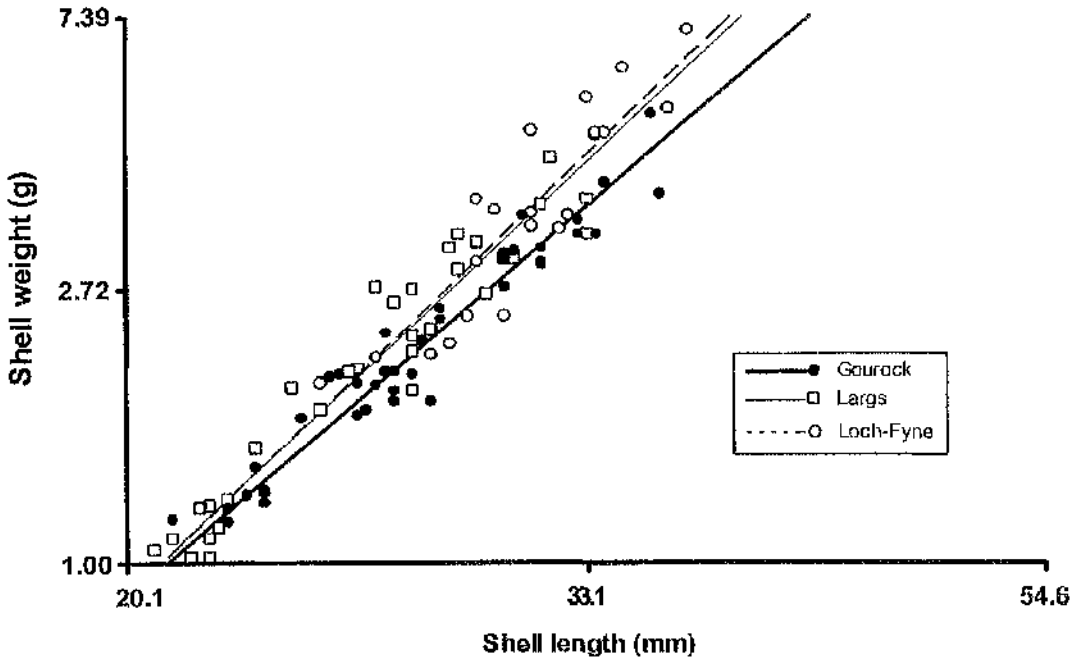

Figure 7.4. The relationship between shell length and shell weight of *Nucella* samples from Gourock (solid circle, thick-solid line), Largs (open square, thin-solid line) and Loch Fyne (open circle, dashed line), on a double natural logarithmic basis.

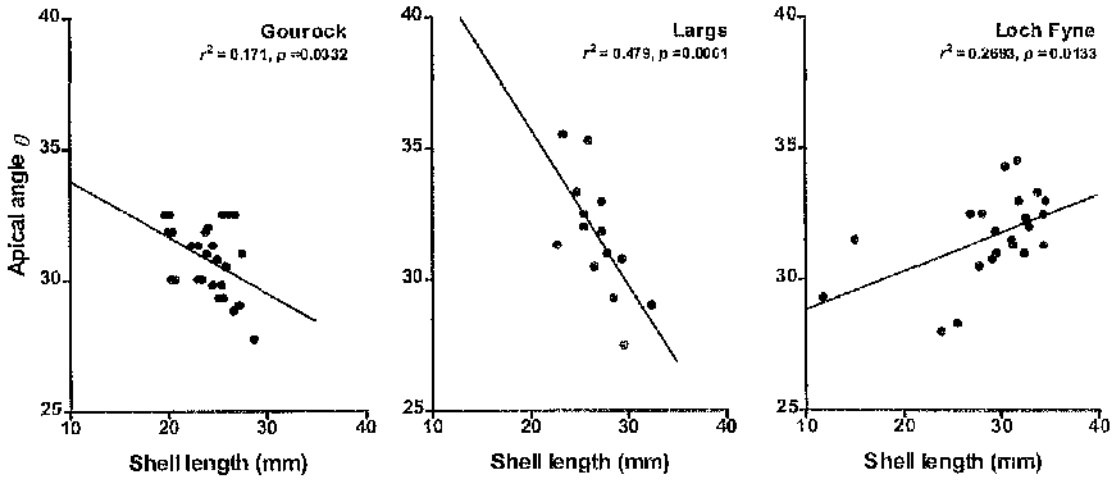

Figure 7.5. The relationship between length and the apical angle of *Nucella* shells from Gourock (a), Largs (b) and Loch Fyne (c).

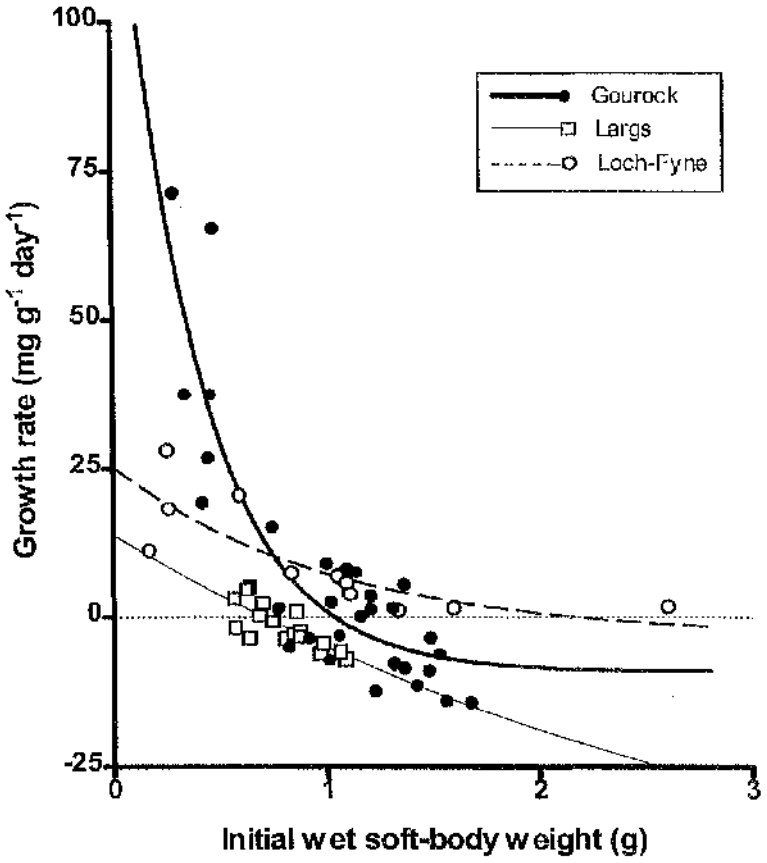

Figure 7.6. The relationship between initial size and the estimated *in situ* growth rate of tagged dogwhelks in Gourock (solid circle, thick-solid line), Largs (open square, thin-solid line) and Loch Fyne (open circle, dashed line).

Metals and MT in Nucella

In general, the concentrations of trace metals in the digestive gland/gonad complex of *Nucella* were dependent on size (Fig 7.7; Table 7.8 and 7.9). For Cd, size-dependent relationship varied with population, the Cd concentration in Gourock samples increased in relation to size but a reverse trend was observed in Largs samples, and Cd concentration in dogwhelks from Loch Fyne was independent of size (slope = 0). For other metals (Cu, Pb and Zn), the concentrations in all samples significantly decreased with increasing size, except Cu and Zn in samples of Gourock and Largs which were independent of size. At population level (including different sizes), profiles of metal concentrations of Cd, Cu and Pb in *Nucella* were significantly different among areas (Fig. 7.7, Table 7.9), but there was no significant difference in Zn between populations. The strong interaction between the concentration versus area and size (Table 7.9; Interaction $p < 0.001$) indicated that the metal concentration profile in *Nucella* not only

depended on the effect of area but also changed with individual sizes in each population.

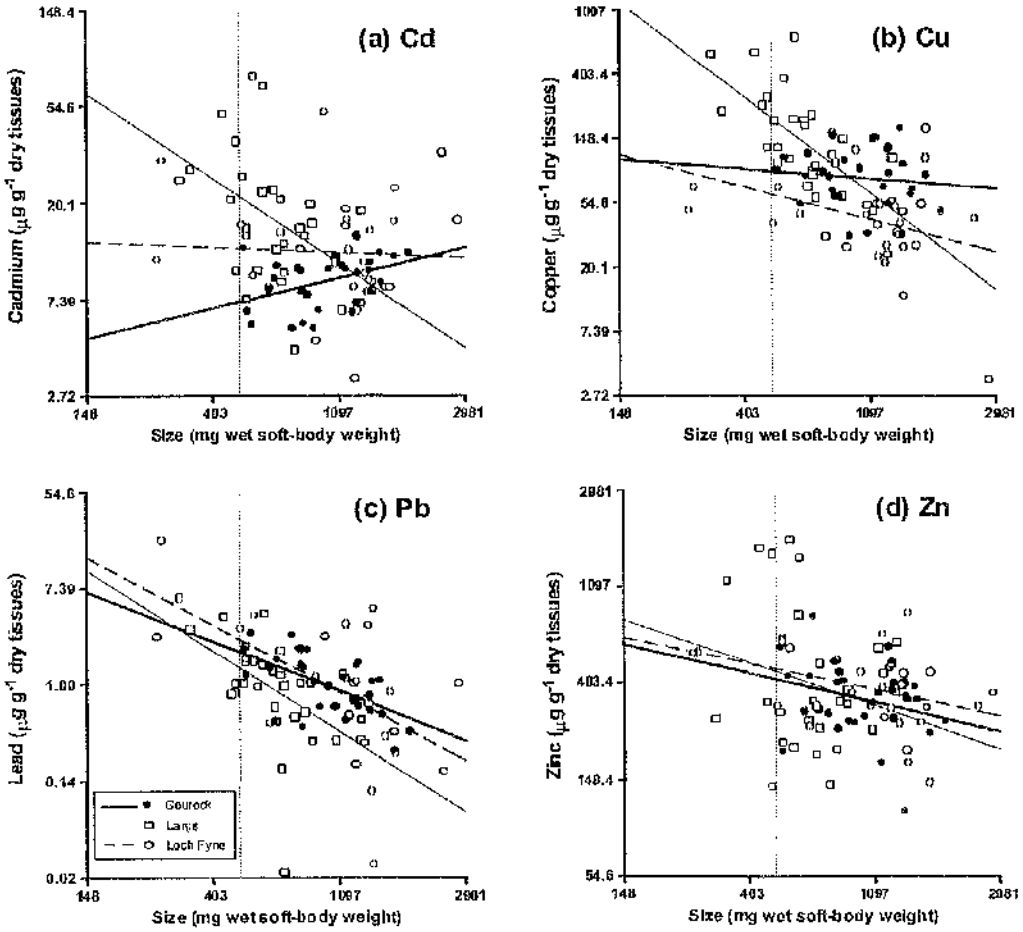

Figure 7.7. The relationship between size and concentration of (a) Cd, (b) Cu, (c) Pb and (d) Zn in the digestive gland/gonad complex of dogwhelks from Gourock (solid circle, thick-solid line), Largs (open square, thin-solid line) and Loch Fyne (open circle, dashed line), on a double natural logarithmic basis.

Table 7.8. Results of the regression analysis between metal concentration in digestive gland/gonad complex and animal size of *Nucella lapillus* (n = 30). Ln-concentration (μg g^{-1} wet tissues) is regressed on Ln-dry soft-body weight (mg).

Area	Ln *a*	SE of *a*	*B*	SE of *b*	*r*	*p*
Cadmium						
Gourock	0.051	0.935	+0.310	0.135	+0.397	0.0297*
Largs	8.519	2.095	-0.878	0.322	-0.458	0.0110*
Loch Fyne	2.868	1.358	-0.055	0.194	-0.053	0.7791
Copper						
Gourock	5.408	1.622	-0.148	0.234	-0.118	0.5336
Largs	14.550	2.025	-1.485	0.312	-0.361	0.0503
Loch Fyne	7.232	1.710	-0.499	0.244	-0.669	<0.0001***
Lead						
Gourock	7.058	1.810	-1.072	0.262	-0.596	0.0005***
Largs	10.720	3.307	-1.670	0.509	-0.527	0.0028**
Loch Fyne	9.646	2.653	-1.402	0.379	-0.573	0.0009***
Zinc						
Gourock	7.895	1.372	-0.299	0.198	-0.274	0.1426
Largs	8.873	2.515	-0.444	0.387	-0.212	0.2612
Loch Fyne	7.818	0.866	-0.270	0.124	-0.381	0.0378*

Ln *a* is the intercept; *b* is the regression coefficient and *r* is the correlation coefficient. Significant correlations are denoted by asterisks *p< 0.05, **p<0.01 and ***p<0.001.

Table 7.9. Analyses of covariance of metal concentration in the digestive gland/gonad complex, versus area and size (wet soft-body mass) where size is the covariate.

		Cadmium		Copper		Lead		Zinc	
	d.f.	M.S.	P	M.S.	P	M.S.	P	M.S.	P
Regression	3	2.093	<0.001***	8.852	<0.001***	10.126	<0.001***	0.668	0.061
Area	2	2.132	0.001**	4.834	<0.001***	3.430	0.016*	0.136	0.596
Size	1	0.347	0.267	6.353	<0.001***	30.162	<0.001***	1.581	<0.016*
Area x size	1	4.231	<0.001***	25.716	<0.001***	29.391	<0.001***	22.419	<0.001***
Residual	86	0.279		0.389		0.795		0.261	

*p< 0.05, **p<0.01 and ***p<0.001.

It had been noted that growth of *Nucella* were very different at sizes < 0.7 g among the populations, with a descending order: Gourock > Loch Fyne > Largs. With a view to studying the effect of *in situ* growth rate on metal accumulation, we compared the concentration of metals at a size with 0.5 g wet soft-body mass (Fig. 7.8). The animals from Largs at this standardised size, accumulated significantly higher Cd and Cu in the tissues than the whelks from other two sites (Fig. 7.8a, b), although there was no significant different in Pb or Zn concentration among all populations. Furthermore, the population of Loch Fyne also had a higher Cd concentration in the tissues than in the dogwhelks from Gourock (Fig. 7.8a). In accord with the results of Cd and Cu concentrations, the highest MT concentration in the Leiblein gland of *Nucella* was observed in the Largs population, while the other two populations had similar levels of MT (Fig 7.9a; Kruskal-Wallis statistic = 41.37, n = 90, $p < 0.0001$). A similar result was noted if converting the MT concentration into content per whole Leiblein gland (Fig 7.9b; K-W statistic = 24.97, $p < 0.0001$). Partial correlation between concentration of MT-inducing metals (Cd, Cu and Zn) in the digestive gland/gonad complex and MT in the Leiblein gland controlling for size, revealed that the MT concentration was strongly correlated with the concentration of Cd or Cu in *N. lapillus* (for Cd: $r = 0.2932$, $p = 0.005$; for Cu: $r = 0.3103$, $p = 0.003$; all cases with d.f. = 87). Unexpectedly, there was a weak negative correlation between MT and Zn concentrations ($r = -0.2480$, $p = 0.019$).

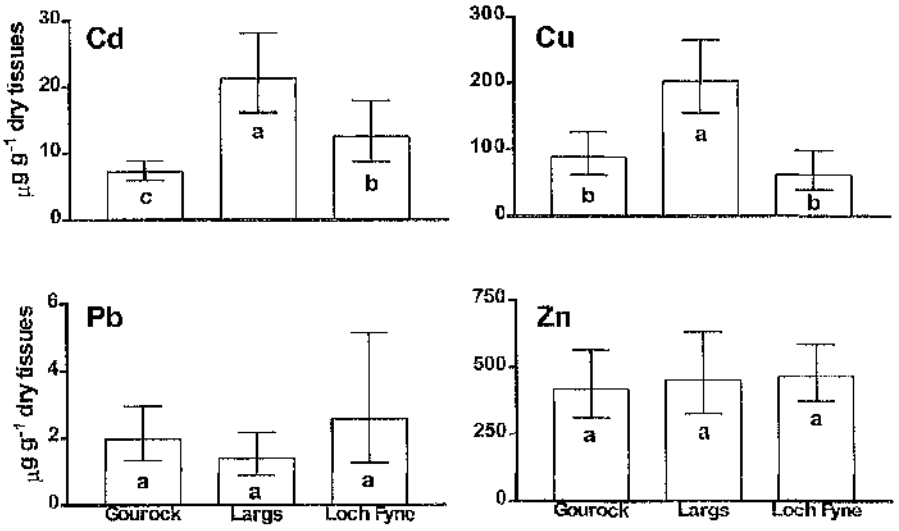

Figure 7.8. Comparisons of the concentration of (a) Cd, (b) Cu, (c) Pb and (d) Zn in the digestive gland/gonad complex of dogwhelks from Gourock, Largs and Loch Fyne, at a standard size (0.5 g wet soft-body weight).

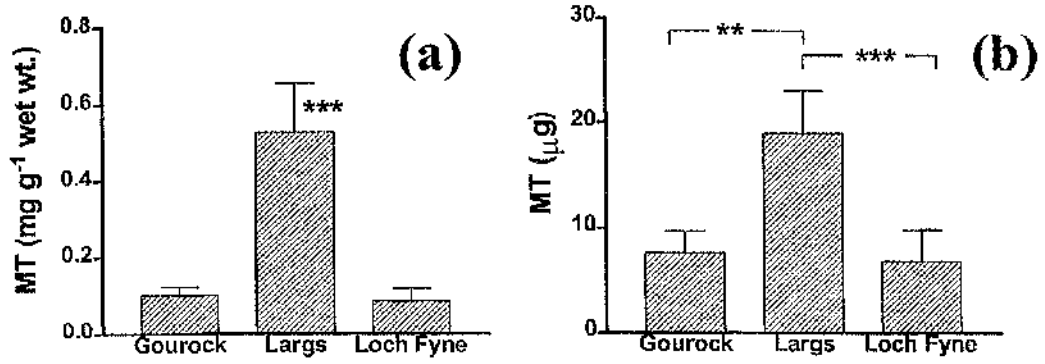

Figure 7.9. Comparisons between the average concentration (a) and content (b) of metallothionein (MT) in the Leiblein gland of dogwhelks from Gourock, Largs and Loch Fyne. Asterisks denote the significantly different means: **p<0.01 and ***p<0.001.

RNA, the RNA/Protein and Glycogen in Nucella

Gourock dogwhelks had a significantly higher RNA concentration in their foot muscle than animals from Loch Fyne (Fig. 7.10a; ANOVA: $F_{2,87} = 5.161$, $p = 0.0076$). and heigher RNA/protein ratios than both of the other two populations (Fig. 7.10b; K-W statistics = 40.09, $p < 0.0001$). Partial correlation analysis with a correction for size also suggested that the RNA/protein ratio decreased with increasing concentration of MT in the Leiblein gland ($r = -0.3186$, $p = 0.015$, d.f. = 87) and with increasing concentration of Cd in the digestive gland/gonad complex ($r = -0.3187$, $p = 0.015$).

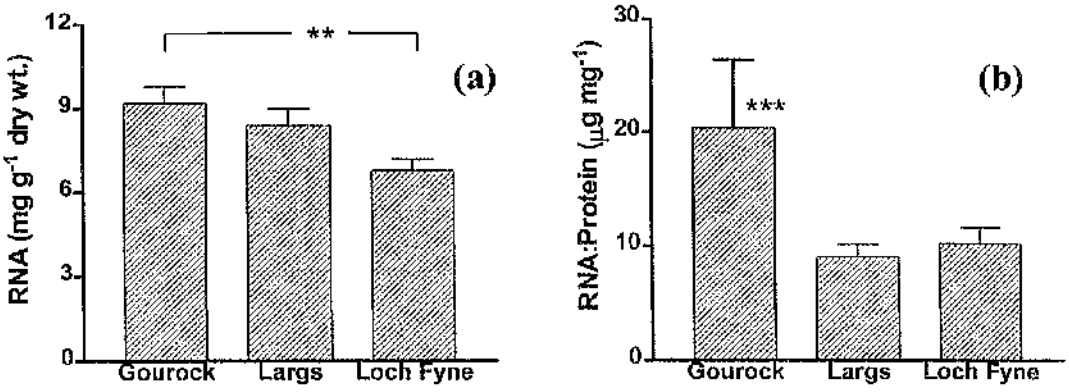

Figure 7.10. Comparisons between the average RNA concentration (a) and the RNA/protein ratio (b) in foot muscle of dogwhelks from Gourock, Largs and Loch Fyne. Asterisks denote the significantly different mean(s): **p<0.01 and ***p<0.001.

Glycogen stores varied with the tissues and areas (Fig. 7.11). In general, the foot mussels stored more glycogen than did the digestive gland. In the foot muscles, the Loch Fyne population showed the lowest glycogen stores (Fig. 7.11a; ANOVA: $F_{2,87}$ = 5.529, p = 0.0055). In contrast, the Loch Fyne population had the highest glycogen stores in the digestive gland/gonad complex (Fig. 7.11b; ANOVA: $F_{2,87}$ = 6.935, p = 0.0016). These results suggested that there might be differences in energy allocation strategy among different populations.

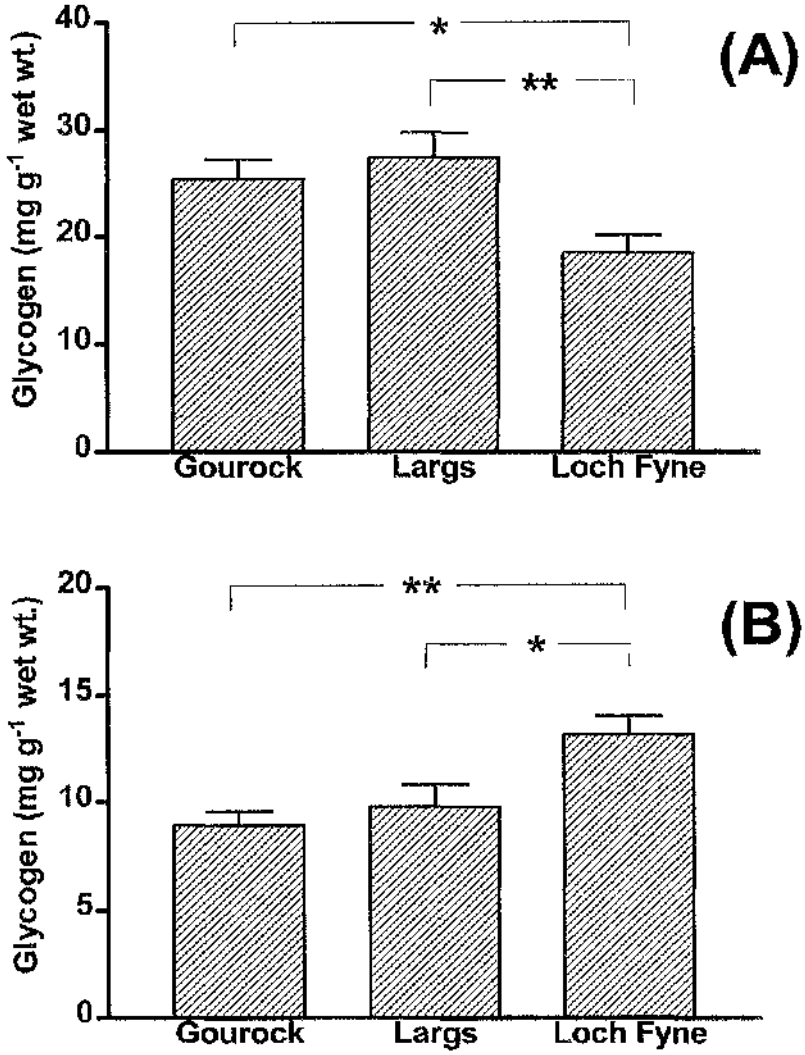

Figure 7.11. Comparisons between the average glycogen concentration in foot muscle (a) and in the digestive gland/gonad complex (b) of dogwhelks from Gourock, Largs and Loch Fyne. Asterisks denote the significantly different means: *p<0.05 and **p<0.01.

DISCUSSION

General Metal Contamination Profiles

Based on the concentrations of metals in Chelex and *M. edulis*, all areas showed similar concentrations of Cd and Cu, but Gourock generally had higher Pb and Zn than the other two sites. TBT contamination was also greater in Gourock, followed by Largs as indicated by the imposex indices of *N. lapillus*. Although *N. lapillus* from Loch Fyne were larger in size (age), they only presented a very low level of imposex. As expected, Loch Fyne is a cleaner area compared to the other two sites, with less TBT, Pb and Zn in the marine environment. Less pollutants present in the marine environment of Loch Fyne, could be one of the major reasons why the dogwhelk population in this site exhibited a longer life span as indicated by a wider size(age)-frequency distribution and a greater proportion of large animals (Fig. 7.2).

According to the data obtained from Chelex and mussels, we should expect the *Nucella* population from Gourock to have higher Pb and Zn concentrations, and all populations to have similar Cd and Cu concentrations. However, metal contamination profiles generated using *N. lapillus* are very different from the patterns indicated by Chelex and mussels. Across all sizes, there was no difference in Zn concentration among populations. The concentrations of Zn were much higher than those of the other metals in the dogwhelks. The high concentrations of Zn in *N. lapillus* may come through the food-chain from barnacles because the levels of Zn in barnacles continue to accumulate throughout life since barnacles store Zn in phosphate granules (Rainbow et al 1990, O'Leary & Breen 1997). Comparisons at a standardised size (0.5 g wet soft-body), indicated that concentrations of Pb were also similar throughout all sites while dogwhelks from Largs showed significantly higher concentrations of Cd and Cu in the tissues. Concentrations of Cu in dogwhelks at this size were similar between Gourock and Loch Fyne while the latter population showed significantly higher Cd than the former. Gourock is a polluted area in the Clyde Estuary while Loch Fyne is a clean area. However, the Cu, Pb and Zn concentrations in *Nucella* (at 0.5 g wet soft-body) from Gourock were not significantly different from those in Loch Fyne's dogwhelks.

Growth Rate Confounding Nucella as Biomonitors of Metal Contamination

The results of *in situ* growth study indicated that growth rate of small dogwhelks (< 0.7 g wet soft-body) was highest in Gourock, followed by Loch Fyne and then Largs. As metal concentration is expressed as μg per g soft-body weight, dogwhelks with slow growth rates may take longer to reach the same body weight as the faster growing ones, and thus accumulate more metals. It is therefore likely that the tissue dilution effect on the metal concentrations had occurred in *Nucella* of Gourock, leading to lower concentrations of Cd and Cu, and similar concentrations of Pb and Zn compared to the other populations. In contrast, growth of *Nucella* from Largs was significantly slower so that their concentrations of Cd and Cu were higher. Therefore growth rate of *Nucella* can primarily explain the differences in the metal contamination profiles between data generated from Chelex/mussels, and *Nucella*.

The population from Gourock not only grew well, but also had higher values of CI and presented the highest average of RNA or the RNA/protein ratio. These parameters have been widely utilised to assess the conditions of organisms such as scallops (Lodeiros 1996) and oysters (Wright & Hetzel 1985) in the field. In general a higher concentration of RNA or the RNA/protein ratio reflect a better growth, nutritional or health state of an organism (Mayer et al 1989, Wo et al 1999). These results suggest that the Gourock population had better nutritional conditions than the dogwhelks from the other two sites.

The population of Gourock grew faster, and had higher values of CI as well as better health and nutritional conditions, indicating that these dogwhelks had better fitness. Better fitness may imply that the animals have better physiological conditions in detoxification and regulation of metals. Low metal concentrations in these fast growers were not only caused by the tissue dilution effect but also might be due to a greater efficiency in detoxification and excretion of trace metals. Nevertheless, dogwhelks from Largs or Loch Fyne had relatively poor growth rates and lower health and nutritional conditions. They took longer to reach the same weight as those from Gourock. Although Gourock is near Largs and has similar metal contamination profiles as suggested by the results of Chelex and mussels, the slower growing dogwhelks in Largs presented the highest concentrations of Cd, Cu and MT. Therefore, growth rate and health conditions (RNA/protein ratio) are important factors, which can confuse the metal contamination profiles, if using marine molluscs such as *N. lapillus* as biomonitors of trace metals.

Factors Influencing the Growth Rate of Nucella

There was no difference in topography, hydrology and the energy level of tidal/wave exposure between the 3 sites – all factors affecting the phenotypes of *N. lapillus* (Etter 1996, Kirby et al 1997). However, we noted that all sites were covered with barnacles *S. balanoides*, and/or *Chthamalus* spp., while mussels *M. edulis* were more frequently found in Loch Fyne and Gourock but not in Largs. Feeding on mussels alone or in combination with barnacles promotes better growth in *N. lapillus* than feeding on barnacles alone (Etter 1996, Leung & Furness submitted). Thus, slow growth rate of *Nucella* from Largs may be directly linked to their feeding on barnacles, a less profitable prey item.

In addition, metal contamination in estuaries is often associated with organic enrichment, eutrophication and organic enrichment may promote growth of marine organisms in a polluted area like Gourock. In a rocky shore at Sandgerdi, Southwest Iceland, highly contaminated with organic wastes from fish factories, dogwhelks grew extremely well reaching a maximum size over 40 mm but with very thin shells (personal observation). A similar phenomenon might be happening in Gourock, though further evidence is necessary.

We also noticed that there were empty shells of *Nucella* and high numbers of shore crabs *Carcinus maenas* in the sampling site of Largs. Higher predation pressure may restrict the foraging activity of dogwhelks, as there is a trade off between foraging and hiding within a refuge (Hughes & Burrows 1994, Vadas et al 1994). It is possible that dogwhelks in Loch Fyne were also under a considerable predation pressure as suggested by low population density and high effort per unit catch (majority of the dogwhelks were hidden under rock or seaweeds). The predators of dogwhelks in Loch Fyne may include shore crabs *C. maenas*, oystercatchers *Haematopus ostralegus*, herring gulls *Larus argentatus* and corkwing wrasses *Crenilabrus melops* (Moore 1938a). Palmer (1990) showed that crab effluent and scent of damaged conspecifics strongly reduced the feeding activity of juvenile dogwhelks, resulting in slower tissue growth and considerable thickening of the shell. In this way, heavier shells of dogwhelks from Loch Fyne and Largs might also be associated with a higher predation pressure.

Any contaminated area, like Gourock, might be unsuitable for certain predatory species to stay or survive, however the dogwhelk, a tolerance species, could have grown normally and possibly under a low predation pressure. Pollution mediated

alteration of community structure can be a potentially influential factor changing predation pressure (DeAngelis 1996) that might be favourable to the population of *N. lapillus*. As there is a growing concern to integrate studies of environmental pollution, toxicology and ecology, researchers are encouraged to consider beyond individual organism and to focus on population or community levels when studying toxicity of contaminants (Lagadic et al 1994, Baird et al 1996, Calow 1996). Further field experiments including behavioural and ecological studies, are required to confirm whether pollution can enhance fitness of dogwhelks by lowering predation pressure.

Toxic effects of Trace Metals on Nucella

Cellular toxicity may result if the rate of metal influx into the cell exceeds the rate of MT synthesis and/or the maximum level of these proteins synthesised by the cell (Viarengo 1985, Di-Giulio et al 1995). Therefore it has been proposed that measurement of MT may provide information about potential health hazards of metals in exposed organisms (Benson et al 1990, Bebianno & Machado 1997), although MT may be influenced by various biotic and abiotic factors (Leung & Furness 1999a, Mouneyrac et al 1999, Mouneyrac et al 2000). A significantly higher MT concentration observed in the Leiblein gland of *N. lapillus* from Largs, is five time greater than that of the other two populations. With the results of metal concentrations, such high MT concentration implies that this population could be under sublethal stresses caused by the trace metals, especially Cd and Cu. Based on the results of partial correlation analysis, MT is a good predictor for either Cd or Cu but not for Zn in this species. In addition, MT is generally responding to the levels of metals (Ag, Cd, Cu, Hg, Zn) in the tissues which can be affected by growth, as demonstrated in the present study. As the slow growing *Nucella* in Largs accumulated more Cd and Cu, these metals could have induced more MT in their tissues. Thus, growth apparently has an indirect effect on MT induction that also follows the pattern of concentrations of Cd and Cu in this species.

Interestingly, the results of partial correlation also suggested that the either Cd or MT concentrations correlated negatively with the RNA/protein ratio. This negative correlation might be indicative of a reduction in food consumption at increased metal burdens, although there was no significant correlation between the concentration of each metal and individual growth rate. The dogwhelks from Largs, at the standardised size (0.5 g wet soft-body), had 21.35 μg Cd g^{-1} and 202.96 μg Cd l^{-1} in the digestive gland/gonad complex, almost twice the concentrations found in the other populations.

Previous laboratory studies have demonstrated that low concentrations of Cd or Cu can inhibit normal metabolism and reduce growth through reducing consumption in gastropods (e.g. Lai & Lam 1994, Cheung & Wong 1988, Gomot 1997). A recent chronic study also showed that the growth rate of Cd-exposed *N. lapillus* decreased significantly with increasing Cd concentration in the tissues (Leung & Furness submitted). However, as mentioned previously, the growth rate of these wild populations of *N. lapillus*, could also be reduced by a poor quality diet and a high predation pressure.

Glycogen represents the readily mobilisable storage form of glucose for most organisms. Lagadic et al. (1994) proposed to use glycogen as a biomarker of environmental pollution because changes in glycogen concentrations are not as transient or sensitive to non-toxicant stress (e.g. temperature and salinity). In the laboratory acute study, waterborne Cd exposure results in reduction of glycogen in *N. lapillus* (Abdullah & Ireland 1986; Leung et al submitted), suggesting that there are costs of combating the toxic effects of Cd. In the present field experiment, the foot muscles stored more glycogen than did the digestive gland/gonad complex, consistent with the laboratory results (Leung & Furness submitted). Higher glycogen stores in the muscles may serve to meet the energy demands for locomotion and mucus secretion, whilst the glycogen in these tissues might be reserved for reproductive purposes as *N. lapillus* can breed throughout the year (Moore 1938b). However, there is no obvious causal relationship between metal pollution levels and glycogen stores in these wild *Nucella* populations. Our previous chronic toxicity study also observed that there was no significant effect of Cd on glycogen concentrations in the foot muscles and digestive gland/gonad complex of fasted or fed *N. lapillus* following exposure to Cd chronically for 80 days (Leung & Furness, submitted). The present results raise a question about the suitability of using glycogen as a stress biomarker in biomonitors, as consumption and reproductive state, apart from the stress caused by chemicals, can influence glycogen stores in *Nucella*.

Implication of the Present Results

In nature, different populations of the *N. lapillus* (e.g. Gourock and Largs), exhibited different accumulation of and responses to trace metal contamination. Much of this variation can be attributable to proximate environmental heterogeneity, including prey composition, predation pressure, hydrology and so on (Lam 1999). Such variation and

uncertainty of toxicity data may be critical for decision making in risk assessment (e.g. formulating the discharge limit for industrial effluents) based on the results of laboratory toxicity tests with a single strain of a species at each trophic level. It is very difficult to modify the conventional toxicity tests in order to include all different strains of the test species and consider all environmental variables, because of limited resources and time. As *in situ* field examinations on different populations of a biomonitor species are more ecologically sound and essential to environmental protection and conservation, field and laboratory experiments should be run concurrently and complementary to each other.

It is evident that growth can confound the monitoring results of trace metals using *N. lapillus*. In fact, even in the most common biomonitor *M. edulis*, their shell morphology, growth rate and health or nutritional condition can be very different between areas. Sukhotin and Maximovich (1994) conducted a transplantation study and showed that growth rate of transplanted *M. edulis* could be reduced by 2-5 times compared to the control, depending on the type of habitat. Additionally, pollution may reduce growth rate of biomonitors. Widdows et al (1995) showed that scope for growth of *M. edulis* declined following the pollution gradients in North Sea, and mainly associated with polyaromatic hydrocarbons. Based on the results from laboratory studies, Wang and Fisher (1997) modelled the metal accumulation by *M. edulis* and noticed that the growth rate and its constant changing according to size, are needed to model and to predict metal concentration and allometry of metal accumulation in the mussels. Any change in growth, therefore, will modify the final metal concentration in the tissue. Nowadays, we compare the metal concentration of the biomonitors at a standardised size between sampling sites, using regression between size (biomass, shell length or shell weight) and metal content (Langston & Spence 1995). However, this method does not resolve the problem of growth-mediated differences in metal concentrations of biomonitors. Although it has been suggested that biomonitoring programmes should use biomonitors with similar size and growth rate, this is not always practical.

CONCLUSION

Researchers should incorporate data of *in situ* growth rate and condition index or the RNA/protein ratio while monitoring trace metals using biomonitors in order to rule out the effect of growth-mediated differences in the metal and MT concentrations. This comprehensive biomarker approach is applicable and indispensable for monitoring programmes using gastropod molluscs as biomonitors. If resources are limited, any monitoring programme should at least include data of condition index, which is easy to measure with a minimal cost.

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CHAPTER 8

General Discussion

METALLOTHIONEIN AS A BIOMARKER OF METAL EXPOSURE

Metallothioneins (MTs) are frequently proposed as a measure of response to metal exposure in marine molluscs (Benson et al., 1990; Livingstone, 1993; Roesijadi, 1994; Bebianno and Machado, 1997; Viarengo et al., 1999). It is clear that sublethal levels of Cd can induce MT in the dogwhelks *Nucella lapillus* (Chapters 2-5) and periwinkles *Littorina littorea* under controlled laboratory conditions (Langston and Zhou, 1987; Bebianno and Langston, 1995). In wild populations, the concentrations of MT in the Leiblein gland of *N. lapillus* are correlated well with Cd or Cu (Chapter 7), while the MT concentrations in the whole *L. littorea* increase in relation to the concentration of Cd (Chapter 6). Since both of these gastropods cannot regulate or excrete Cd efficiently (Chapter 2; Bebianno and Langston, 1998), they detoxify the accumulated Cd through MT synthesis. Further, half life of Cd induced MT in these gastropods (40 d in the whole animal of *N. lapillus* (Chapter 2); 69 d and 160 d in the gills and kidney of *L. littorea* (Bebianno and Langston, 1998)), is longer than those in bivalves and mammals (Chapter 2). Longer MT turnover rates in these gastropods provide longer detectable periods for the changes in MT levels due to the contaminants in marine environments. Therefore, the present results provide further support for the use of MTs as biomarkers for exposure of trace metals, especially for Cd, in these marine gastropod species.

The rate of Cd-MT synthesis is dependent on tissue types in *N. lapillus* (Chapter 2). Higher rates of MT synthesis are noted at the Leiblein gland and followed by kidney of *N. lapillus* (Chapter 2), while the gills and kidney of *L. littorea* showed higher rates of Cd-MT induction (Bebianno and Langston, 1995). In both species, high basal MT levels in the digestive gland were observed, possibly involving in Cu metabolism during turnover of haemocyanin (Chapter 2, Fig. 2.5; Bebianno, 1990). As the Leiblein gland of *N. lapillus* is easy to dissect and the most sensitive tissue for Cd-MT induction, use of this tissue of *N. lapillus* for MT analysis has been proven useful by the laboratory and field experiments (Chapters 3-5, & 7).

FACTORS AFFECTING MT LEVELS

As certain metals such as Cd, Cu, Zn, Hg, Ag and Au principally induce MTs, the factors affecting the concentration of these metals may directly or indirectly influence the concentration of MT. Both Cd accumulation and Cd-MT synthesis of *N. lapillus* increased in relation to water temperature (Chapter 3). These results indicate that the level of MT in biomonitors like *N. lapillus* may be subject to seasonal (e.g. summer vs. winter) and geographical (e.g. temperate vs. tropics) variations. Therefore, temperature should be considered if comparing MT or metal levels using mollusc populations from different latitudes with different temperature profiles.

The earliest monitoring studies have shown that animal size (biomass, shell weight or shell length) is an important independent variable influencing metal levels in marine gastropods (Nickless et al., 1972; Stenner and Nickless, 1974; Boyden, 1974). The relationship between size and the concentrations of various metals of *L. littorea* or *N. lapillus* is dependent on the metal and sampling site (Chapters 6 & 7). Across all data (regardless of sampling sites), the concentrations of MT generally decreased with increasing animal size of *L. littorea* and *N. lapillus*, respectively. In the laboratory experiment (Chapter 3), concentrations of Cd or MT in the tissues of *N. lapillus* exposed to Cd, were also generally inversely correlated with size (depending on tissue type and temperature). Higher rates of MT synthesis (weight specific) in small animals are apparently due to higher metabolism, protein synthesis and growth rate in younger individuals.

It has been widely suggested that growth rate can influence the resultant metal concentrations in biomonitors (Phillips and Rainbow, 1993; Langston and Spence, 1995). Previous field studies noted that metal and/or MT concentrations of molluscs varied according to seasonal changes in biomass (e.g. weight loss in winter or after spawning; and weight gain in summer) (Baudrimont et al., 1997; Bordin et al., 1997; Serra et al., 1999; Mouneyrac et al. 2000). The present laboratory results showed that the fasted *N. lapillus* had significantly higher Cd and Cd-MT concentrations than the animals fed with barnacles or mussels (Chapter 4), indicating that tissue wastage during fasting increases the metal concentrations whereas a tissue dilution effect on Cd and Cd-MT occurs in fed individuals. Furthermore, feeding mussels can promote better growth rate than feeding barnacles alone in *N. lapillus*. Therefore,

nutritional state and prey type, which have substantial effects on the growth rate and condition index, indirectly affect the concentrations of MT and metals in this predatory gastropod. Later, a field study (Chapter 7) reconfirms these laboratory results, and indicates that growth rate is an important factor and it can confound the use of *N. lapillus* (and *L. littorea*, Chapter 6) as biomonitors of trace metal contamination. Therefore, the growth rate of mollusc biomonitors should be considered in biomonitoring program.

Other possible factors

The effect of sex on Cd-MT induction in the Leiblein gland of *N. lapillus*, is not significant (Chapter 4). Further work is needed to study the MT concentrations in the gonad and other tissues of *N. lapillus* collected during the breeding seasons (April-May and July-August; Feare, 1970). Other biotic factors such as the effect of predation pressure (Chapter 7), and aging on metal accumulation and MT induction by *N. lapillus* are worth exploring in the future.

In coastal marine environments, there are mixtures of chemicals (e.g. hydrocarbons, metals, pesticides, agricultural and sewage treatment effluents) present in seawater and sediment. These chemicals may interact with each other, and may alter toxicity of trace metals (e.g. Cd and Hg) synergistically or antagonistically. Such uncertainties of combined toxic effects of chemicals demand further research studies, although other methods such as quantitative structure activity relationship (QSAR) models could assess the toxicity of individual chemical compounds based on their chemical and physical properties (Lipnick, 1995). In Chapter 5, it is demonstrated that hydrogen peroxide can also induce MT-like proteins in *N. lapillus* and Cd-MT may serve as an antioxidant and protect the animals from oxidative stress. Nevertheless, there is a need to characterise this oxidative stress-induced MT which is likely different from those induced by metals. In addition, magnitude and significance of such effects on induction of the MT-like proteins under natural conditions (low Cd and H_2O_2 concentrations) have yet to be established. Clearly, interaction between metals and other pollutant(s) is an important area to ecotoxicology of trace metals (Phillips and Rainbow, 1993; Langston and Spence, 1995), for example, investigation of the combined effects of diluted diesel or fuel oils (commonly leaked or discharged from the engines of ships) and metals on MT induction by molluscs.

Another important environmental factor, which has not been reported in this thesis, is salinity. In general, salinity is inversely correlated with the bioavailability of metal ions to bivalve molluscs (Phillips and Rainbow, 1993), so that the animals may have higher concentrations of metals and MT in their tissues at low salinity. However, Mouneyrac et al. (1998) observed that the effect of salinity on the MT levels of wild resident oysters, *Crassostea gigas*, was not significant. Notwithstanding, *N. lapillus* may not respond similarly to the bivalves, as dogwhelks are predatory gastropods, and have different rates of metal accumulation and MT synthesis. Therefore, a study on the Cd-MT induction of *N. lapillus* under different salinity (11, 22, 33‰) and fluctuating-salinity (11-33‰ or 22-33‰) has been conducted at Sandgerdi Marine Laboratory, Iceland (the project is in collaboration with University of Iceland, and funded by BIOICE; samples await further analysis).

In oyster *C. virginica*, pre-exposure to Cd can greatly enhance MT synthetic rate and promote quicker responses to subsequent metal exposure (Unger and Roesijadi, 1996; Roesijadi et al., 1997). If these were true, animals born and living in a metal contaminated area should have faster up-regulation of MT synthesis than the animals living in clean areas. In terms of detoxification, fast MT induction is beneficial by reducing the high-risk window (low MT but high metals). Nevertheless, there is virtually no field validation of this hypothesis.

CAN WE USE MT AS A BIOMARKER OF METAL TOXICITY?

During this study, attempts have been made to investigate whether there is any relationship between MT and other fitness biomarkers including glycogen stores, condition index, growth rate, the RNA/protein ratio so that the maximum or threshold level of MT indicating cellular toxicity, could be identified (Chapters 3-4, & 7). In the acute toxicity study (Chapter 3), oxygen consumption and glycogen stores of *N. lapillus* were greatly reduced by exposure to Cd for 20 d at 10°C, while the concentration of MT in these animals was significantly increased. These results are promising that MT may be correlated to the decline of energy storage, although decline of glycogen could also be due to a shift to anaerobic respiration (spending more glycogen reserves) by Cd exposure. Nonetheless, in the chronic or field study (Chapters 4 & 7), there were no significant effects of the Cd-exposure or degree of metal contamination on the glycogen stores of *N. lapillus*. Feeding rate varied

between individuals and will directly affect the glycogen stores; and different populations may differ in strategy of energy allocation (e.g. Loch Fyne population stored more glycogen in gonad/digestive gland). For further study, analysis of glycogen and MT should be carried out using the same tissues including homogenised tissues from whole organism as well as separated tissues in order to allow correlation analysis between these two parameters.

Growth rate and nutritional condition (showed by the RNA/protein ratio) have indirect effects on MT concentrations of *N. lapillus*, since a tissue dilution effect on metals and MT occurs in fast growing individuals (Chapters 4 & 7). Regarding to the relationship between MT and fitness, *N. lapillus* of Largs had significantly higher metals (Cd and Cu) and MT levels while they showed lower growth rate and nutritional conditions. High metal accumulation and MT induction may indicate toxic effects such as inhibition of consumption and growth. However, such high metal and MT levels in Largs *N. lapillus* can be primarily explained by poor growth and nutritional conditions which are directly influenced by feeding on a less-profitable diet, barnacles and high predation pressure (Chapter 7). In future, a time-course laboratory study should be conducted in order to correlate growth rate (individual or population) and MT synthesis of *N. lapillus*. Hopefully, the MT responses can be correlated with early changes in Darwinian fitness that may have consequences for the whole population as suggested by Depledge et al. (1995).

There are several unanswered questions: what is the level of MT that can serve as an early warning signal for cellular toxicity? How can we determine such a value? The present study and other previous studies on MT induction in molluscs commonly used relatively high concentrations of Cd (100-500 $\mu g\ l^{-1}$). However, the range of average Cd levels occurring in coastal environments, is 0.27-5.7 $\mu g\ l^{-1}$ (Phillips, 1980). Recently, Roesijadi (1999) has demonstrated that the rate of MT synthesis in oysters *Crassostrea virginica* under laboratory exposure to Cd is much higher than that of wild animals. Although high MTs were measured in a natural population of *C. virginica* of a Cd-contaminated area, they actually synthesised MT at a basal rate, and their MTs were stabilised by Cd present in tissues (Roesijadi, 1999).

The maximum of MT concentration is probably a warning signal for cellular toxicity caused by metals (Fig. 8.1). But the values estimated based on high metal concentration do not reflect the actual picture in the biomonitors under natural

environments. To improve the accuracy of estimated maximum levels of MT, we should use more realistic concentrations of metals (e.g. 2-10 folds of normal concentrations) for exposure study under controlled laboratory conditions and design the experiment as a long term study (e.g. 1-2 years) (i.e. making the gap between the curves (L) and (F) as close as possible). Alternatively, *in situ* field study should be conducted although other factors such as seasonal changes of biomass may influence the levels of MT in the biomonitors.

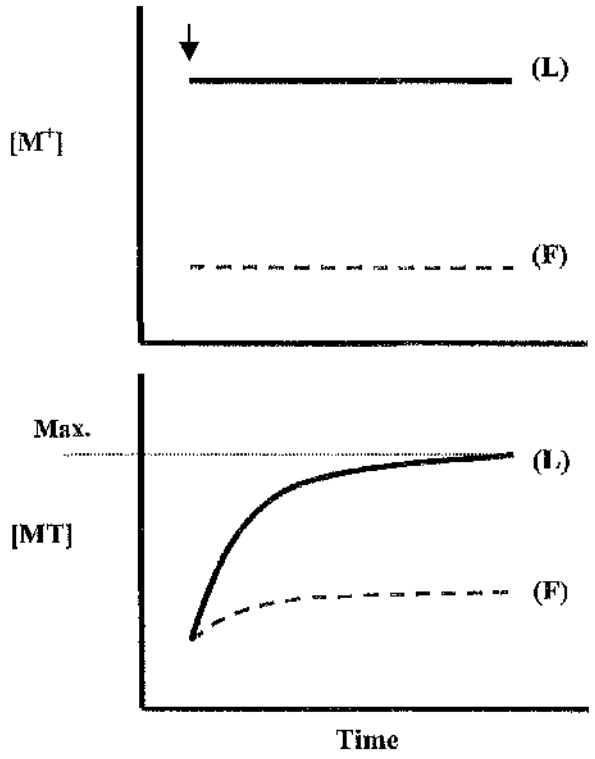

Fig. 8.1. Schematic diagram showing the differences in waterborne metal concentration [M+] and MT level [MT] in a biomonitor between the laboratory experimental conditions (L) and the natural environmental conditions (F). An arrow indicates the point of experimental metal concentration applied. "Max." denotes the estimated maximum of MT level by the laboratory experiment.

STANDARDISATION OF PROCEDURES FOR MT QUANTIFICATION

The present results of MT concentrations in *N. lapillus* and *L. littorea*, are compared with data obtained using differential pulse polarography (DDP) (Table 8.1). The values of MT determined by the silver saturation method (SAM), are generally comparable to those obtained by DPP, except that our MT values of *L. littorea* are relatively lower. Lower MT of *L. littorea* measured by SAM could be due

to the fact that our samples might be less contaminated with metals, and whole soft body tissues were used for MT analysis. Nevertheless, such differences between the methods might also be attributed to (1) differences in the sensitivity of the method and (2) different mammalian MT used as standard for calibration. Geret et al. (1998) showed that isolation procedure may has a substantial effect on the concentration of MT measured. Previous comparison studies indicated that DPP is relatively more sensitive than metal saturation method (Onosaka and Cherian, 1982; Wagemanni et al., 1994). As different methods can provide different values of MT, comparisons between data from literature are difficult (Geret et al., 1998). Therefore, it is urgent to develop a method for MT quantification with high precision, accuracy and specificity (Nordberg, 1998). We should standardise the procedures for MT isolation and quantification, and develop standard reference materials for mollusc MTs, in order to allow data comparisons for biomonitoring programs (W. J. Langston, personal communication, 1998). Furthermore, commercially available MT reference materials should also be checked with regard to purity (Nordberg, 1998).

Table 8.1. Comparison the present MT results with data obtained from literature.

Species	Tissues	MT Conc. mg g^{-1} dry wt	Method*	Standard	Source
L. littorea	Digestive gland	11.9	DPP	Rabbit liver MT	Bebianno & Langston (1989)
	Remaining tissues	2.6			
	Digestive gland	8.4 ± 1.3	DPP	Rabbit liver MT	Bebianno et al. (1992)
	Remaining tissues	3.3 ± 0.6			
	Whole soft tissues	0.2 to 1.1 (depending on animal size)	SAM	Horse kidney MT	This study (Chapter 6; Fig. 6.2a)
N. lapillus	Digestive gland	5.29	DPP	Rabbit liver MT	Bebianno & Langston (1989)
	Remaining tissues	1.95			
	Gills	1.91	SAM	Horse kidney MT	This study (Chapter 1; Fig. 1.4a)
	Gland of Leiblein	2.97			
	Kidney	2.06			
	Digestive gland	2.35			
	Gonad	3.52			
	Other tissues	0.53			

*DPP: differential pluse polarography; SAM: silver saturation method.

METALLOTHIONEIN AS A MONITORING TOOL

Measuring MTs in marine gastropods can indicate levels of exposure to certain metals, for example, Cd and Cu for *N. lapillus* and Cd for *L. littorea*. Undoubtedly, quantification of MTs in biomonitors is a promising monitoring tool for indicating metal bioavailability, and to a lesser extent for toxicity. As there is high individual variation in MT levels (due to differences in diet, growth, consumption and size) observed in wild populations of these two gastropods, careful design of sampling strategy (e.g. optimal sample size, time- or space-bulking sampling), and accurate MT determination are desirable if using MT as a monitoring tool. Water temperature and biological data including growth rate, condition index, food type and availability, and reproductive status are essential for interpretation of MT data, and should be incorporated into the monitoring program. Analytical methods used for quantifying different MT-isoforms in tissue samples of molluscs are currently available (Dallinger et al., 1997; Chassaigne and Lobinski, 1998; Lobinski, 1998; Chapter 1). As a result, we can accurately measure specific MT such as Hg-MT and Cd-MT for monitoring target toxic metals and minimise any interference of MT-like proteins induced by oxidative stresses. With the commercial available reference materials for mollusc MTs, these new techniques will enhance the usefulness of MTs as biomarkers of trace metal contamination in the near future.

With consideration of temperature, size, growth rate, and seasonal variation of biomass, we can model the behaviour of MT synthesis in a particular biomonitor species (e.g. *N. lapillus*) and use this model to predict the levels of the metal contamination and toxicity in different sampling areas based on the MT data. However, more research efforts are required to improve our understanding of the mechanisms of MT synthesis within an organism under natural conditions, and the relationship between MT levels and metal toxicity; and to establish how MT changes with size and biomass (to obtain various constants for the relationships) before formulating the mathematical model.

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