FIELD AND LABORATORY STUDIES ON HEAVY METAL UPTAKE AND TISSUE DISTRIBUTION, ATPASE ACTIVITY AND METALLOTHIONEIN IN TISSUES OF THE NORWAY LOBSTER *NEPHROPS NORVEGICUS* (L.)

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CANDIDATE'S DECLARATION

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

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SUMMARY

1). Heavy metal concentrations in sea water and sediment vary widely in different parts of the world. An important factor affecting heavy metal concentrations in an area is man-made contamination.

2). Heavy metals inhibit or alter many biological processes in decapod crustaceans and toxic effects of heavy metals vary depending on experimental animals, metals and concentrations used. The most important factors which affect toxicity and accumulation of heavy metals by marine animals are the growth, sex, age and physiological condition of marine animals, salinity and temperature of sea water, chemical characteristics of metals and interaction among metals. Tolerance to heavy metals and detoxification of heavy metals are also important factors influencing toxicity of heavy metals to marine animals.

3). The thesis outlines consequences of heavy metal pollution for marine animals and humans who consume these. I then describe the Clyde Sea area, its commercial importance and man-made contamination and the biology of an important commercial species the Norway lobster *Nephrops norvegicus*.

4). Natural concentrations of cadmium, copper and zinc in tissues of *Nephrops norvegicus* from the Clyde Sea area were highest in the hepatopancreas and gill, whereas mercury concentrations were found in highest levels in the gill and tail muscle. Iron concentrations were highest in the gill. Concentrations of metals showed variation among different months of the year with highest levels tending to occur during moult. Metal concentrations were also influenced by carapace length with pronounced size-related increases in mercury concentrations in the tail muscle and cadmium in the hepatopancreas. There were considerable differences in mean concentrations of some metals in the tissues of male and female *Nephrops*.

5). Toxicity studies showed that organic and inorganic mercury, copper, cadmium, lead and zinc (0.1, 0.1, 0.1, 1, 1 and 2 mg l^{-1} respectively) were toxic to *Nephrops norvegicus*. At those concentrations toxicities ranged widely among the metals, the

most toxic metals to the animals being organic mercury (100 % mortality in three days), inorganic mercury (100 % mortality in one week) and copper (100 % mortality in two weeks). The other metals did not cause 100 % mortality over a 30-day period.

6). The non-essential metals (organic mercury, inorganic mercury, cadmium and lead) were accumulated by tissues of *Nephrops norvegicus* after exposure to sublethal concentrations of the metals dissolved in sea water for 30 days. The highest concentrations of both mercury compounds were accumulated in the gill tissue while the highest concentrations of cadmium were in the gill and hepatopancreas. Lead concentrations were highest in the gill and carapace. Exposure to a sublethal concentration of copper showed increases in copper concentrations in the carapace, gill, tail muscle and ovary, whereas there was no increase in the hepatopancreas and external eggs. Exposure to a sublethal concentration of zinc also showed increases in zinc concentrations of the carapace, hepatopancreas gill, and ovary, whereas there was no increase in the tail muscle and external eggs.

7). There were differences in the accumulation of organic and inorganic mercury from sea water by tissues of *Nephrops norvegicus*. Except in the gill tissue, organic mercury was accumulated more than inorganic mercury. There were also sex related differences in the accumulation of the metals as male *Nephrops* accumulated higher concentrations of organic and inorganic mercury in the hepatopancreas than female *Nephrops*. Carapace length showed significant effects on metal accumulation as accumulation of both mercury compounds and cadmium was higher in the gills of smaller animals than that of larger animals. The adsorption of the metals onto the carapace surface was found to be very important in determining the metal concentrations of the carapace.

8). Concentrations of mercury increased in all the tissues of *Nephrops norvegicus* after feeding with a food source containing high concentrations of cadmium and

mercury. These increases in the tissues showed positive relationships with feeding rate. Cadmium concentrations increased only in the hepatopancreas. Cadmium concentrations in the hepatopancreas and carapace showed positive relationships with feeding rate. Concentrations of copper in the gill and tail muscle were increased after feeding, though none of the tissue copper concentrations showed a positive relationship with feeding rate. Zinc and iron concentrations in the tissues of *Nephrops* did not increase after feeding and, there was no positive relationship with feeding rate in any tissues.

9). Distribution of mercury and cadmium in the gill, hepatopancreas and tail muscle of *Nephrops norvegicus* differed among treatments. Mercury burdens in control animals were mainly in the tail muscle. After feeding, the hepatopancreas and tail muscle shared the total mercury burdens. Organic and inorganic mercury distributions also varied among the tissues after uptake from sea water, with organic mercury being more evenly distributed among tissues than inorganic mercury, the latter being predominantly in the gill. Much of the cadmium burden was always in the hepatopancreas in all the three treatments. Assimilation of organic mercury from food by tissues was higher than assimilation of inorganic mercury.

10). Characterization of ATPases such as total ATPase, Na,K-ATPase, total Mg-ATPase, oligomycin sensitive and insensitive Mg-ATPase in the gill of *Nephrops norvegicus* showed that maximum activities of the ATPases were in sodium, potassium, magnesium and ATP concentrations of 100, 20, 4 and 6 mmol l^{-1} respectively. 1 mmol l^{-1} ouabain inhibited 100 % Na,K-ATPase activity, while 100 % of oligomycin sensitive Mg-ATPase was inhibited in oligomycin concentration of 0.3 mmol l^{-1}. The activities of the gill ATPases were maximum when temperature was at or near to 37 ^{o}C. Na,K-ATPase and oligomycin sensitive Mg-ATPase activities were more sensitive to temperature than oligomycin insensitive Mg-ATPase the latter being still active (50 %) at a temperature of 70 ^{o}C. Size of *Nephrops*

showed significant effects on the activities of gill ATPases as the activities of Na,K-ATPase and oligomycin sensitive Mg-ATPase had negative relationships with carapace length. Storage of the gills was found to reduced the activities of gill ATPases, even storage at -70 ^{o}C. The activities of the Na,K-ATPase (34 and 45 %) and oligomycin sensitive Mg-ATPase (31 % and 40 %) were reduced after one and two months storage at -70 ^{o}C, respectively.

11). Control male *Nephrops norvegicus* showed higher activity of Na,K-ATPase than control female *Nephrops norvegicus*. After exposure to sublethal concentrations of cadmium, copper and zinc dissolved in sea water, the activity of Na,K-ATPase was inhibited significantly in male animals by the metals but not in female animals. However, the activity of total Mg-ATPase was inhibited significantly in female animals, but not in male animals. There was no other significant alteration on the activity of ATPases after exposure to the metals. Na,K-ATPase activity had a negative relationship with gill copper in males, while this ATPase had positive relationships with cadmium and zinc concentrations in female animals.

12). After exposure to sublethal concentrations of cadmium, copper and zinc, cadmium concentrations were increased significantly in the gill and hepatopancreas of both male and female *Nephrops norvegicus* in relation to increases in exposure concentrations. Concentrations of copper and zinc increased in the gills of male animals but not in female animals. In the hepatopancreas neither copper nor zinc showed any change in concentration with exposure in either sex suggesting regulation of these metals by *Nephrops norvegicus*.

13). There were positive relationships between the concentrations of cadmium and metallothionein in both the gill and hepatopancreas of male and female *Nephrops norvegicus*. Copper concentrations in the hepatopancreas also showed a significant positive relationship with metallothionein in male animals but not in female animals. Ratios of cadmium in the metallothionein fraction of the hepatopancreas increased in

both male and female animals, while copper and zinc ratios did not change in the hepatopancreas of either sex.

14). The activity of ATPases in the gill of male *Nephrops norvegicus* was determined from 10 stations in the Clyde Sea. Because many *Nephrops norvegicus* in the Clyde Sea were infected by a parasite (*Hematodinium perezi*), investigations were carried out by separating the animals as normal (blue blooded) and infected (white blooded). Activities of total ATPase, total Mg ATPase, Na,K-ATPase, oligomycin sensitive and insensitive Mg-ATPase were significantly affected by the infection namely white blooded animals had higher activities of the ATPases than normal animals. Stations also affected significantly the activities of the ATPases, except for oligomycin insensitive Mg-ATPase activity. All the ATPase activities had negative correlations with concentrations of copper and zinc in the blood.

15). The blood colour, station and carapace length also affected some gill and blood parameters such as ion concentrations in the gill and blood, % gill water and blood osmolality in *Nephrops norvegicus* caught in 10 stations from the Clyde Sea. Blood copper and zinc concentrations were much lower in the white blooded animals than in normal animals.

16). Concentrations of copper, zinc and metallothionein in the gill of *Nephrops norvegicus* caught in 11 stations from the Clyde Sea were found to be affected by the infection: namely infected animals had higher concentrations of metallothionein but showed lower copper concentrations. Conversely, cadmium concentrations were not affected by the infection but levels differed significantly among stations. The ratio of copper to metallothionein in the gill was reduced three times in the infected animals. Copper and metallothionein concentrations showed negative relationships, possibly resulting from independent effects of stress due to parasitic infection.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Heavy metals are normal constituents of sea water and many are essential for life in the marine environment. Under normal conditions these metals are supplied to the marine environment by rivers, atmospheric dumping or wind blown materials following volcanic activities and the weathering of rocks. At the present time, however, additional quantities of metals enter estuaries and coastal waters from industrial effluents, sewage and from atmospheric pollution. Although some metals such as copper, zinc and iron are essential for marine animals, all are toxic in high concentrations.

1.1 HISTORY OF HEAVY METAL POLLUTION

Man has benefited from the use of heavy metals for many hundreds of years. Therefore, the history of metal pollution begins with the use of metals by Man. At this point one should describe the difference between contamination and pollution. Clark (1989) describes **contamination** as the presence of elevated concentrations of metals in water, sediment or organism, i.e. concentrations that are above the natural background level for the area and the organism, whereas **pollution** is the introduction by Man, directly or indirectly, of metals to the marine environment resulting in deleterious effects such as harm to living resources, hazards to human health ; hindrance of marine activities including fishing ; impairment of the quality for use of sea water ; and reduction of amenities. In other words, contamination may provide a warning signal, but it does not constitute pollution unless, first, it is caused by human activities and second, it has some damaging effects. Although metals have been being used for a long time, a considerable contribution of metals to the environment began after industrialization in this century which may mean that the history of metal pollution begins early in this century.

1.2 CHEMICAL AND PHYSICAL PROPORTIES OF SOME HEAVY METALS

1.2.1 MERCURY

Mercury is an element with no known biological function. It has an atomic number of 80, an atomic mass of 200.59, a specific gravity of 13.55 g/cm^3, a melting point of -39.8 ^{o}C and a boiling point of 357 ^{o}C. Mercury occurs naturally as six isotopes; ^{202}Hg (30%), ^{200}Hg(23%), ^{199}Hg (17%), ^{201}Hg(13%), ^{198}Hg(10%) and ^{204}Hg(7%), as well as trace amounts of ^{196}Hg and the relatively stable radioisotope ^{203}Hg (Burg and Greenwood, 1991). Elemental mercury is usually referred to as mercury vapour when present in the atmosphere or as metallic mercury in the liquid form. This form is toxicologically important because it has a relatively high vapour pressure and a certain water (about 20 μg l^{-1}) and lipid solubility (5-50 mg l^{-1}) (Burg and Greenwood, 1991). Mercury occurs in ionic form as Hg^{2+} (mercuric salts) and Hg^{+} (mercurous salts). The former readily form complexes with organic ligands, notably sulfhydryl groups. In contrast to $HgCl_2$, which is both highly soluble in water (69 g l^{-1} at 20 ^{o}C) and highly toxic, Hg_2Cl_2 is less soluble (2 mg l^{-1} at 25 ^{o}C) and correspondingly less toxic. The least soluble mercuric form is cinnabar (HgS) which has a water solubility of 10 ng l^{-1} (Weast, 1978). Organic mercury compounds consist of diverse chemical structures in which mercury forms a covalent bond with carbon. The group is limited to alkylmercurials (methyl and ethylmercury), arylmercurials (phenylmercury) and the family of alkoxyalkyl mercury diuretics. Organic mercury cations form salts with inorganic and organic acids, e.g. chlorides and acetates, and react readily with biologically important ligands, notably sulfhydryl groups. They also pass easily across biological membranes perhaps since the halides (e.g. H_3CHgCl, although it is not clear whether

this exists in a stable form in tissues at physiological pH) and dialkylmercury are lipid soluble (Clarkson et al., 1977).

1.2.1.1 Sources, Production, and Uses of Mercury

Mercury is found in igneous rocks of all classes and has an ubiquitous distribution. In nature, mercury occurs in a variety of physical and chemical forms. Normal soils typically contain 20-150 ppb Hg, but near known geological deposits the level can reach as high as 80 % (Burg and Greenwood, 1991). Mercury is mined as cinnabar (mercury sulphide). Generally, mercury binds strongly to the organic components in soil so that mobility by leaching is minimal and contamination of ground water is unlikely unless mercury leaches from a municipal landfill (US EPA, 1984).

Elemental and inorganic mercury compounds are used in the manufacture of scientific instruments (thermometers, barometers), electrical equipment (switches, rectifiers, oscillators, electrodes, batteries, meters, mercury vapour lamps, x-ray tubes, lead and tin solder), dental amalgams and synthetic silk. In the chemical industry mercury is used as a fluid cathode for the electrolytic production of acetic acid, chlorine and sodium hydroxide (Burg and Greenwood, 1991). In the past mercury has been used in the plating, tanning and dyeing, textile, photographic, and pharmaceutical industries. It has been used for the preparation of drugs and disinfectant, and arylmercury compounds have been used as disinfectants, fungicides, antiseptics, herbicides, preservatives and as a denaturant for ethyl alcohol (Baeyans et al., 1979 ; Langston, 1990 ; Burg and Greenwood, 1991).

The annual production of mercury world-wide reached a peak of 10,600 tonne in 1971 falling to a little over 6000 tonne in 1987 (Clark, 1989). Mercury is released to

the environment in different forms such as mercury(II) oxide, mercury(II) sulphide (cinnabar), mercury chloride, mercury nitrates, mercury sulphates, mercury(II) thiocyanate, chloride and dithiocarbamate, borate and oleate of phenylmercury, and chloride, silicate and phosphate of alkylmercury compounds (Burg and Greenwood, 1991). These inputs are estimated to amount to about 5000 tonne/year world-wide, but a further 3000 tonne/year is derived from burning fossil fuels. One also should not forget the natural inputs of mercury, of about 3500 tonne/year from the weathering of rocks, but especially about 25,000-150,000 tonne/year from volcanic areas, as gases (Clark, 1989).

1.2.2 CADMIUM

Cadmium is another heavy metal with no known biological function. It is a relatively volatile element though less volatile than mercury or lead. Cadmium has an atomic number of 48 and an atomic mass of 112.4. It has a boiling point of 767 ^{o}C and a density of 8.64 g/cm^{3} at 20 ^{o}C. Cadmium is a silver-white, lustrous and ductile metal. There are eight naturally occurring isotopes of cadmium; ^{114}Cd (29%), ^{112}Cd(24%), ^{111}Cd(13%), ^{110}Cd, ^{113}Cd, ^{116}Cd, and ^{108}Cd. Cadmium belongs, along with zinc and mercury, to the second subgroup of the Periodic Table; it has an oxidation state of +2 in all compounds. With a normal electrochemical potential of -0.40 relative to the hydrogen electrode, it is slightly more noble than zinc. The cadmium ion (r=103 pm) is very close in size to the calcium ion (r=106 pm). Therefore some similarities can be seen between cadmium and calcium (Stoeppler, 1991).

1.2.2.1 Sources, Production, and Uses of Cadmium

Cadmium commonly occurs in an isomorphic form in zinc minerals such as zinc blende (ZnS) with cadmium contents from 0.1 to 0.5 %, and galmei ($ZnCO_3$) with cadmium contents up to a maximum of 5 %. Phosphate rocks also show a broad range of cadmium contents with an average of approximately 15 mg kg^{-1}. Pure cadmium minerals such as greenockite (hexagonal CdS), hawleyite (cubic CdS), otavite (CdCO3), monteponite (CdO), and cadmoselite (CdSe) occur very rarely. Cadmium is mainly extracted (>95 %) from cadmium-enriched by-products obtained from the roasting of zinc minerals and purified by electrolytic deposition or vacuum distillation at a temperature of 420-485 ^{o}C (Stoeppler, 1991).

Cadmium has a variety of uses. Electrodeposited cadmium has excellent properties for protecting iron and steel against corrosion-even a thickness of 0.008 mm is sufficient for protection. Another increasingly important use is in rechargeable nickel-cadmium batteries. Other uses include cadmium pigments (cadmium sulphide, cadmium selenide and mixtures of both) which are generally very stable thermally (e.g. for plastic materials in cars). Cadmium soaps made with saturated and unsaturated fatty acid play an important role as temperature and light stabilisers mainly for PVC (e.g. for plastic window profiles). A small percentage of cadmium (cadmium sulphide-copper sulphide) is used in solar cells for direct conversion of light into electrical energy. Cadmium is also used in nuclear reactors as a neutron absorber and in various alloys with such metals as tin, copper, and aluminium (Stoeppler, 1991). World production of cadmium has been increased from 16800 tons/year in 1970 to 19700 tons/year in 1986 (Clark, 1989 ; Stoeppler, 1991).

1.2.3 LEAD

Lead is also a biologically non-essential metal and is ubiquitously distributed in nature. Although its natural concentrations are not high, in the last fifty years great amounts of lead have been extracted, concentrated and used by Man, and re-emitted into the environment (Ewers and Schlipkoter, 1991). Lead is known to have been used by Man prior to 2000BC (Goyer, 1991).

Lead has an atomic number of 82, an atomic mass of 207.1. It is a bluish-white, soft metal with a density of 11.34 g/cm^3, a melting point of 327 ^oC, and a boiling point of about 1740 ^oC. Natural lead consists of 52 % ^{208}Pb, 24 % ^{206}Pb, 23 % ^{207}Pb, and 1 % ^{204}Pb. In most organic compounds lead is in the +2 oxidation state. The salts of Pb(II), lead oxides and lead sulphide, are not readily soluble in water, with the exception of lead acetate, lead chlorate, and to some extent, lead chloride. Inorganic Pb(IV) compounds are unstable and strong oxidising agents. Because of their use as antiknock agents in petrol, tetramethyllead and tetraethyllead are the most important organolead compounds. Both are colourless liquids with boiling points of 110 ^oC and 200 ^oC, respectively. At these temperatures or slightly below, they start to decompose.

1.2.3.1 Sources, Production, and Uses of Lead

Lead is represented in almost all constituents of the Earth's crust. It can be found in all environmental media and in all components of the biosphere. The most important lead minerals are galena (lead sulphide), cerussite (lead carbonate), and anglesite (lead sulphate). In 1982 globally about 3.7 million tonne of lead were produced from mined ores, though consumption of refined lead was about 5.2 million tonne world-

wide. About 40 % of all lead consumed is used for the production of lead-acid batteries. The production of tetramethyllead and tetraethyllead accounts for approximately 10 % of the world lead consumption. Lead based pigments are used as a protective coating for steel structures, for painting used on highways and for exterior uses. Lead chemicals are also used in glassware and ceramics and as stabilisers in plastics. Other uses of lead could involve lead sheets, cable sheeting, solder, ammunition, bearing alloys, type metal, tubes, weight and ballast which account for 20 % of uses (Ewers and Schlipkoter, 1991).

Lead and its compounds may enter the environment at any point during mining, smelting, processing, use, or disposal. Global emissions were about 500,000 tonnes/year around 1919, about 2 million tonnes/year around 1940, and about 4.5 million tonnes/year around 1970 (Nriagu, 1989). Estimates of the dispersal of lead emissions into the environment indicate that the atmosphere is the major initial recipient. Mobile and stationary sources of lead emission tend to be concentrated in areas of high population density, and near smelters. From these emission sources, lead moves through the atmosphere to various components of the environment (Ewers and Schlipkoter, 1991).

1.2.4 COPPER

Copper is an ubiquitously distributed metal. It is very easily complexed and is involved in many metabolic processes in living organisms. Thus, copper is an essential metal for life. The respiratory pigment (haemocyanin) of many molluscs and higher crustaceans contains copper.

Copper has an atomic number of 29 and atomic mass of 63. It has been known for about 10,000 years and occurs in metallic form or in compounds as Cu(I) or Cu(II). The red metal has a density of 8.93 g/cm^3, a melting point of 1083 ^{o}C and a boiling point of about 2590 ^{o}C. Natural copper consist of an isotopic mixture of 69.1 % ^{63}Cu and 30.9 % ^{65}Cu. Except for silver, copper is the best common conductor for heat and electricity. Compounds of Cu(I) and Cu(II) and Cu complexes have very different properties from the metal.

1.2.4.1 Sources, Production, and Uses of Copper

Copper is almost always extracted from ores found in underground or open-pit mines. The most important ores contain, besides small amounts of metallic copper, Cu_2S, CuS, $CuFeS_2$, CuO, $Cu_2CO_3(OH)_2$. Copper is used in electrical applications, water piping, stills, roofing material, and kitchenware; for chemical and pharmaceutical equipment; as a pigment; and as a precipitant of selenium. Alloys of copper include those with zinc (brass), tin (bronze), nickel (monel metal), aluminium, gold, lead, cadmium, chromium, etc. Copper sulphate is used to supplement pastures deficient in the metal; as an algicide and molluscicide; as a mordant; in electroplating and, as a component of Fehling's solution, to estimate reducing sugars in urine. Cupric oxide has been used as a component of paint for ship bottoms. Copper chromates are pigments, catalysts for liquid phase hydrogenation, and potato fungicides (Scheinberg, 1991).

The movement of relatively high concentrations of copper from the Earth's crust into the soil depends on weathering, the process of soil's formation, drainage, oxidation-reduction potentials, the amount of organic matter in the soil, and, perhaps most important, the pH. Almost all copper carried into the ocean is precipitated,

accounting for its lower concentration there than in freshwater (Scheinberg, 1991).

1.2.5 ZINC

Zinc is an essential metal for life incorporated in many enzymes in vertebrates and invertebrates.

Zinc has been used for many purposes for a long time by Man. It is a bluish-white, rather soft metal, which solidifies in hexagonal crystals. It has an atomic number of 30, an atomic mass of 65.39 and a density of 7.14 g/cm^3. Zinc melts at 419.6 ^oC and boils at 907 ^oC. Natural is zinc composed of five stable isotopes (48.6 % ^{64}Zn, 27.9 % ^{66}Zn, 4.1 % ^{67}Zn, 18.8 % ^{68}Zn and 0.6 % ^{70}Zn). Zinc, oxidation state +2, has a strong tendency to react with acidic, alkaline, and inorganic compounds. Because of its amphoteric properties zinc forms a variety of salts. Zinc chlorate, Zn-chloride, the sulphates and nitrates are readily soluble in water, whereas the oxide, carbonate, the phosphates and silicates, the sulphides and organic complexes are particularly insoluble in water.

1.2.5.1 Sources, Production, and Uses of Zinc

Zinc occurs in almost all minerals in the Earth's crust with a median concentration of about 70 mg kg^{-1}. The principal ores used for production are the sulphides sphalerite (zinc blende, cubic ZnS) and wurtzite (hexagonal ZnS) and their weathering products smithsonite ($ZnSO_4$, trigonal) and hemimorphite (2 $ZnOxSiO_2xH_2O$, rhombic or trigonal) (Ohnesorge and Wilhelm, 1991). Zinc oxide is the most important zinc compound. It is used in rather large amounts in the production of photocopy paper, of chemicals and paints. It is also used in floor coverings, for glasses, enamels,

fabrics, plastics, lubricants, and in rayon manufacture. Zinc oxide is a long serving, well-known pharmaceutical compound for external application for burns and skin infections as a powder and ointment. Other zinc compounds used could be summarised as follows; uses in soldering, welding fluxes, fire proofing, pesticidal wood protectant, dry batteries, cauterising agent in medicine, as phosphors in X-ray and TV screens, and luminous watch faces, as lubricants, for the water proofing of textiles, papers, and concrete. Organozinc compounds are used as fungicides or antiseptics (Ohnesorge and Wilhelm, 1991). The world production of zinc was estimated about 6 million metric tons between 1980 and 1982.

1.3 HEAVY METAL LEVELS IN THE MARINE ENVIRONMENT

Under natural (uncontaminated) conditions metal concentrations in the marine environment are very low. Because of the use of metals in different fields of industry, agriculture, medicine, etc., concentrations of metals may be increased in the marine environment, especially in estuaries and coastal waters which are much more vulnerable to the impact of pollution than the open ocean. Therefore, concentrations of metals may vary greatly with locality and time. For example, the range of heavy metals in ocean water in $\mu g\ l^{-1}$ is reported as follows ; Hg 0.004-0.012, Cd 0.0002-0.025, Cu 0.025-0.64, Pb 0.006-0.015, Zn 0.0007-0.588 (Burton and Statham, 1982). The levels of the metals, however, are higher in coastal water and especially in estuaries since they are often contaminated by human activities. Concentrations of heavy metals in some of estuaries and coastal waters around the world are given in Table 1.1. In Table 1.1, high concentrations of metals represent the areas where human activities were prime factors. Concentrations of heavy metals in sediments are normally higher than in the sea that overlies them. The most important factors affecting metal concentrations of sediments are also dependent on

the sources of contamination. Oceanic levels of heavy metals in sediments are also lower than those from coastal waters and estuaries (Chester and Stoner, 1974). Sediment concentrations of uncontaminated areas are reported as follows; Hg 0.03, Cd 0.2, Pb 25, Cu 10 and Zn 100 $\mu g\ g^{-1}$ (Bryan and Langston, 1992). Concentrations of heavy metals in the sediments of some estuaries and coastal waters are given in Table 1.2. In Table 1.2, high concentrations of metals in sediments are also found in areas where human activities were also prime factors.

Concentrations of heavy metals in marine animals are rather complicated to generalise. They depend upon whether the metal is essential for a class of marine animals. Copper, zinc and iron are metabolic requirements by marine animals. However, one group of marine animals may employ a metal for a prime physiological function, and thus contains that metal in high levels while other groups may not need it in high levels but only in trace levels. For example, copper in crustacea is used for the respiratory pigment 'haemocyanin' and is found in high levels whereas in fish, levels are in trace amounts since they use iron in 'haemoglobin' for this purpose. Under natural conditions non-essential metals (Hg, Cd Pb) are generally present at very low concentrations. However, concentrations in aquatic animals are heavily dependent on the concentrations of metals in water. An increase in the concentration in water could lead to a significant increase in the metal concentration in the tissues of marine animals. Therefore, animals from polluted areas often have higher levels of non-essential metals in their tissues than animals from relatively clean areas. (Langston 1990 ; Bryan and Langston 1992). Some species of marine animals may naturally contain high concentrations of a non-essential metal than other group of animals (Clark, 1989). An essential feature affecting concentrations of metals in animal tissues is the ability of animals to bioaccumulate or to regulate metal concentrations.

Table 1.1. Range of heavy metal concentrations (μg l^{-1}) in sea water and estuaries from different areas.
()= mean, *= dissolved, #= particulate, the others are ranges of total concentrations.

Location	Mercury	Cadmium	Lead	Copper	Zinc	Reference
The great Barrier Reef (Aust.)		<0.01-0.06	<0.06	0.11-0.24	0.03-0.35	Denton and Burdon-Jones (1986)
Coastal North Sea (U.K.)		(0.2)	(0.25)	(2.0)		Balls and Topping (1987)
Mersey Estuary (U.K.)	0.05-0.185*					
	0.03-0.007#					Campbell et al. (1986)
Poole Hatchery intake (U.K.)		3.30-3.80	1.00-5.00	(4.0)	22.6-26.0	Boyden (1975)
Darwin Harbour (Australia)		0.10-0.40	0.20-2.30	0.30-2.60	0.70-4.00	Peerzada and Ryan (1987)
Bristol Channel (U.K.)		0.40-9.40	0.35-13.0	0.60-5.40	2.70-44.0	Abdullah and Royle (1974)
North Sea and U.K. Coastal	0.003-0.08					
Poole Horbour	0.009-0.07					Langston (1990)
Poole Horbour		0.30-42.0		0.20-28.0	1.00-174	Abdullah and Royle (1974)
Southern Bight of the North Sea		0.026-0.185*		0.30-1.89*	1.40-18.3*	
		0.01-0.11#		0.36-2.65#	0.60-6.66#	Nolting (1986)
Thana Creek, Bombay Harbour	0.079-0.32					Zingde and Desai (1981)
North Sea and Scheldt Estuary	0.030-0.29					Baeyens et al. (1979)
East Coast of Britain		0.01-0.06*	0.01-0.13*	0.12-0.58*		
		0.01-0.06	0.03-0.26	0.12-0.66		Balls (1985)
Belgian and Dutch Coast		0.21-0.13	0.36-1.72	0.46-2.02		Mart et al. (1980)
Humber Estuary (U.K.)		0.02-0.60*	0.01-0.05*	0.32-3.30*		
		0.02-0.06	0.07-2.40	0.33-4.00		Balls (1985)
Rosignano Solvay (Italy)	(0.0092*)	0.01-0.03*	0.01-0.10*	0.05-0.23*	0.37-1.40*	
	(0.0034#)	(<0.007)#	0.03-0.20#	0.03-0.13#	0.39-1.41#	Seritti et al. (1987)
Baltic Sea	0.001-0.006	0.03-0.07	0.02-0.11	0.31-0.95	1.30-3.30	Brugman (1981)
Derwent Estaury (Tasmania)	<0.10-16	0.50-15.0	4.00-16.0	10.0-27.0	6.0-1500	Bloom and Ayling (1977)
Restronguet Creek (U.K.)		0.70-38.0	<2.00-4.0	3.0-176.0	22.0-20460	Bryan et al. (1985)
Belleduna Harbour (Canada)		0.11-125		0.36-0.91	1.68-260	Loring et al. (1980)

Table 1.2. Range of heavy metal concentrations (μg g^{-1}) in sediment in the marine environment from different areas.
()= mean concentrations.

Location	Mercury	Cadmium	Lead	Copper	Zinc	Reference
Scheldt Estuary (Belgium)		7.4-15	(182)			Panurakul and Baeyens (1991)
Herradura Bay (Chile)		<5.0-20		<50-210		Trucco et al. (1990)
The Saguenay Fjord (Canada)	0.03-1.2	0.01-0.5		1.3-28	36-236	Pelletier and Canuel (1988)
The Gulf of Nicoya (Costa Rica)	(0.023)	(0.163)	(5.78)	(8.95)	(86.5)	Dean et al. (1986)
The Gulf of Venice (Italy)	0.1-3.0	0.5-4.0	10-68	2.5-45	2.0-450	Donazzolo et al. (1981)
Tasucu Delta (Turkey)		(<0.5)	(<5.0)	16-26	26-47	Sanin et al. (1992)
The Forth (Scotland)		0.03-0.3	20-50	10-75	60-160	Davies (1987)
Northeasthern Irish Sea	0.1-29					Rae and Aston (1981)
Baltic Sea	0.01-9.0	<0.01-8.1	2.0-400	1.0-283	(2090)	Brugman (1981)
Bristol Channel/Severn Estuary	(0.48)	(1.1)	(88)	(54)	(255)	Bryan et al. (1985)
Mersey Estuary (U.K.)	(6.2)	(3.9)	(205)	(144)	(255)	Langston (1986)
Restronguet Creek (U.K.)	(0.46)	(1.53)	(341)	(2398)	(2821)	Bryan and Langston (1992)
Tamar Estuary (U.K.)	(0.83)	(0.96)	(235)	(330)	(452)	Bryan and Langston (1992)
Poole Estuary (U.K.)	(0.81)	(1.85)	(96)	(50)	(165)	Bryan and Langston (1992)
Solway Estuary (U.K.)	(0.03)	(0.23)	(25)	(7.0)	(59)	Bryan and Langston (1992)
S.East Coast of India	(0.04)	(0.06)	(30)	(46)	(68)	Subramanian and Mohanachandra (1990)

1.4 BIOACCUMULATION OF METALS BY MARINE INVERTEBRATES

The concentrations of metals in aquatic animals vary because they reflect the net effect of two competing processes, that of uptake and of depuration. The ambient concentration of metal in the water will itself vary because of changes in the rate of addition of metals and their losses from the water. Bioaccumulation of metals is mainly dependent on the availability of metals from the environment. The principles which govern the transport of most substances into biological tissues also apply to metal transport. Metals which exchange rapidly (e.g. Co) are accumulated less efficiently than metals which exchange slowly (e.g. Hg), as would be expected if a balance between influx and efflux provided the underlying control on metal uptake (Luoma, 1983). Two aspects of metal uptake which are unique, however, may strongly influence interpretation of metal availability studies. In some circumstances metal concentrations never reach a steady state (i.e. life-long net uptake may occur). Also the ultimate level of bioaccumulation is not always solely a function of transport rates. Life-long net accumulation of metals is indicated by a positive correlation between metal concentrations and size of organism in many species (Luoma, 1983).

Uptake of heavy metals by aquatic animals is generally known to be by two uptake routes, which are uptake from water and from food. However, metals could also be accumulated from sediment. Bryan and Langston (1992) indicated that uptake of metals from sediment in deposit-feeding animals may occur following the ingestion of particles, or, in some cases, by pinocytosis of particles at the body surface. Concentrations of heavy metals in the sediments are usually higher than those in the overlying water by about three to five fold. With such high concentrations, the bioavailability of even a minute fraction of the total sediment metal assumes considerable importance, especially in some filter-feeding and burrowing organisms.

Furthermore, several metals, including mercury and lead, may be transformed in sediments to organo-metallic compounds having increased bioavailability and toxicity (Bryan and Langston, 1992). Uptake from water is perhaps the most important route for many or all heavy metals because aquatic animals accumulate them continuously. Concentrations of free metal ion appear to be the most important control on metal uptake from solution (Luoma, 1983). Studies on aquatic animals showed that heavy metals, especially non-essential metals such as Hg, Cd and Pb, are accumulated from the solution with no regulation and in proportion to environmental concentrations. They are also accumulated from the food and the concentration of the tissues increases. There is evidence, however, that tissue concentrations of essential metals such as Cu and Zn can be regulated by decapod crustaceans, at least up to a threshold environmental level. Net accumulation of these metals only begins after this mechanism breaks down at concentrations which are higher than these threshold levels. More information is given in Chapters 3, 4 and 6.

1.5 TOXIC EFFECTS OF HEAVY METALS

Toxic effects of metals occur after excretory, metabolic, storage and detoxification mechanisms are no longer able to match to uptake rates. This capacity, however, may vary greatly between phyla, species, populations, even individuals, and may depend on the developmental stage of the organism as well (Langston, 1990). Therefore, toxic effects and bioaccumulation of metals are well correlated. Studies on aquatic animals show that heavy metals affect many biological processes such as regeneration, ecdysis, development, respiration, reproductive performance, behaviour, hatching and osmoregulation (Table 1.3).

Table 1.3. Effects of sublethal concentrations of heavy metals on crustaceans.

Species		Metal	Effect	Reference
Grass shrimp,	*Palaemonetes pugio*	Hg	shorter intermoult period	Kraus et al. (1988)
Brine shrimp,	*Artemia salina*	Hg	reduction in adult lifespan	Cunningham and Grosch (1978)
Fiddler crab,	*Uca pugilator*	Cd	retards limb regeneration and ecdysis	Weis (1985)
Xanthid crab,	*Rhithropanopeus harrisii*	Hg	reduced plasticity and retard development	McKenney et al. (1982)
Shore crab,	*Carcinus maenas*	Hg	disruption of endogenous rhythms	Depledge (1984)
Fiddler crab,	*Uca annulipes*	(Hg,Cd,Zn,Cu)	inhibition of the rate of respiration	Uma Devi and Prabhakara (1989)
Grass shrimp,	*Palaemonetes pugio*	Cd	reduction of locomotor activity	Hutcheson et al. (1985)
Grass shrimp,	*Palaemonetes pugio*	Hg	reduced predator avoidance	Kraus and Kraus (1986)
Copepod	*Eurytemora affinis*	(Cu,Cd)	reduced growth, swimming and	Sullivan et al. (1983)
Fiddler crab,	*Uca pugilator*	Zn	retard of limb regeneration	Weis (1980)
Brine shrimp,	*Artemia franciscana*	Hg	inhibition of the emergence and hatching	Go et al. (1990)
Shore crab,	*Carcinus maenas*	Cu	reduced osmolality and ion levels	Bjerregaard and Vislie (1986)
Rock crab,	*Cancer irroratus*	Cd,Pb	inhibition of gill ATPases in vitro	Tucker and Matte (1980)
Crayfish,	*Procambarus clarkii*	Pb	reduced oxygen uptake and damage to gill	Torreblaca et al. (1989)
Lobster,	*Homarus americanus*	Zn	inhibition of gill Na,K-ATPase in vivo	Haya et al. (1983)
Pink shrimp	*Penaeus duorarum*	Cd	pathological black gills	Couch (1977)
Lobster	*Homarus americanus*	Cd	histopathological alterations in tissues	Odense and Annand (1981)
Pallas	*Corophium volutator*	Hg	inhibition of burrowing in sediment	Erdem and Meadows (1980)
Fiddler crab	*Uca pugnax*	Hg	inhibition of limp regeneration	Callahan and Weis (1983)
Prawn	*Macrobranchium kistnensis*	Cu	severe histological alteration in gill	Ghate and Mulherkar (1979)
Prawn	*Cardina sp.*	Cu	severe histological alteration in gill	Ghate and Mulherkar (1979)
Isopod	*Jaera nordmanni*	Hg,Cu,Cd	ultrastructural changes in organelles	Bubel (1976)
Norway lobster	*Nephrops norvegicus*	Cd+Cu+Zn	inhibition of gill Na,K-ATPase in vivo	Chapter 5

1.6 FACTORS AFFECTING BIOACCUMULATION AND TOXICITY OF HEAVY METALS

1.6.1 Physiological Conditions of Marine Animals

Seasonal variations in the physiological conditions of aquatic organisms primarily arise from the reproductive cycle (such as the maturation of the gonads and gametes and spawning) and changes in the growth rate. These include substantial changes in the balance within the organism between protein, lipids and carbohydrates, as well as affecting body weight, water content and the gonad condition of the organism (Mance, 1987). Changes of physiological conditions during different seasons of year are more pronounced in crustacean which must moult in certain periods. Concentrations of copper and zinc in decapod crustaceans can change depending on moult stages of animals (Engel and Brouwer, 1987). Loss of metals in the shell of crustaceans during moult can also contribute an important variation to the total metal concentrations of the animals.

1.6.2 Growth, Age, and Sex

The concentration of a metal in an organism is a function of the balance between the rate of accumulation and depuration. It is also a function of the rate of change in the body mass as the rate of growth will determine the quantity of tissues through which the net gain or loss of metal will be distributed (Mance, 1987). Thus, in general, animals which have fast growth rates show lower tissue concentrations of non-essential metals than animals which have slower growth rate (Phillips, 1980). Age is also known to be important in the interpretation of the metal content of marine animals directly or indirectly by weight or physical dimensions (Mance, 1987). Mercury is different from most heavy metals because tissue concentrations have been

consistently shown to increase with age and size. Therefore size and age of marine animals are very important factors to take into account especially when a relationship exist between metal concentration in tissues and size and/or age of aquatic animals (Phillips, 1980). Sex of marine animals could also be a significant factor since male and female animals could have different growth rates (Mance, 1987 ; Howard, 1989). Additionally, spawning has a considerable direct impact upon body content. The extent of this depends upon the relative sizes and therefore weight loss from the gonads of the two sexes. The faster growing sex can be expected to contain lower concentrations of metals. The Norway lobster, *Nephrops norvegicus* is very good example for this. Male animals grow much faster than females. Thus, the same age of males and females would have different sizes males being larger than females (Howard, 1989). Mercury a shows positive relationship with size and therefore concentrations in the tail muscle of each sex in same size group are also different, namely females present higher levels than males (Davies and McKie, 1983 ; Lima, 1984).

1.6.3 Salinity and Temperature

Although it is not universal, uptake of metals generally increases with an increase in temperature and decrease in salinity. The higher toxicity of metals such as Hg, Cd, Cu, Pb and Zn at low salinities is generally attributable to more rapid accumulation rates and therefore it is very important to consider this effect in estuaries (Langston, 1990). Phillips (1980) indicated that salinity affects the physiology of aquatic animals as well as having effects on metal accumulation. Phillips (1980) also indicated that aquatic animals accumulate metals at higher rates in higher temperature as well as showing higher loss in high temperature.

1.6.4 Chemical Characteristics of Metals

Uptake of metals by marine animals can be affected by the chemical form of metals. Hg, Sn and As are known to occur in methylated forms in the environment. The best known examples of methylation affecting metal accumulation and toxicity are for Hg and As (Luoma, 1983). Organic mercury accumulates to higher concentrations in the tissues and is more toxic to animals than inorganic mercury (Chapter 3). Oxidation state also affects the availability of Hg, Se, As, and Cr. In general, Hg^{o} is considerably more available than Hg^{2+} because of the lipid solubility of the former. Se^{2+} is more available to mussels than Se^{6+} and Cr^{6+} is more available to organisms in general than Cr^{3+} (Luoma, 1983). Chelation is also an important factor which affects accumulation and toxicity of heavy metals. Chelation is the formation of a metal ion in association with a charged or uncharged electron donor referred to as a ligand. The ligand may be monodentate, bidentate, or multidentate; that is, it may be attach or co-ordinate using one or two or more donor atoms. Chelating agents are generally non-specific in regard to their affinity for metals. They will mobilise and enhance the excretion of a rather wide range of metals to varying degrees, including essential metals such as calcium and zinc. Chelating agents include; BAL (British Anti Lewisite), DMPS (2,3-dimercapto-1-propanesulfonic acid), Calcium EDTA, Penicillamine (Goyer, 1991).

1.6.5 Enhanced Tolerance to Metals

Aquatic animals can develop enhanced tolerance against toxic effects of heavy metals by living in slightly contaminated waters. Tolerant animals can often survive in environmental metal concentrations that animals from relatively clean environments can not tolerate (Uma Devi, 1987 ; Uma Devi and Prabhakaro Rao, 1989 ; Kraus et al., 1988). The reason for this could well be related to processes of induction of

metal-binding proteins which form after exposure to heavy metals. Studies have shown that marine animals which live in elevated levels of heavy metals can bind these metals to special low molecular weight proteins 'metallothioneins' which can be an efficient detoxification tool against heavy metal toxicity (Chapter 6). Therefore, animals from contaminated areas could be more resistant to heavy metal toxicity and show higher tissue concentrations than animals from clean areas. They could also have higher levels of metallothionein since metallothionein levels increase with heavy metal exposure (Chapter 6). Studies have also shown that protection against toxic effects of cadmium depends on presynthesized metallothioneins (Kito et al., 1982 ; Goering and Klaassen, 1984).

1.6.6 Interaction of Heavy Metals

Interactive effects among metals may be a very important factor influencing metal availability to organisms and toxicity. There are several types of possible interactions: (1) enhancement of uptake (synergistic effect) (2) competitive displacement on metal-specific binding proteins in tissues; and (3) competitive inhibition of uptake at the environmental interface (antagonistic effect) (Luoma, 1983). In particular there is consistent evidence of competition between a number of metals such as Cd-Zn, Cu-Ag, Cu-Mn, Cd-Se and Hg-Se (Bryan et al., 1985 ; Sunda et al., 1981 ; Pelletier, 1985). Studies with crustaceans showed that cadmium can be antagonistic to the biological uptake of zinc and copper (Negilski et al., 1981 ; Devineau and Amiard-Triquet, 1985). Selenium can also show an antagonistic effect to the toxic effects of mercury (Lucu and Skreblin, 1981). However, cadmium and selenium showed a synergistic relationship (Bjerregaard, 1982 ; 1985). Mercury and cadmium did not increase or decrease each other's accumulation but they showed effects in additive ways (Weis, 1978).

1.7 DETOXIFICATION OF HEAVY METALS IN MARINE ANIMALS

Homeostatic mechanisms of an animal are a crucial factor in its natural environment. Terrestrial animals are generally only exposed to metals in their diet or in the air they breathe, whereas aquatic animals are exposed to dissolved and particulate metals in the medium in which they are immersed as well as in their diet. The surface is larger, consequently uptake of metals is often far greater in aquatic animals. In aquatic habitats, the effects of environmental variables on metal uptake are much more important since variation in salinity, pH, redox conditions, etc., can affect both the chemical speciation of the metal and the physiology of the animal (George, 1982). Therefore, the intracellular concentrations of metal ions must be regulated for the maintenance of essential life processes. Since metals may be more readily available to aquatic organisms and many appear unable to regulate the accumulation, they provide good examples of detoxification mechanisms for dealing with intracellular concentrations of non-essential metals and excess levels of essential metals (George, 1982). Heavy metals are detoxified as; nucleoprotein complexes (Cu, Pb), phospholipid complexes (As, methylated), selenium complexes (Hg, and methyl-Hg, possibly Cd), metallothionein (Cd, Cu, Hg, Zn, and possibly Ag), lipofuschin granules, 3degrees lysosomes (As, Au, Cd, Cu, Fe, Hg, Pb, Pu, Th, Zn), phosphate granules (Fe, Zn, Al, Cu, Sr and trace of Hg, Pb), blood cell vesicles (Cu with S, Zn with P) and O_2 transport proteins (Fe, Cu) (see George, 1982). Metallothionein which could be one of the most important detoxification mechanisms of heavy metals will be discussed widely in chapter 6.

1.8 WELL KNOWN HEAVY METAL POLLUTION INCIDENTS IN THE MARINE ENVIRONMENT

The Minamata Bay disaster in Japan is one of the best known examples of heavy metal pollution. The town of Minamata had a population of 50,000 for whom seafood was a staple component of the diet. In 1932, Minamata Bay began receiving mercury waste from a factory which used mercuric oxide as a catalyst in the production of acetaldehyde and vinyl chloride. This continued until 1968 (Mance, 1987). The factory discharged mercury waste at concentrations as high as 120 ppm of which 5 % of this was methyl mercury (Miettinen et al., 1970 ; Salvatore et al., 1977). According to Langston (1990) the factory put 80 tonne of mercury into the Bay between 1932-1968 which caused sediment concentrations of several hundreds ppm, with water concentrations of as high as 3.6 ppm.

Mercury poisoning was seen in great numbers of people, especially fishermen and their families whose main component of their diet was seafood. Common problems caused in the town were; death, impaired vision, loss of motor co-ordination and other neurological abnormalities. Hormonal and enzymatic disturbances were also common (Salvatore et al., 1977). The first recorded human case was that of a 5 year old girl suffering from delirium, speech disturbance and difficulties in walking as a result of brain damage in 1956 (Mance, 1987). There were 800 verified victims of Minamata disease, with 107 fatalities and a further 2800 possible additional victims by 1975.

A second outbreak of mercury poisoning in Japan occurred in 1965 among fishermen living near the mouth of the Agamo River. Like the Minamata tragedy, this was caused by the contamination of fish by mercury in an effluent, in this case, from a factory 60 km upstream (Clark, 1989).

Cadmium was the reason for the itai-itai disease in Japanese villages on the Jintsu River. This disease caused 100 deaths with painful effects on joints and bones. It was attributed to contamination of rice by cadmium from the effluent from a zinc smelter (Clark, 1989). Stoeppler (1991) indicated that patients had taken up 300-480 μg of cadmium per day. The symptoms of disease were severe renal tubular damage and pronounced osteomalacia causing great pain in back and legs.

1.9 PUBLIC HEALTH STANDARDS

After recognition of hazards of heavy metal toxicity, maximum acceptable concentrations of heavy metals in seafood have been suggested. The World Health Organisation (WHO) recommended a maximum tolerable consumption of mercury in food of 0.02 mg of methyl mercury and 0.3 mg of total mercury per week. Standards adopted for maximum permitted mercury levels in seafood are 0.5 $\mu g\ g^{-1}$ (ppm) in the USA and Canada, 0.7 $\mu g\ g^{-1}$ in Italy, 1.0 $\mu g\ g^{-1}$ in Japan, Germany, Sweden, and Switzerland (Clark, 1989). The WHO established the toxicological threshold of cadmium as 0.525 mg ingested per week. Actual weekly uptake, however, should be no more than 0.1 to 0.25 mg Cd, in extreme cases 0.5 mg (Hapke, 1991). Clark (1989) indicated that except in the case of shellfish from contaminated water, seafood contains no more cadmium than other foods and does not represent a special hazard. He also indicated that there is no danger to humans of copper poisoning from seafood-the lethal dose is about 100 mg-but the human taste threshold for copper is low, 5.0-5.7 ppm, and the taste repulsive. Lead does not play an important part in aquatic food chains. Lead concentrations in fish depend upon the amount of lead pollution in the marine environment. Clark (1989) indicated that contamination of sea and seafood does not appear to be a matter of concern, though it is held responsible for serious damage to health on land. A weekly dietary intake of 3 to 4 mg is accepted as toxicologically harmless because absorption is also low (Hapke, 1991).

To obtain seafoods which contain heavy metals in acceptable low levels and to protect marine life, heavy metal concentrations in the marine environment can be monitored against Environmental Quality Standards (EQS). EQS levels of metals were divided into two different categories as estuary and coastal waters. McLusky (1989) summarised these levels as (in $\mu g\ l^{-1}$) ; Cd < 5.0 and < 2.5, Cu < 5.0 and < 5.0, Hg < 0.5 and < 0.3, Pb < 25.0 and < 25.0, Zn < 40.0 and < 40.0 in estuaries and coastal waters respectively.

1.10 CLYDE SEA AREA AND HEAVY METAL CONTAMINATION

The Firth of Clyde (Figure 1.1) on the west of Scotland is a major seaway and vacation area. This area consists of the estuary of the River Clyde and the area to the seaward. The Clyde Sea area has a connected water area of 1856 km^2, a volume of about 100 km^3, and the land surface draining into it is 5360 km^2 in extent. It is a vital waterway where sea lanes converge on the west of Scotland's most highly developed urban and industrial areas (Steele et al., 1973).

The Clyde Sea supports important commercial fisheries and receives domestic, industrial and agricultural wastes from a catchment area with a human population of about two million (Steele et al., 1973). Pollution sources are numerous. Sewage sludge consisting of primary settlement material, activated sludge and industrial waste is daily dumped off Garroch Head by Glasgow Corporation and liquid is discharged into the River Clyde. Untreated sewage from inland as well as coastal towns is also discharged along much of the coast of the Firth (Steele et al., 1973 ; Mackay, 1986). Sewage sludge contaminated with a variety of heavy metals has been dumped in the Firth of Clyde for some years at a rate of 1 million tons per year (Mackay, 1972 ; Clark and Davies, 1989). Topping (1974) indicated that the Clyde Sea area also receives considerable inputs of heavy metals from atmospheric deposition.

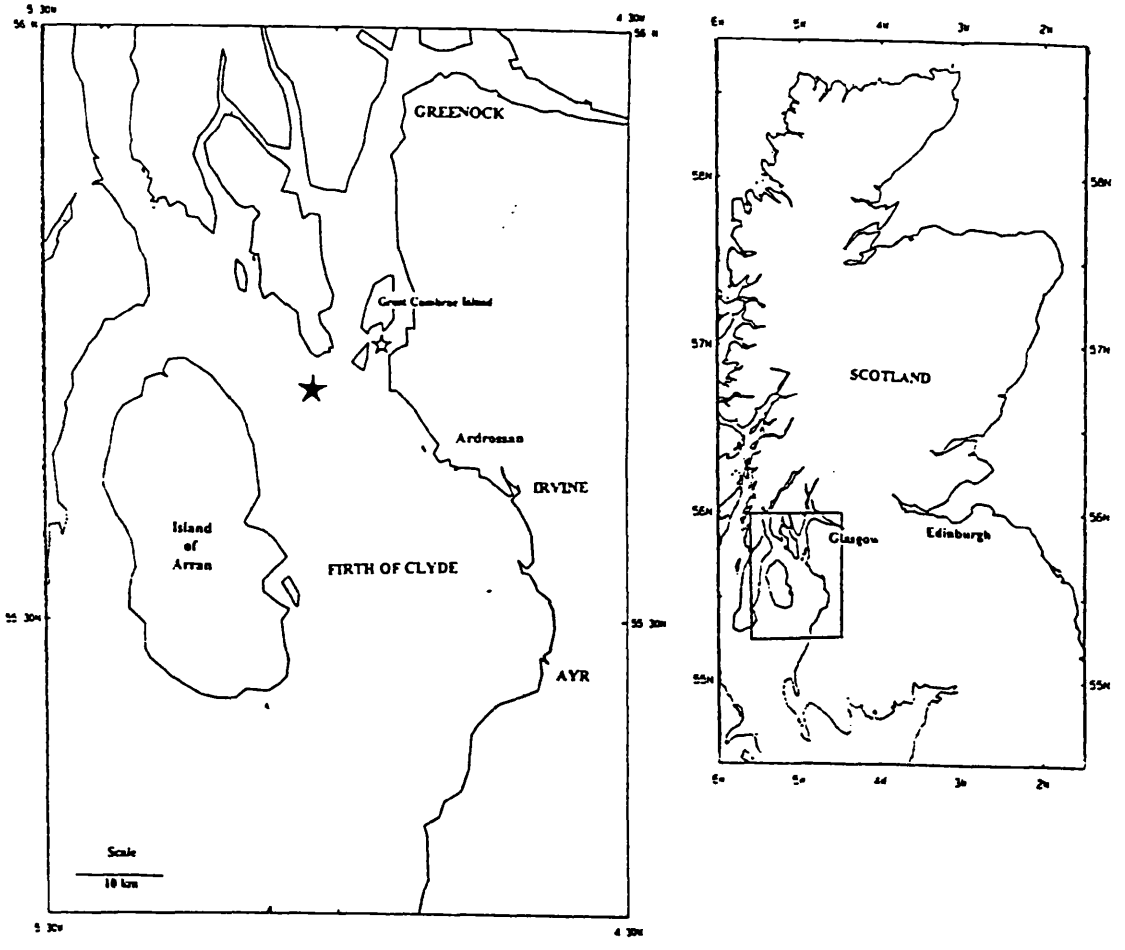

Figure 1.1. A map of the Clyde Sea. The filled star indicates the sewage sludge dumping area, while the unfilled star indicates the sampling area of the Norway lobster *Nephrops norvegicus* in Chapters 2-6.

The Garroch head dumping area is a circle, radius 0.93 km, centred on the position 55°39' 48"N 05°00' 48"W. The sewage is from the city of Glasgow after mixing of primary and secondary, undigested, sludges, originating from full scale biological treatment plants using either filtration or activated sludge processes. Concentrations of heavy metals in the surface sediment showed increases in concentrations towards the centre of the disposal area. This was also accompanied by a change in sediment colour from brown at the edge to black/brown in the centre (Clark and Davies, 1989).

1.11 THE NORWAY LOBSTER, *NEPHROPS NORVEGICUS* (L.)

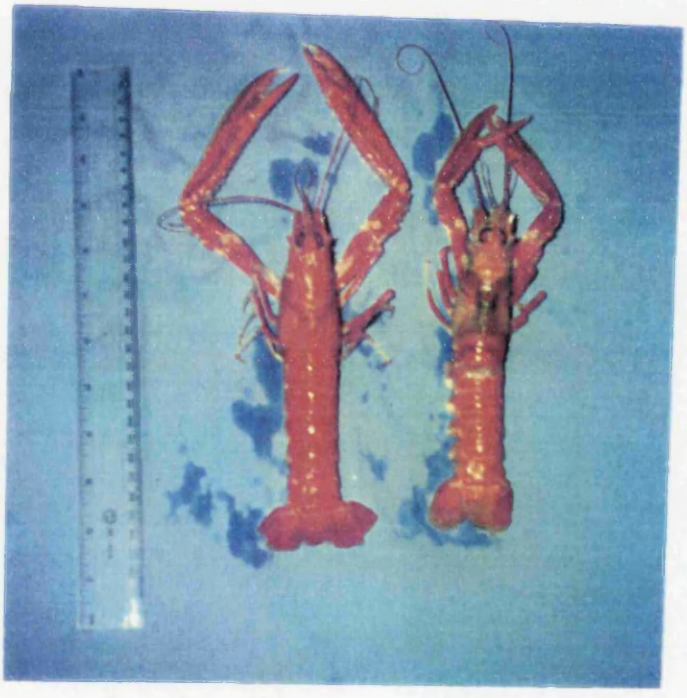

Figure 1.2. A picture of male (left) and female (right) Norway lobster *Nephrops norvegicus*.

The Norway lobster, *Nephrops norvegicus* (Figure 1.2) is a member of a group of animals, the Crustacea, characterised by possessing a hard, jointed, external shell which encases the body and limbs. It is closely related to the common lobster *Homarus gammarus*, from which it is readily distinguished by its smaller size, orange red colour, long slender chelae (claws) and prominent kidney-shaped eyes. The fore part of the body is covered by a continuous shell, the carapace, while the hind part (tail or abdomen) is divided into six flexible segments. The carapace length is the measurement most often used in denoting the size of *Nephrops*. The body bears 20 pairs of jointed appendages, specialised for performing different functions. The antennae are sensory and are used for gathering information about the environment. The large claws can be used for defence and for catching items of prey, which are passed to the mouth, where different mouthparts cut them up and manipulate them into the digestive tract. The four pairs of legs are used for walking, and together, with the claws, for digging the burrow. The sexes are separate and can easily be distinguished one from the other. The first pair of pleopods on the tail are rigid in the male animals and form a forward-pointing tube which is used to pass a sperm package to the female during mating. The sperm then fertilise the eggs when they are laid. In the female the first pair of pleopods is flexible and feather-like. The pleopods in females on the abdomen are used to carry the eggs. Any detached limb is regenerated and grows with moult.

The Norway lobster, *Nephrops norvegicus* is widely distributed on the continental shelf of Europe. Its consumption by Man has increased significantly in recent years in the U.K. and other European countries. For example, although landing of *Nephrops* from waters in north-east Europe was 25,000 tonnes in 1965, this reached 55,545 tonnes in 1985. *Nephrops* is the most important shellfish species in the United Kingdom and the Scottish fishery for this animal is the most important fishery of its type in Europe. Landings by Scottish vessels in 1987 were over 16,500 tonnes with a

value of over 32.6 million pounds (Howard, 1989).

The female *Nephrops* attains maturity at an overall body length of 73-78 mm (carapace length 21-23 mm), when it is three years old, while the male *Nephrops* appears to reach maturity after three years (CL 25 mm). During late summer and early autumn the ripe ovary in mature females can be seen through the carapace as a dark, green-black area. In Scottish waters the mature females generally spawn every year, the eggs being fertilised and spawned from August to November. The dark-green eggs being fertilised and spawned are carried on the pleopods of the female's tail and remain there for about nine months while they develop. The number of eggs carried increases with an increase in the size of the animal. A female of 25 mm carapace length carries about 500 eggs and one of 35 mm CL about 1,500. As the embryo develops within the egg there is a gradual change in colour from dark-green, through pale green, to a pinkish brown colour just prior to hatch. Hatching starts in late April and continues until August. The Norway lobster is an active forager. The larvae are carnivorous, actively preying on a wide range of planktonic organisms. The juveniles and adults feed on a wide variety of material, including molluscs, annelid worms, Crustacea, echinoderms and small fish. They can browse on very small organisms like the microscopic Foraminifera found in the mud, catch active prey by snapping with their claws, or search for food on or within the surface of the mud.

In order to grow, *Nephrops* must first cast their hard outer shell, a process known as moulting. The process takes about 30 minutes and is followed by a marked increase in size as the animal absorbs water. The new shell reaches full hardness about two weeks after the moult, and no further growth can then occur until the next moult. Moulting occurs at any time of the year, although peak periods occur in March and April and from July to November (Howard, 1989). Peak periods of moulting activity are generally more pronounced in females, which must moult before mating takes place.

Mating occurs when the female is in the "soft" condition, when she is impregnated by a hard male In Scottish waters juvenile Norway lobsters grow rapidly, attaining a mean total length of 52 mm (14 mm CL) after one year, having moulted 10 times. After this initial burst, growth rates slow down, and the inter-moult period increases. On attaining sexual maturity the females moult only once a year whilst, in general, the males of comparable size continue to moult at least twice a year until they reach a total length of about 100 mm (30 mm CL). After this size Norway lobster generally moult once a year, except for the largest animals (above 45 mm CL), for which there may be an interval of up to three years between moults (Howard ,1989). Male *Nephrops* grow much faster than female animals and at the same age the male and female animals have different sizes males being larger than females (Davies and McKie, 1983 ; Howard, 1989).

Nephrops norvegicus is one of the member of a complex community of burrow-dwelling species which includes several fish species, a small crab and many other invertebrates. The distribution of the Norway lobster is dependent upon the availability of a sea bed composed of fine cohesive mud in which it can build burrows. Some areas have very fine sediments with a high (90 %) content of silt and clay particles, while others have much coarser sediments with 40 % silt and 60 % sand particles. *Nephrops* are found within this range of sediments, but as the sediment changes, so does the structure of the population, with large animals at low population densities in fine sediments and smaller animals in high population densities with coarser sediments. The burrows extend 20-30 cm below the mud surface and range from simple tunnels with a single opening, through the more typical forms with a wide sloping front entrance and a smaller rear entrance, to complex tunnels with more than two openings. Norway lobsters are found in depths ranging from 15 m to 800 m (Howard, 1989). The density and spacing of burrows varies considerably from one animal in every 5 m^2 to 4 animals per m^2 in most recognised grounds.

1.12 GENERAL AIMS OF THIS STUDY

As has been explained above, heavy metals can accumulate in tissues of marine animals and interfere with many of biological functions in marine animals. They can also be lethal to marine animals at higher levels. When heavy metals enter the food-chain at elevated levels in the marine environment, they can also reach humans. *Nephrops norvegicus* is a very important commercial food for human consumption in many countries and the Clyde Sea is continuously contaminated with metals. There is little information available on heavy metal concentrations in *Nephrops norvegicus* or on the distribution of metals among tissues in relation to sex, size and season. In this study concentrations of metals (Hg, Cd, Cu, Zn and Fe) were measured in the tissues such as the gill, hepatopancreas, tail muscle, carapace, ovary and external eggs of *Nephrops* caught from the South of the Isle of Cumbrae in the Clyde Sea and the effects of sex, size and season on the metal concentrations of the tissues were investigated. Toxicity of metals dissolved in seawater was studied to find out lethal and sublethal concentrations of metals (methyl Hg, Hg, Cd, Cu, Zn and Pb) and influences of sex and size on metal accumulation and tissue distribution were investigated using sublethal concentrations of the metals. Accumulation of metals from food was also studied and comparison of tissue distribution among tail muscle, gill and hepatopancreas of heavy metals after exposure to metals in sublethal concentrations in seawater and food were investigated using triangular diagrams to investigate the dominant uptake route of metals into the tissues under laboratory conditions. There is also no information in the literature on the effects of heavy metals on physiological functions in *Nephrops norvegicus* such as responses of gill ATPases to heavy metal pollution. Characterisations of gill ATPases were first, carried out and effects of sublethal concentrations of heavy metals (Cd, Cu and Zn) in combination with the activities of gill ATPases such as Na,K-ATPase, Mg-ATPase and its oligomycin sensitive and insensitive components were investigated in relation to

contamination gradient in male and female *Nephrops* under laboratory conditions. There are suggestions that heavy metals can be detoxified by induction of low molecular weight proteins such as metallothionein in the tissues of marine animals when they are exposed to elevated levels. However, there is no information in the literature indicating whether *Nephrops norvegicus* is able to induce metallothionein when exposed to heavy metals. Induction of metallothionein in the gill and hepatopancreas of the animals was investigated in relation to contamination gradient after exposure to heavy metals (Cd, Cu and Zn) in combination in male and female animals in laboratory conditions. The relationship between metal and metallothionein concentrations was also investigated. Studies were carried out under field and laboratory conditions to be able to apply the laboratory experiments to natural conditions. Size and sex of the animals were taken into account to be able to correct any error which might come from these parameters. ATPase activity in the gill of male animals was investigated in field samples using animals from ten stations (including the sewage dumping ground) of the Clyde Sea to find out if there were any variations in the activities of gill ATPases in relation to contamination gradient. Some measurements on the metal and ion concentrations of the gill and blood were also carried out. Metallothionein concentrations in the gill and hepatopancreas of male animals in different stations in the Clyde Sea area (including the sewage dumping ground) were also investigated in relation to contamination gradient. Heavy metal concentrations of the tissues were also measured and the possible relationship between heavy metal and metallothionein concentrations were investigated. Because *Nephrops norvegicus* in the Clyde Sea are infected by a fatal parasite related to *Hematodium perezi* (flagellata) in recent years, studies such as ATPase activity and metallothionein in the tissues of *Nephrops norvegicus* from different stations in the Clyde Sea were also investigated for effects of the infection on some parameters in the tissues of the infected and normal animals. Chapters of this study are as follows;

Chapter 1 = General Introduction.

Chapter 2 = Heavy Metal Concentrations of the Tissues of the Norway Lobster, *Nephrops norvegicus*; Effects of Sex, Size and Season.

Chapter 3 = Effects of Sex and Size on Metal Accumulation and Tissue Distribution of Heavy Metals from Sea Water by the Norway Lobster, *Nephrops norvegicus*:; Toxicities of Heavy Metals.

Chapter 4 = Accumulation of Heavy Metals from a Food Source and Comparative Routes of Mercury and Cadmium Accumulation and Tissue Distribution of Heavy Metals from Food Source and Sea water by the Norway Lobster, *Nephrops norvegicus*.

Chapter 5 = Characterization of Gill ATPases and Effects of *In Vivo* Exposure to Cadmium, Copper and Zinc on the Activities of the Gill ATPases in the Norway Lobster, *Nephrops norvegicus*.

Chapter 6 = The Induction of Metallothionein in the Gill and Hepatopancreas of the Norway Lobster, *Nephrops norvegicus* After Exposure to Cadmium, Copper and Zinc.

Chapter 7 = Gill ATPase Activities and Some Parameters in the Gill and Blood of the Norway Lobster, *Nephrops norvegicus* from Contaminated and Uncontaminated Areas of the Clyde Sea; Effects of a Parasite Infection.

Chapter 8 = Heavy Metal and Metallothionein Concentrations in the Gill of the Norway Lobster, *Nephrops norvegicus* from Contaminated and Uncontaminated Areas of the Clyde Sea; Effects of a Parasite infection.

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CHAPTER 2

HEAVY METAL CONCENTRATIONS IN SOME TISSUES OF THE NORWAY LOBSTER *NEPHROPS NORVEGICUS*: EFFECTS OF SEX, SIZE AND SEASON

2.1 INTRODUCTION

Metals occur naturally in sea water and many, such as copper, cobalt, iron, manganese, nickel, selenium and zinc are used for essential purposes by marine organisms. Crustaceans require copper, zinc and iron for their respiratory pigment, enzyme or body components and must accumulate them from ambient water and food to respond to the demands of metabolism. White and Rainbow (1985 ; 1987) estimated total requirements of some essential metals in decapod crustaceans to be 84, 71 and 27 $\mu g\ g^{-1}$ Cu, Zn and Fe respectively. Levels of essential metals in tissues can be regulated by decapod crustaceans at concentrations of dissolved metals below a threshold level. At metal concentrations above this threshold concentration, the regulation mechanisms break down and these metals are accumulated in proportion to the dissolved metal concentrations (Bryan, 1964 ; Bryan, 1967 ; White and Rainbow, 1982 ; Rainbow, 1985 ; Rainbow and White, 1989). Thus, the concentrations of essential metals in water may not be life threatening to the animals until threshold concentrations are reached. However, metals are released in industrial effluents, sewage and atmospheric pollution (Nolting, 1986 ; Mance, 1987 ; Langston, 1990 ; Guieu et al., 1991). Many experimental studies have shown that excess amounts of essential metals can cause elevated concentrations in crustaceans and cause mortality depending on concentration, time and species (Eisler and Hennekey, 1977 ; Ahsanullah and Arnott, 1978 ; Price and Uglow, 1979 ; Jonhson and Gentile, 1979 ; Ahsanullah et al., 1981 ; Devineau and Amiard-Triquet, 1985 ; Bjerregaard and Vislie, 1986 ; Nugegoda and Rainbow, 1988) or interfere with normal processes of metabolism of crustaceans (Table 1.3).

Mercury and cadmium have no known role in biological systems. In addition to being present at low natural levels, they are contaminants of aquatic systems that are

released by anthropogenic activities such as, from chlor-alkali plants, the use of fungicides, pesticides, antifouling preparations, mining and smelting facilities (Campbell et al., 1986 ; Mance, 1987 ; Langston, 1990). Berk and Colwell (1981) showed that mercury can be bioaccumulated by marine animals through the food-chain. The dominant route for accumulation of cadmium in Crustacea was found to be by food (Jennings and Rainbow, 1979 ; Davies et al, 1981). It is well known that accumulations of non-essential metals are greatly dependent on concentrations in ambient water, period of exposure and species. There is no evidence that tissue concentrations of non-essential metals can be regulated by crustaceans. These metals are accumulated and stored and concentration factors may reach many thousand fold (Nimmo et al., 1977 ; Jennings and Rainbow, 1979 ; Meadows and Erdem, 1982 ; Devineau and Amiard-Triquet, 1985 ; Riisgard and Famme, 1986 ; Krishnaja et al., 1987).

The Norway lobster, *Nephrops norvegicus* is a widely distributed crustacean on the continental shelf of Europe. There are important inshore fisheries in countries bordering these waters and the consumption of Norway lobster has increased in recent years. In North-west Europe alone, landings of Norway lobster have increased from 25,000 tonnes in 1965 to 55,000 tonnes in 1985. In economic terms, the Norway lobster is the most important shellfish species in the United Kingdom (Howard, 1989). The Clyde Sea area receives anthropogenic inputs of pollutants (Mackay et al. 1972 ; Steel et al. 1973 ; Mackay, 1986), like most British waters (Allen and Rae, 1986 ; Campbell et al., 1986 ; Nolting, 1986 ; Langston, 1990 ; Cossa and Fileman, 1991) and supports a large fishery for *Nephrops* (Bailey et al. 1986). This chapter reports the concentrations of mercury, cadmium, copper, zinc and iron in the tissues such as the carapace, hepatopancreas, gill, tail muscle, ovary and external eggs of *Nephrops norvegicus* sampled in different months of the year

from the South of the Isle of Cumbrae in the Clyde Sea Area. Male and female animals were separated and size (carapace length) of the animals was taken into account in expressing the metal concentrations of the tissues. This study will also help for later studies if sampling period, sex and size of *Nephrops norvegicus* are important factors to consider in further experimental and field studies.

2.2 MATERIALS AND METHODS

The following methods of animal capture, dissection and heavy metal analysis have also been used in chapters 3 and 4 of this thesis unless otherwise indicated.

2.2.1 SAMPLING OF THE ANIMALS

All of the Norway lobsters, *Nephrops norvegicus*, were caught from south of the Isle of Cumbrae, Clyde Sea, Scotland (Figure 1.1).

2.2.1.1 Experimental Animals

Experimental animals were caught by trapping in creels, and transferred to a fibreglass stock holding tank which contained 1 tonne circulating sea water. The animals were allowed to acclimatise for a week. The animals were retained in circulated sea water which was continuously filtered, oxygenated and cooled. The experimental sea water was routinely tested for aquarium criteria by the aquarium technician and was approximately ; salinity 32 per thousand, pH.7.7, nitrite 0.33 mg NO_2 l^{-1}, nitrate 12.5 mg NO_3 l^{-1}.

2.2.1.2 Field Animals

Nephrops which would be used to measure natural concentrations of the metals were caught by trawling and brought to the laboratory. They were frozen at -20 ^{o}C for later use if analyses could not be done immediately. The following procedure was applied to the both natural and experimental animals.

2.3 DISSECTION

Carapace length (from the rear of the eye socket to the mid dorsal edge of the carapace) was measured to the nearest 0.1 cm and male and female lobsters separated. Then, samples were carefully dissected using clean equipment to separate the tissues; carapace, hepatopancreas, gill, tail muscle, ovary and egg mass. Male testis was removed in order not to mix to with the hepatopancreas. Each tissue sample was weighed using a Precisa 300MC (Metagram Instruments Ltd., Aspley Guise, Buckinghamshire) top-pan balance and put in a marked clean glass petri dish. All samples were placed in an oven which was set to 60 ^{o}C to dry the samples. Drying was conducted for at least six days to ensure that all samples achieved a constant dry mass. Dry tissues were used to measure metal concentrations in both natural and experimental animals using the following techniques.

2.4 TRACE METAL ANALYSIS

2.4.1 Total Mercury Determination

Total mercury concentrations in tissues of Norway lobsters, *Nephrops norvegicus* were measured using a cold vapour, atomic absorption spectrophotometry technique,

incorporating a Data Acquisition Ltd. DA 1500-DP6 Mercury vapour detector. All mercury analyses were done over two days. Samples were subjected to the following procedure before mercury measurements were obtained.

1-) In the first day, dry tissue samples of 0.050-0.300 g were weighed to the nearest 0.001 g using a Precisa 300 MC (Metagram Instruments Ltd., Aspley Guise, Buckinghamshire) top-pan balance and placed in clean digestion tubes. 4 ml concentrated sulphuric acid and 1 ml concentrated nitric acid (Analytical grades) were added to the tubes to begin digestion. Samples were placed in a thermostatic water bath which was set to 57 ^{o}C (Grant Instruments Ltd., Cambridge, Barrington). Tubes were shaken occasionally to aid sample digestion.

2-) After tissue digestion had finished (about three hours), the tubes were taken from the water bath and put onto a bench to cool at room temperature. Then the tubes were placed in a refrigerator to cool to 4 ^{o}C.

3-) Potassium permanganate solution was made up in a dark glass bottle as 5% (25 g potassium permanganate added (Spectrosol grade) to 500 ml distilled water) and placed onto a magnetic stirrer for at least three hours. This solution was cooled in a refrigerator for 30 minutes before using it.

4-) 2 ml aliquots of 5% potassium permanganate were added to the cooled samples using a graduated syringe. After each 5% potassium permanganate addition, the tubes were placed back in the refrigerator for 10 minutes to prevent heating and frothing. In total, 14 ml of 5% potassium permanganate solution was added to each sample which effectively oxidised the tissue present. The tubes were left in the refrigerator overnight.

5-) 2% potassium permanganate solution (12 g potassium permanganate added to 600 ml distilled water) was made up in a dark glass bottle and put onto a magnetic stirrer overnight.

6-) 50% sulphuric acid solution was prepared by carefully adding, a few ml at a time, 300 ml of acid to 300 ml distilled water in a conical flask placed in a cold water bath. The flask was covered and left overnight.

7-) Reducing agent was prepared using 85 g tin (II) chloride in 250 ml distilled water and 250 ml hydrochloric acid in a conical flask. This mixture was aerated overnight.

8-) On the following day, any precipitates in the samples were dissolved with 30 % hydrogen peroxide solution by adding dropwise, with great care since it causes heating and frothing.

9-) Each sample was transferred into a 25 ml volumetric flask and made up to volume with distilled water. Digesting tubes were rinsed with distilled water and the rinse was included in the 25 ml. After repeatedly inverting the volumetric flask to ensure complete mixing, part of each sample was poured into a numbered 10 ml beaker to await analysis.

10-) Standard mercury solutions were prepared using mercury (II) nitrate. For this, 100 μl of the 2% potassium permanganate solution and 100 μl mercury (II) nitrate (BDH standard solutions) were added to a 100 ml volumetric flask and made up to volume with distilled water. Three replicate standard solutions were made up in this way, inverted repeatedly to ensure complete mixing and poured into beakers to await

analysis.

11-) The 2% potassium permanganate solution was mixed with the 50% sulphuric acid in a dark glass bottle and cooled in the refrigerator for 30 minutes.

12-) Mercury determination was conducted by adding 20 ml of the acidified potassium permanganate solution, 25 ml distilled water and 1 ml of sample to a Dreschel flask ; this mixture was reduced with 10 ml of the reducing agent and any free mercury so produced drawn through magnesium perchlorate drying agent and into the analyser. 'Background' mercury levels in chemicals used were measured by repeating the above procedure, but not using the 1 ml of sample. All readings from the analyser were subsequently corrected for the 'blank' (background) readings. Calibrations of the analysis were performed with replicate analyses of standard mercury (II) nitrate solutions; 100 μl (equivalent to 100 ng of mercury) of the standard solution was analysed as above. The relationship between the reading obtained from the analyser and the amount of mercury in the standard solution was linear and therefore, only one concentration of mercury (II) nitrate solution was analysed. The above procedure allowed up to 40 samples to be analysed; blank and standard readings were checked regularly during the course of sample analysis. All chemicals used were of 'Spectrosol', 'Analar' or 'Puranal' analytical grades throughout. The mercury vapour detector was allowed to equilibrate to its working temperature for at least 2 hours prior to every set of analyses.

The following steps were followed to determine the mercury concentration in a particular sample.

1-) Blank readings were subtracted from standard readings, and the mean 100 ng

standard reading obtained.

2-) The blank reading was subtracted from the sample reading and the amount of mercury (in ng) determined as follows;

$$\frac{\text{Reading}}{\text{Mean standard / 100}}$$

3- The mercury concentration ($\mu g\ g^{-1}$) in a tissue is given by....

$$\frac{\text{ng mercury * 25}}{\text{sample dry weight (g) * 1000}}$$

2.4.2 Organic Mercury Determination

Extraction of organic mercury in the tissues of *Nephrops norvegicus* was carried out using an adaptation of the method of Uthe et al. (1972).

1-) Dry tissue samples were weighed using the same equipment. Tail muscle was finely ground, while hepatopancreas was mashed with acid washed sand. The ground samples were mixed with 10 ml 0.1 M copper sulphate solution (25 g copper sulphate added to 1 litre distilled water), 5 ml acidic sodium bromide solution (250 g sodium bromide added to 565 ml distilled water, to which was added 89 ml of sulphuric acid and distilled water making a total volume of 806 ml with distilled water) and 10 ml of toluene in a centrifuge tube. The tubes were covered with cling

film to prevent evaporation of toluene and their contents mixed very well by shaking by hand. Methyl mercury was released as methyl mercury bromide into the organic (toluene) phase.

2-) Samples were centrifuged for 15 minutes at ca. 4000 revolutions per minute. 5 ml of toluene was taken with a graduated syringe and transferred to a second smaller centrifuge tube.

3-) 2 ml of 0.005 M sodium thiosulphate (stock 0.05 M solution, 12.4 g sodium thiosulphate added to 1 litre distilled water; 0.005 M solution prepared from this solution by diluting it 10 times) and 5 ml of the toluene sample were mixed thoroughly. By this process methyl mercury bromide was converted into methyl mercury thiosulphate which passed into the aqueous phase.

4-) The toluene/sodium thiosulphate mixture was centrifuged for 10 minutes at ca. 2000 revolutions per minute. 1 ml of sample from the aqueous phase was taken with a graduated syringe and placed in a Kjeldahl flask. This sample in the flask was placed in a water bath at 50 ^{o}C for 1 hour to drive off toluene as the analyser was found to be sensitive to toluene. The 1 ml of extracted sample contained 25 % of the methyl mercury present in sample.

5-) After extraction of methyl mercury, the mercury content was read following the same procedure explained earlier. All chemicals used were of 'Puranal, Analar or Spectrosol' analytical grade throughout.

Extraction method efficiency and reproducibility were previously compared by performing extraction of standard solution of methyl mercury standard from the same

laboratory (Thomson, 1991). He indicated that there was no significant difference between direct measurement of methyl mercury standards and extracted samples.

2.4.3 Determination of Metals Other than Mercury

Concentrations of cadmium, copper, zinc, lead and iron in the tissues of *Nephrops norvegicus* were determined using the following technique.

1-) 0.050-1.00 g of dry tissue samples (0.5-1.0 g of tissue samples which were expected to show low concentrations, e.g. tail muscle) of the animals were weighed (to the nearest 0.001 g) using the same equipment. They were transferred to 50 ml clean digestion flasks. 10 ml nitric acid was added to each flask. The flasks were then placed on a hot plate (temperature adjustable porcelain B290 J.Bibby Science Products Ltd., Stone, Staffordshire, England) in a fume cupboard.

2-) The hot plate was initially set to number 6 (100 ^{o}C), and the samples were left at this temperature for at least four hours, shaking occasionally. After all the samples were dissolved at that temperature, the hot plate's setting was increased to number 7 (200 ^{o}C). Samples were boiled at this temperature for 20 minutes.

3-) Digested samples were taken off the hot plate and the flasks were put on a bench to cool for one hour. The cooled sample solutions were poured into 25 ml clean volumetric flasks and made up to volume with double distilled water. The digestion flasks were rinsed with the same water and these rinsings made up part of the 25 ml. The volumetric flasks were inverted repeatedly to ensure complete mixing and each sample was poured into a clean plastic bottle (50 ml volume) to await analyses.

4-) Concentrations of the metals in the tissues of the lobsters were measured by flame atomic absorption spectrophotometry (Philips PU9200 spectrophotometer, Pye Unicam Ltd., York Street, Cambridge, England). The instrument is an automated analytical instrument which incorporates advanced processing facilities and operates under a central control microprocessor. An array of sensors provides input and output data to the processing system which constantly monitors the instrumental conditions and provides analytical results. The instrument burns a mixture of air and acetylene gases and uses one hollow cathode lamp and one background correction (deuterium) lamp. Readings of the metals were done by the instrument in 0.5 nm bandpass, scanning from 200 nm at a rate of 10.0 nm/min. Lamp current and wavelength were changed by the instrument automatically depending on the chosen metal. Zinc, iron, cadmium, copper and lead were read by the instrument at wavelengths of 213,9 nm, 248.3 nm, 228.8 nm, 324.8 nm and 283.3 nm respectively.

5-) Standard solutions of the metals were prepared by diluting with double distilled water, standard solutions for atomic spectroscopy (BDH Chemicals Ltd., Poole, England). Standards were prepared in the following ranges to obtain the best linear line drawn by the instrument. When standards do not give a linear calibration, the instrument gives a warning sound so that standards could be repeated or reprepared. The following concentrations of the metals were prepared for standard calibrations. These levels showed linear relationships with absorbance values.

Cd = 50, 100, 500 and 1000 $\mu g\ l^{-1}$

Cu = 500, 1000, 5000 and 10000 $\mu g\ l^{-1}$

Zn = 250, 500, 1000 and 2500 $\mu g\ l^{-1}$

Fe = 1250, 2500, 5000 and 10000 $\mu g\ l^{-1}$

Pb = 50, 100, 500 and 1000 $\mu g\ l^{-1}$

After calibration of the instrument was completed by using the above standards, one or two standards were also repeated in every ten samples to check validity of the instrument. The following steps were applied to calculate the concentration of a metal in a tissue. Absorbance values and concentrations of the metals in aspirated solutions were obtained from the instrument. The concentration values were multiplied by dilution factor (25 ml) and divided by dry weight of tissue.

During the digestion of samples, two reference materials (TORT 1 lobster hepatopancreas, National Research Council, Canada) were also included and their metal concentrations were measured in the same way as that of samples. Mean values and standard deviations of reference material measured with the above instrument and method are given together with their certified values in the following table.

Table 2.1. Concentrations of metals and standard deviations in the reference material by methods explained above. Concentrations are given as μg metal g^{-1} dry weight (R.C. = Reference concentrations, P.C. = Present concentrations of the lobster hepatopancreas determined by the above method and instrument).

	Mercury	Cadmium	Lead	Copper	Zinc	Iron
R.C.	0.33±0.06	26.3±2.1	10.4±2.0	439±22	177±10	186±11
P.C.	0.36±0.04	23.9±0.4	10.7±0.6	398±13	170±4.2	175±8.8

2.5 STATISTICAL ANALYSES OF DATA

Trace metal concentrations in the tissues of 288 *Nephrops norvegicus* were statistically analysed to investigate if there were seasonal differences. As levels of some metals (e.g mercury) are known to be different between sexes, the male and female animals were separated. Trace metal concentrations may vary with size (e.g.

mercury, Davies and McKie, 1983), so carapace lengths of the animals were also investigated.

Linear regression analyses (e.g. Draper and Smith, 1981) were used to investigate the relationship if any, between trace metal concentrations (c) and carapace length (l) and month (m). For each metal, tissue and sex, 5 models were considered to find the best description of the data.

Model 1, $c = a \pm$ error.

Metal concentration shows no relationship with either month or carapace length.

Model 2, $c = a + b(l - \hat{l}) \pm$ error.

Metal concentration varies linearly with carapace length; this relationship is the same in each month.

Model 3, $c = a_m \pm$ error.

Metal concentration varies with month; there is no relationship with carapace length.

Model 4, $c = a_m + b\,(l - \hat{l}) \pm$ error.

Metal concentration varies linearly with carapace length. The slope is the same in each month. However, the intercept varies with month.

Model 5, $c = a_m + b_m\,(l - \hat{l}) \pm$ error.

Metal concentration varies linearly with carapace length. The slope and intercept both vary with month.

In all of these models, $\hat{l}$ is the mean carapace length of the whole sample (4.2 cm).

The parameter a_m is the mean concentration in month m (adjusted if necessary to correspond to *Nephrops* length of 4.2 cm), and parameter b_m is monthly variation in mean concentration or slope, then $\{a_m\}$ and $\{b_m\}$ reduce to a and b respectively. The errors are assumed to be independent and normally distributed with zero mean and constant variance; this assumption appears consistent with the data.

The process of model selection is as follows. Model 5 was first fitted to the data. To test whether model 4 was an adequate simplification, model 4 was then fitted to the data and the residual sums of squares from both models were compared using an F-test (Draper and Smith, 1981). If model 4 was an adequate simplification, then it is possible subsequently to test whether we can simplify still further (e.g. model 2 or 3) by further F- tests. In this way, the most suitable model for each data set can be obtained. Tissues which showed trace metal concentrations according to model 1 and model 5 were not further examined in seasonal graphs. They are shown only as mean metal concentrations and standard deviations of tissues, because metal concentration does not depend on either month or size in model 1 and month and size show an interaction in metal concentration in model 5. Tissues which were suitable for model 3 and model 4 are shown in seasonal variation graphs giving estimated mean values of metals in the tissues and standard errors. In the case of model 3, mean metal concentrations and standard error are shown in graphs since carapace length is not a significant factor to take into account. But in case of model 4, concentrations of metals were adjusted for carapace length (I chose the mean carapace length of the whole sample 4.2 cm as a standard) and associated standard errors. In this way, expected concentrations of the metals were calculated. Metal concentrations (on a dry weight basis) of male and female animals were reported as mean levels and standard deviations of the whole sample throughout the sampling period (Table 2.3). However, they could not be compared statistically due to effects of size and season.

Where required for comparisons with other published studies, wet mass equivalent concentrations can be estimated by using the wet to dry mass conversion ratios determined for each tissue (Table 2.4). The relationships between size and metal concentrations of tissues could not be shown on graphs using all samples because carapace length of samples differed significantly among months and also metal concentrations in most tissues varied among months.

2.6 RESULTS

Carapace length and number of male and female *Nephrops norvegicus* caught in different months of the year are given in Table 2.2. Mean concentrations of the metals in the tissues of male and female *Nephrops* are also given with associated standard deviations (Table 2.3).

Table 2.2. Following table shows numbers of male and female animals captured in different months of a year. Mean carapace length (cl) of samples are also given in this table.

Date	Male		Female	
	No	mean cl	No	mean cl
December 1989	24	4.8	6	5.0
January 1990	7	5.4	9	4.1
February 1990	6	4.4	2	5.3
March 1990	120	3.8	12	3.5
May 1990	20	3.7	18	3.6
July 1990	2	4.5	5	5.5
August 1990	6	4.5	26	5.6
October 1990	13	4.0	15	5.3

Table 2.3. Mean concentrations (μg g^{-1} d.w.) of the metals in the tissues of male and female *Nephrops norvegicus* throughout the sampling period and standard deviations (). Carapace length of total samples were 4.0±0.66 (n=198) and 4.7±1.06 (n=91) cm for male and female animals respectively.

Tissue	Sex	Mercury	Cadmium	Copper	Zinc	Iron
Carapace	Male	0.127 (0.08)	1.68 (2.36)	48.6 (25.8)	37.5 (17.0)	214 (156)
Carapace	Female	0.115 (0.07)	1.85 (1.56)	48.0 (23.8)	31.9 (16.2)	156 (148)
Hepatopancreas	Male	0.295 (0.14)	10.30 (7.93)	503 (242)	242 (91.0)	138 (84.0)
Hepatopancreas	Female	0.223 (0.11)	14.25 (11.6)	731 (265)	237 (74.0)	102 (53.0)
Gill	Male	0.417 (0.25)	8.43 (5.89)	250 (105)	161 (104)	916 (999)
Gill	Female	0.753 (0.35)	13.52 (6.62)	207 (106)	156 (148)	1196 (998)
Tail muscle	Male	0.374 (0.12)	1.12 (1.07)	24.9 (11.9)	58.8 (9.73)	37.1 (45.9)
Tail muscle	Female	0.600 (0.27)	1.69 (1.44)	27.6 (14.6)	63.6 (13.1)	21.2 (24.1)
Ovary	Female	0.082 (0.05)	1.85 (1.01)	126 (37.9)	99.5 (21.2)	36.7 (30.0)
External eggs	Female	0.123 (0.11)	2.26 (1.61)	113 (60.7)	131 (33.4)	411 (889)

Table 2.4. Ratios of wet and dry weight of *Nephrops* tissues and standard deviations.

	Carapace	Hepatopancreas	Gill	Tail muscle	ovary	ex.egg
No	107	109	113	122	34	18
ratio	2.24	2.90	9.65	4.70	3.67	4.35
sd	0.37	0.83	1.96	0.49	1.45	1.15

Table 2.5. The following table shows the results of linear regression analyses. The most suitable models for the tissues of each sex are described in this table with assosiated P values. CL=Carapace length, C&CL = Relationship (+ or -) between carapace length and metal concentration in a tissue. int = Interaction between carapace length and month.
* = P < 0.05, ** = P < 0.01, *** = P < 0.001.

TISSUES	METAL	SEX	C&CL	CL P	MONTH P	MODEL
Carapace	Hg	Male		ns	***	3
Hepatopancreas				ns	***	3
Gill				int	int	5
Tail muscle			+	***	***	4
Carapace	Hg	Female		int	int	5
Hepatopancreas				ns	***	3
Gill			+	*	***	4
Tail muscle			+	***	***	4
Carapace	Cd	Male		int	int	5
Hepatopancreas			+	***	***	4
Gill				ns	**	3
Tail muscle				int	int	5
Carapace	Cd	Female		int	int	5
Hepatopancreas			+	***	ns	2
Gill				ns	**	3
Tail muscle				int	int	5
Carapace	Cu	Male		ns	**	3
Hepatopancreas			+	*	***	4
Gill			-	*	ns	2
Tail muscle			-	***	***	4
Carapace	Cu	Female		ns	**	3
Hepatopancreas			+	*	*	4
Gill			-	**	ns	2
Tail muscle			-	*	***	4
Carapace	Zn	Male	-	*	*	4
Hepatopancreas				ns	***	3
Gill				ns	ns	1
Tail muscle				ns	***	3
Carapace	Zn	Female		ns	*	3
Hepatopancreas				ns	*	3
Gill				ns	ns	1
Tail muscle				int	int	5
Carapace	Fe	Male		ns	***	3
Hepatopancreas				ns	**	3
Gill				ns	***	3
Tail muscle			-	*	ns	2
Carapace	Fe	Female		ns	***	3
Hepatopancreas				ns	ns	1
Gill				ns	ns	1
Tail muscle				ns	ns	1

2.6.1 Mercury

Table 2.3 shows that female animals had higher mean concentrations of mercury in their gill and tail muscle than male animals, while males showed higher mean concentrations of mercury in their hepatopancreas. Mercury concentrations of carapace were similar between sexes. For males the order of tissue concentration was gill > tail muscle > hepatopancreas > carapace. For females this order was gill > tail muscle > hepatopancreas > ext. eggs > carapace > ovary. Regression analysis showed all the tissues which statistically analysed showed seasonal variations of mercury concentrations, except for the gill of male animals and carapace of female animals whose interactions were found between size and month (Table 2.4). Significant seasonal variations are shown in Figures of 2.1 and 2.2. Positive relationships were found between carapace lengths and mercury concentrations of tail muscle for male and female animals ($P<0.001$) (Table 2.5). The mercury concentrations in gills of female animals also showed a positive relationship with carapace length ($P<0.05$).

2.6.2 Cadmium

Cadmium concentrations in female animals were also higher than in male animals in all tissues. Tissue concentration order for males was hepatopancreas > gill > carapace > tail muscle, whereas this order for females was hepatopancreas > gill > ex. egg > ovary > carapace > tail muscle (Table 2.3). Regression analyses revealed that male and female animals showed positive relationships between carapace length and cadmium in their hepatopancreas ($P<0.001$) while gills of the both sexes did not show any size dependent variations in cadmium concentrations. Cadmium concentrations in the carapace of both male and female animals, however,

showed interaction between size and month (Table 2.5). Male animals had seasonal variations of cadmium in their gill and hepatopancreas ($P<0.01$ and $P<0.001$, respectively) while female animals had only variation of cadmium concentration in their gill ($P<0.01$). These seasonal variations are shown in Figures 2.3 to 2.4.

2.6.3 Copper

Copper concentrations in the hepatopancreas were higher in females than in males, but in the gill tissue of males showed higher concentrations than females, while in the carapace and tail muscle levels were similar (Table 2.3). Tissue concentration order for males was hepatopancreas > gill > carapace > tail muscle, whereas this order for females was hepatopancreas > gill > ovary > ex. egg > carapace > tail muscle. Regression analysis showed that except for carapace, copper concentrations in all tissues had significant relationships with carapace length. These relationships were positive in the hepatopancreas while they were negative in the gill and tail muscle of both male and female animals (Table 2.5). Seasonal patterns of the tissues from male and female animals also showed similarity between male and female animals. All the tissues except the gill showed significant variations between months (Table 2.5). These variations are shown in Figures 2.5 to 2.8.

2.6.4 Zinc

Concentrations of zinc in the tissues of males and females were very similar (Table 2.3). Tissue concentration order in males was hepatopancreas > gill > tail muscle > carapace, while the order for females was hepatopancreas > gill > ex. egg > ovary > tail muscle > carapace. Regression analysis revealed that zinc concentration in the carapace of male animals showed a significant relationship with

size ($P<0.05$) whereas the other tissues did not show any size related differences in zinc concentrations (Table 2.5). The carapace and hepatopancreas showed seasonal variations in zinc concentration in male and female animals. Male tail muscle also showed significant seasonal variations in zinc concentration ($P<0.001$) while levels in female tail muscle showed an interaction between size and season (Table 2.5). The gill of both sexes did not show seasonal variation in zinc concentration. Significant seasonal variations are shown in Figures 2.9 to 2.12 .

2.6.5 Iron

Table 2.3 shows that iron concentrations in the carapace, hepatopancreas and tail muscle of males were higher than in females. Tissue concentration order for males was gill > carapace > hepatopancreas > tail muscle, whereas the order for females was gill > ex. egg > carapace > hepatopancreas > ovary > tail muscle. The carapace, hepatopancreas and gill tissues of males showed seasonal variation in iron levels, while females showed seasonal variation in iron levels only in the carapace (Table 2.5). A negative relationship between carapace length and iron concentration was found ($p<0.05$) in the tail muscle of males but not in females, while the other tissues did not show any size related relationship (Table 2.5). Significant seasonal variations in iron concentrations are given in Figures 2.13 to 2.15.

Figure 2.1. Seasonal mercury variations in tissues of male Nephrops norvegicus

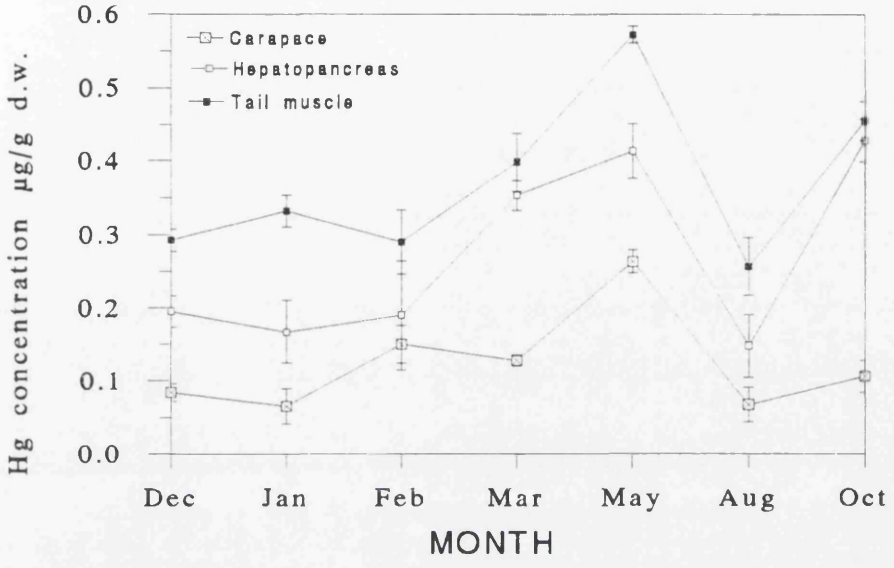


Figure 2.2. Seasonal mercury variations in tissues of female Nephrops norvegicus

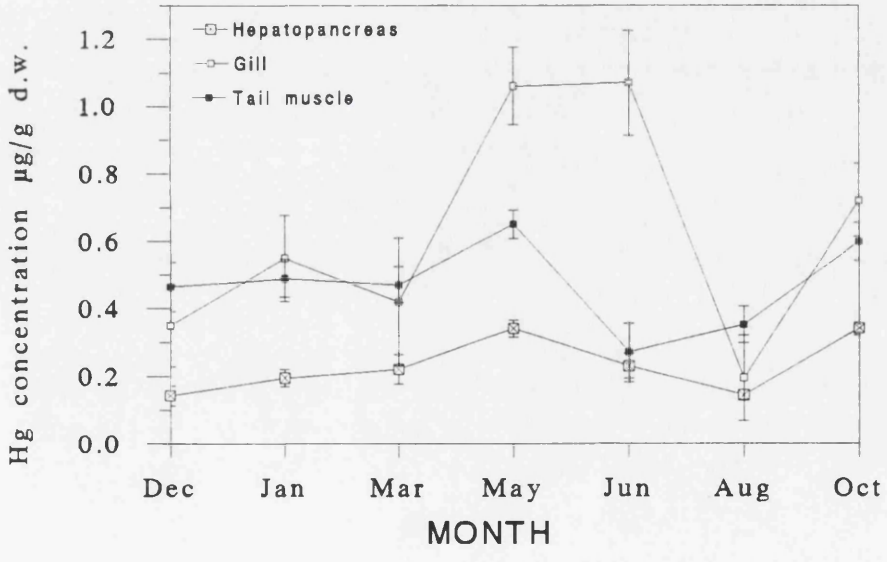


Figure 2.3. Seasonal cadmium variations in tissues of male Nephrops norvegicus

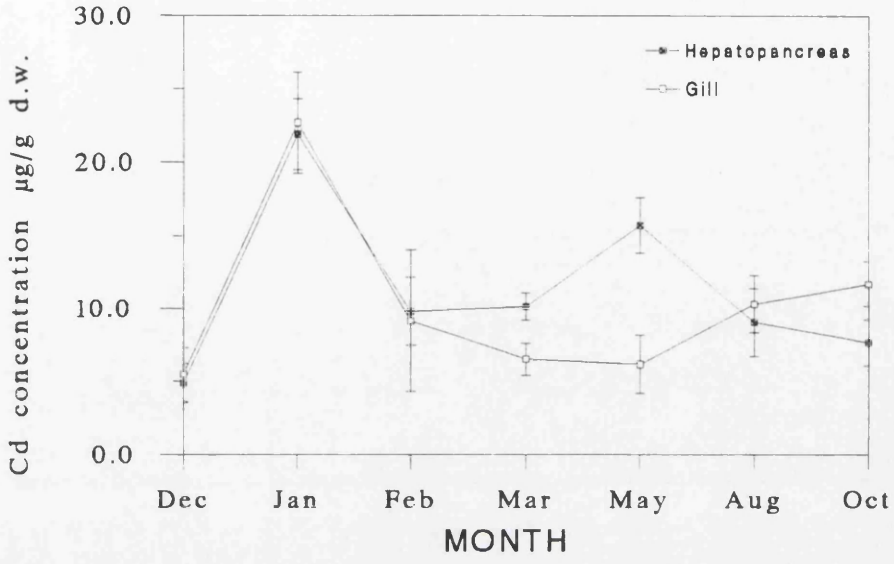


Figure 2.4. Seasonal cadmium variation in the gill of female Nephrops norvegicus

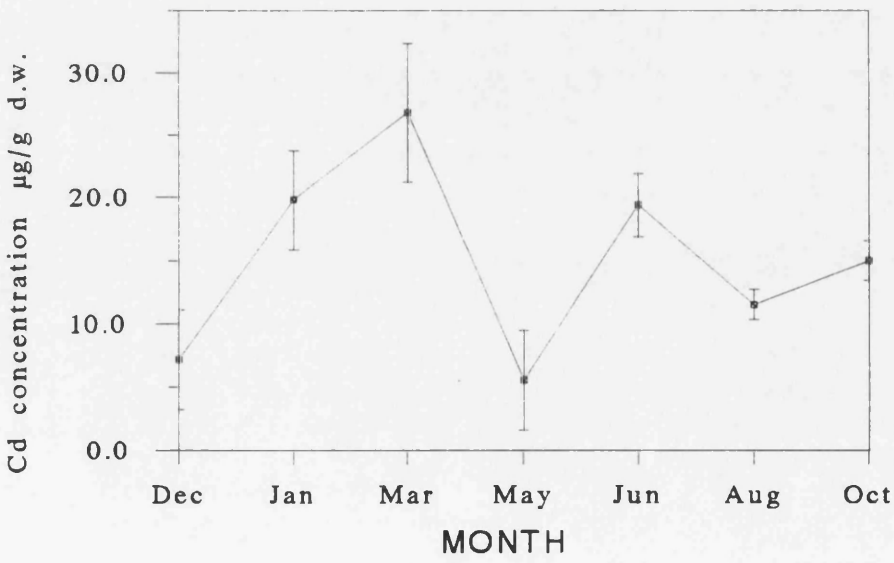


Figure 2.5. Seasonal copper variations in tissues of male Nephrops norvegicus

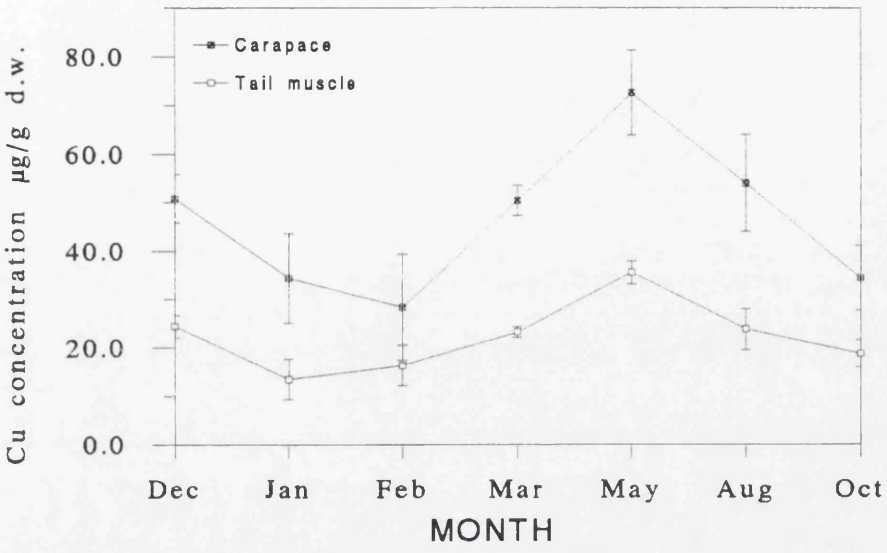


Figure 2.6. Seasonal copper variation in the hepatopancreas of male Nephrops norvegicus

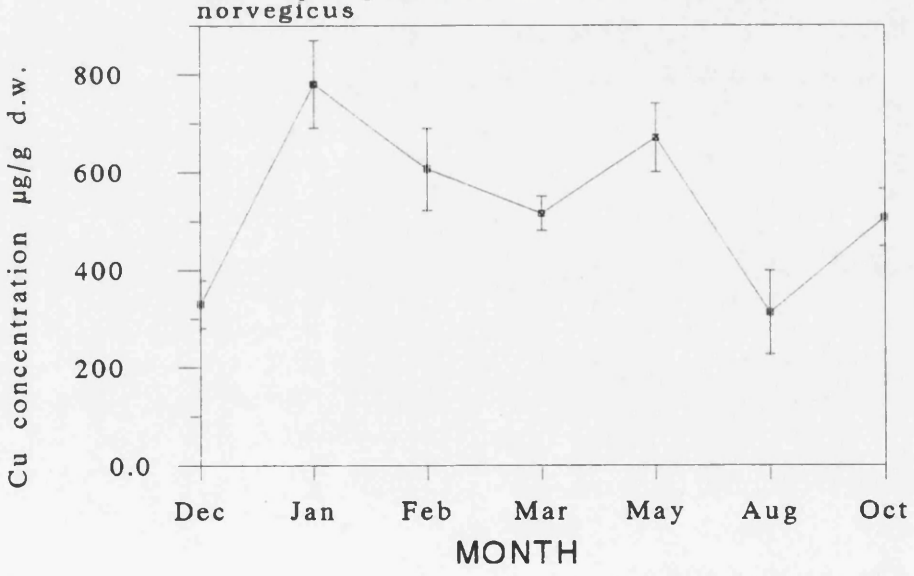


Figure 2.7. Seasonal copper variations in tissues of female Nephrops norvegicus

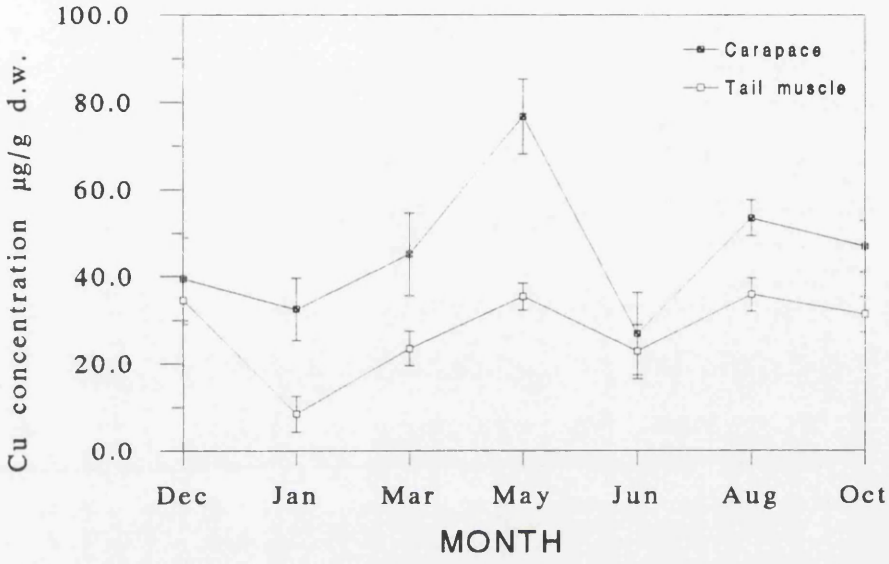


Figure 2.8. Seasonal copper variation in the hepatopancreas of female Nephrops norvegicus

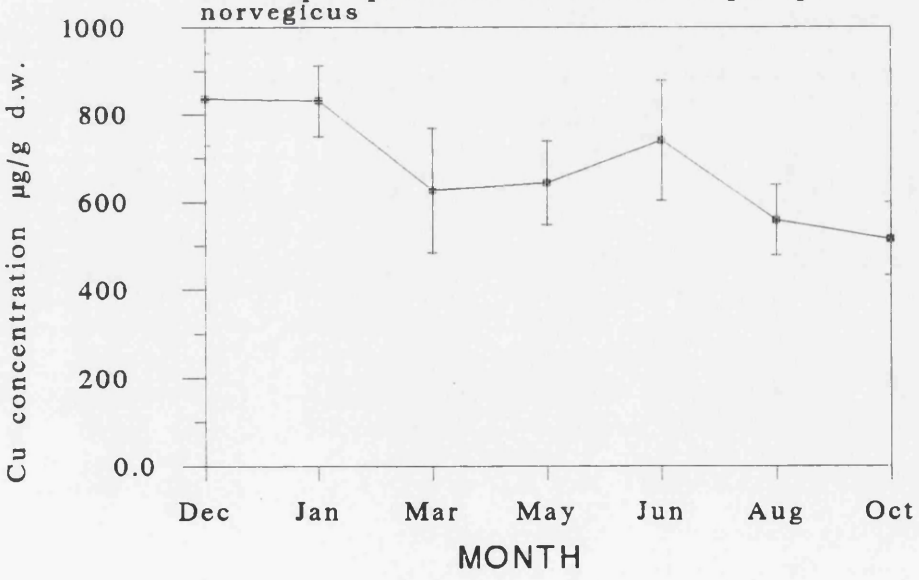


Figure 2.9. Seasonal zinc variations in tissues of male Nephrops norvegicus

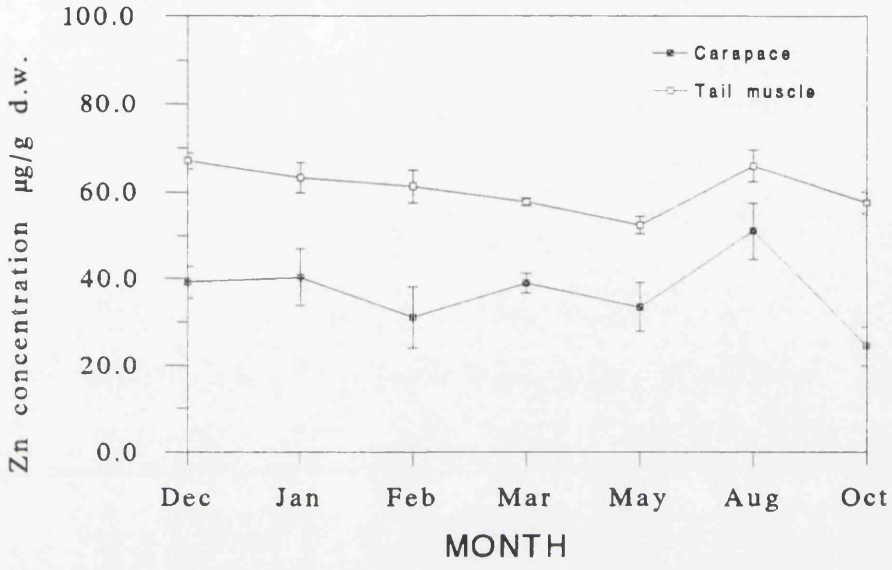


Figure 2.10. Seasonal zinc variation in the hepatopancreas of male Nephrops norvegicus

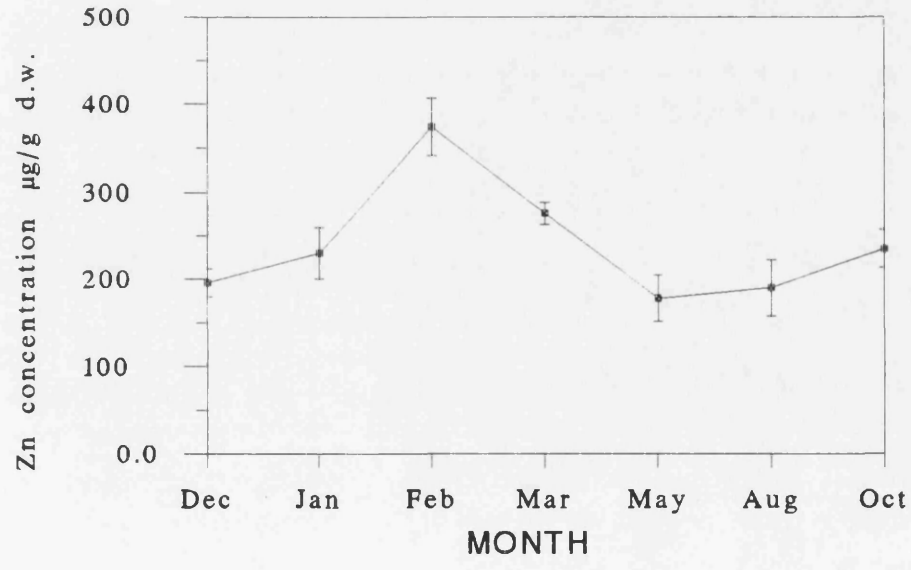


Figure 2.11. Seasonal zinc variation in the carapace of female Nephrops norvegicus

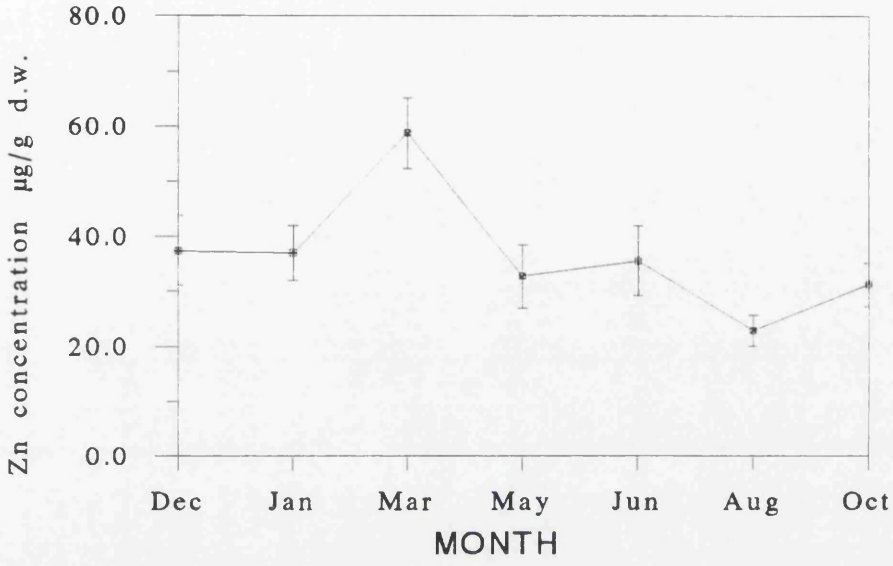


Figure 2.12. Seasonal zinc variation in the hepatopancreas of female Nephrops norvegicus

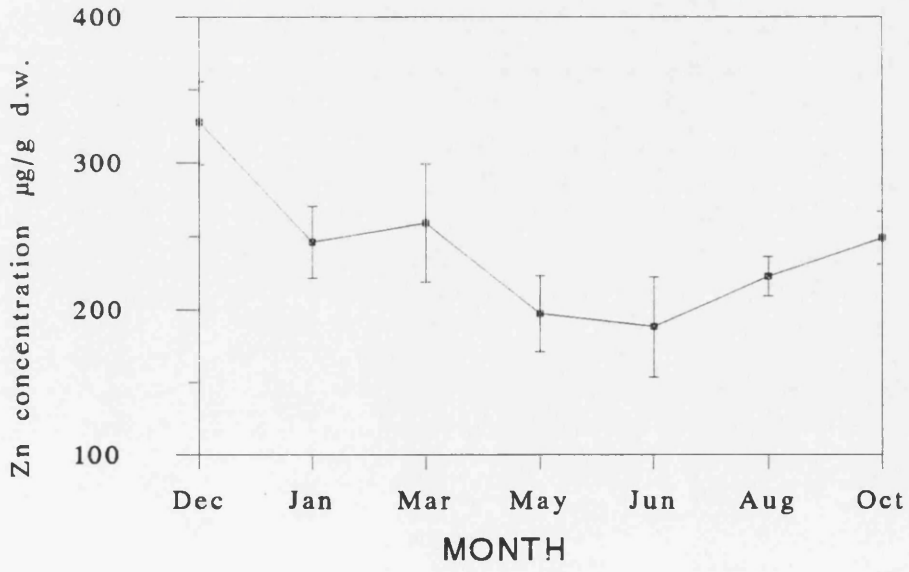


Figure 2.13. Seasonal iron variations in tissues of male Nephrops norvegicus

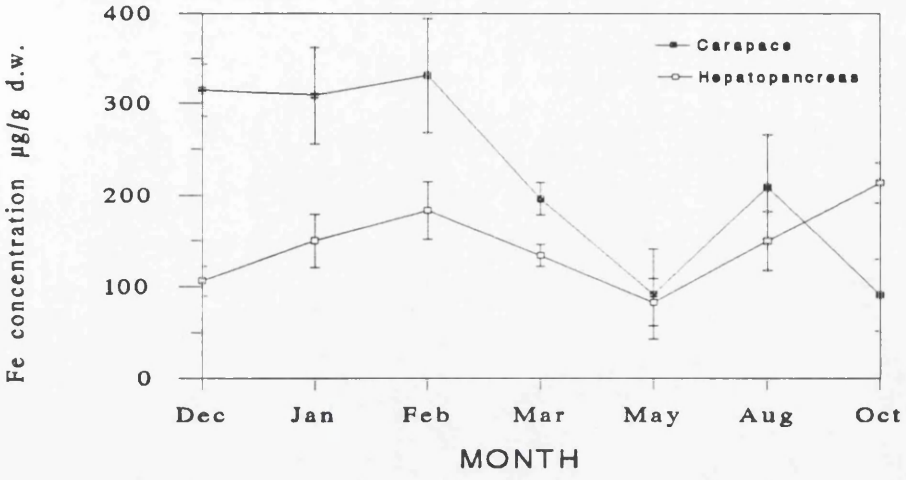


Figure 2.14. Seasonal iron variation in the gill of male Nephrops norvegicus

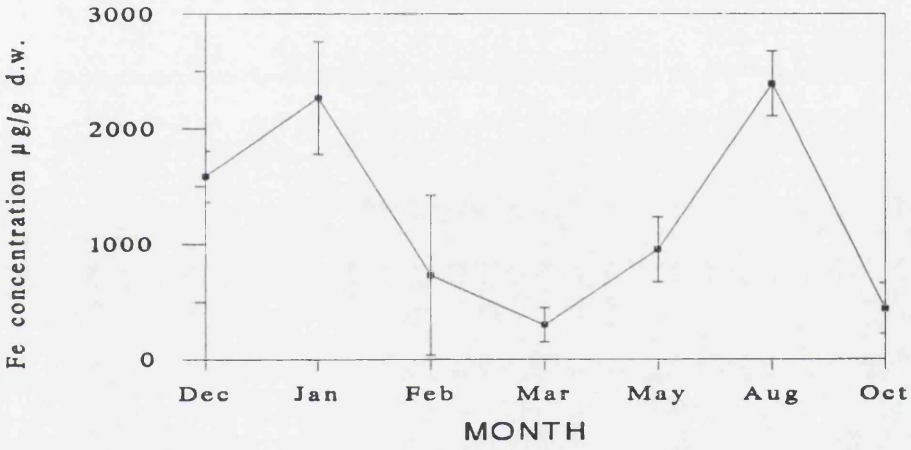
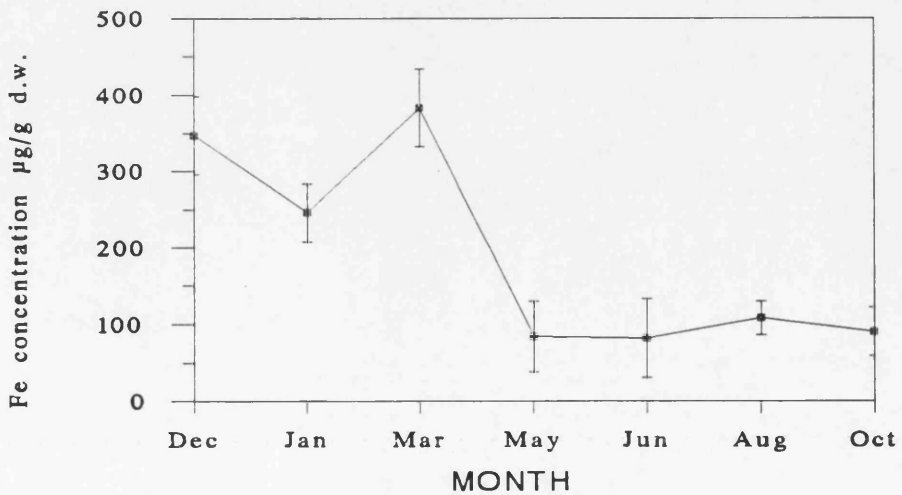


Figure 2.15. Seasonal iron variation in the gill of female Nephrops norvegicus



2.7 DISCUSSION

Mean mercury concentrations in the tail muscle of *Nephrops norvegicus* in this study were similar to levels presented by Davies and McKie (1983) from the Clyde sea area and Lima (1984) from Portuguese waters. Studies in Italian waters found higher levels of mercury in the tail muscle of the animals (Renzoni, 1980 ; Viviani, 1983 ; Capelli et al., 1983). Santoro and Koepp (1986) found higher concentrations of mercury in muscle and hepatopancreas of the blue-claw crab, *Callinectes sapidus* from New Jersey, USA. A positive relationship between body size and mercury concentration in the tail muscle of *Nephrops norvegicus* is an agreement with results of Davies and McKie (1983) ; Lima (1984) ; Renzoni (1980) ; Capelli et al. (1983). Davies and McKie (1983) and Lima (1984) found differences in mercury concentrations of tail muscle in the same size of female and male *Nephrops*. In this study differences between sexes were not statistically compared due to seasonal and size dependent variations. Mean values of tissue metal concentrations were given in Table 2.3. This table clearly shows the difference in tail muscle mercury concentrations between the sexes. This difference can be related to growth rate differences between male and female *Nephrops*. Male *Nephrops norvegicus* grow much faster than females (Davies & McKie, 1983 ; Howard, 1989). Therefore, the same ages of animals would have different sizes; males being larger than females of the same age. This means that females of the same size as males have lived in ambient mercury concentrations for a longer time. However, higher levels of mercury were found in the hepatopancreas of male *Nephrops* than were found in females. Data in Chapter 4 show that males have higher feeding rates than females and distribution of mercury to the hepatopancreas is more by the food route than by direct uptake from water, so higher concentrations of mercury in the hepatopancreas of males might come from greater food intake. Although *Nephrops norvegicus*

moults at any time of the year, peak periods occur in March and April, and from July to November. Peak periods of moulting activity are generally more pronounced in females, which must moult before mating takes place (Howard, 1989). Results showed that mercury levels in all tissues varied seasonally. Female carapace and male gills showed interactions between size and season which did not allow analysis of seasonal and size dependent variations individually. Moulting might cause the biggest variation in mercury levels in tissues, but this may also be influenced by varying conditions of ambient sea water and diet. Tugrul et al (1980) indicated that mercury levels can vary in marine organisms with season. Sivadasan and Nambisan (1988) also showed that mercury levels varied with season in the prawn, *Metapenaeus dobsoni*.

Cadmium distribution among the tissues of *Nephrops norvegicus* was similar to that reported in some other studies. Ray et al. (1981) found that in the lobster, *Homarus americanus* from contaminated and uncontaminated areas of Canada and USA the hepatopancreas contained more than 90 % of the body burden of cadmium. High concentrations of cadmium were also present in the green gland and gills. Davies et al. (1981) also found highest concentrations of cadmium in the hepatopancreas of the crab, *Cancer pagurus*. They also indicated that tissue cadmium levels showed great variations in the same tissue of different animals. Overnell and Trewhella (1979) found high levels of cadmium in the hepatopancreas of *Cancer pagurus*, as found in this study for *Nephrops norvegicus*. Ray et al. (1981) indicated that there can be wide variations in cadmium concentrations in the same tissues of different animals, even within the same size class. They also indicated that individuals with high cadmium levels in one tissue generally tend to have high levels in other tissues. Positive correlation between size and hepatopancreas cadmium in this study also agrees with the results of their study. Uthe et al. (1982) found great variations and

very high concentrations of cadmium in American lobster, *Homarus americanus*. They indicated that two different sites of Belleduna Harbour, New Brunswick, Canada showed different cadmium concentrations in tissues from the lobsters. Data from both areas are quite high when compared to the present data for the hepatopancreas, though tail muscle levels of cadmium from both sites were low, as for tail muscle of *Nephrops*. White and Rainbow (1989) indicated that the variations in cadmium concentrations of *Systellaspis debilis* could be related to dietary enrichments of cadmium from different areas. Cadmium concentrations in the tail muscle of *Nephrops* were also different in studies from different areas. For example, Murray (1981) could not detect (<0.2 μg g^{-1}) cadmium in the tail muscle of *Nephrops norvegicus*, and Capelli et al. (1983) found mean concentrations of 0.14 μg g^{-1} in the tail muscle, whereas Viviani (1983) found concentrations of only 0.053-0.087 μg g^{-1}. But Schuhmacher et al. (1990) found Cd concentrations in *Nephrops* to be between 0.161-0.547 μg g^{-1} w.w. from coastal waters of Spain. Differences among the other studies and the present study or any other crustacean in cadmium concentrations could be due to different concentrations of cadmium in sea water or food. Size and sex of samples or season which the animals were caught could be important to record since true comparisons can not be done otherwise. In the present study, hepatopancreas cadmium showed size and season effects in males, while it showed only a size effect in females. Higher concentrations of cadmium in the hepatopancreas of female animals could be due to higher carapace length of the animals. It has been shown that food can be a dominant uptake route for cadmium, especially for the hepatopancreas (Jennings and Rainbow, 1979 ; Davies et al., 1981). As indicated earlier, male *Nephrops* have higher feeding rates than females. So, seasonal changes in diet conditions would affect cadmium concentrations of males' hepatopancreas more than that of females' hepatopancreas. Seasonal variations in cadmium concentrations of the gill should come from seasonal variation

of ambient water condition, because food does not affect cadmium concentrations of the gill (Davies et al., 1981 and Chapter 4).

Copper concentrations in the tail muscle of *Nephrops norvegicus* have previously been found to be similar to the present results (Murray, 1981 ; Capelli et al., 1983). Copper concentrations vary greatly between tissues of decapod crustaceans, even in the same species (Bryan, 1964 ; 1967 ; 1968 ; Murray and portman, 1984). Nugegoda and Rainbow (1988) indicated that decapod crustaceans from different locations can show big differences. Two prawn species *Pandalus montagui* and *Palaemonetes varians* showed enormous variations between the species from different habitats. Copper concentrations in the tissues of *Nephrops* vary far more than those for zinc, as Bryan (1964) found for *Homarus vulgaris* though the distribution of copper among the tissues of *Nephrops* was similar to those for other decapod crustaceans which have been summarised by Bryan (1968). Mean copper concentrations in the hepatopancreas of *Homarus americanus* were higher than found in the hepatopancreas of *Nephrops*, whereas ovary and shell copper concentrations were lower than found in this study for ovary and carapace. Cuadras et al. (1981) found higher levels of copper in the tissues of the hermit crab, *Dardanus arrosor* than found in *Nephrops*, whereas Arumugam and Ravindranath (1983) found lower concentrations of copper in the hepatopancreas of *Scylla serrata* than found in this study. They also indicated that there was not any significant difference between male and female animals in the concentrations of copper. Darmono and Denton (1990) found a positive relationship between size and copper concentrations in decapod crustaceans, whereas White and Rainbow (1987) and Rainbow and Abdennour (1989) found positive relationships between copper concentrations and dry weights in crustacean species. In the present study, size negatively affected concentrations of copper in the gill and tail muscle in male and female animals, while the

hepatopancreas showed a positive size effect in both sexes. The gill tissue did not show any seasonal effects in either sex, while the carapace and tail muscle showed seasonal variations in both sex. Bryan (1967) indicated that most of the copper in muscle tissues in Crustacea is the result of contamination of cellular spaces by the blood. Seasonal changes in the blood concentrations of copper could affect copper concentrations of the tail muscle. Moulting also affects copper concentrations of the tissues, especially the carapace and hepatopancreas. Belleli et al. (1988) indicated that haemocyanin concentrations from the Mediterranean lobster, *Palinurus elephas* differ between the sexes and change in relation to the period of the year. Haemocyanin is a protein which contains copper. Changes of haemocyanin concentrations may indicate changes in copper concentrations of the tissues from the lobster as well. Engel (1987) also found significant variations in copper concentrations of the haemolymph and digestive gland of the crab, *Callinectes sapidus* among moult, premoult and intermoult stages, having hihgest levels in premoult stage and lowest in soft crab stage.

Zinc concentration is relatively less variable in the same tissues of *Nephrops norvegicus* when it is compared to the other essential metals studied. But variations among the different species of decapod crustaceans can be enormous. Ober et al. (1987) found zinc concentrations in decapod crustaceans to be between 36-464 $\mu g\ g^{-1}$ d.w., and Bryan (1968) found variations to be between 18-49 $\mu g\ g^{-1}$ w.w. among a large variety of species. Zinc concentrations in the tissues of *Nephrops norvegicus* were found to be within this range. The concentrations of zinc in *Nephrops* seem to be higher than at least in some tissues of *Homarus vulgaris*, such as hepatopancreas, gill and carapace, while the others are more or less similar (Bryan, 1964). Murray (1979) and Capelli et al. (1983) showed similar zinc concentrations in the tail muscle of *Nephrops norvegicus* to the present results. Darmono and Denton (1990)

found the lowest concentrations of zinc to be in the muscle tissues of juvenile banana prawn *Penaeus merguinsis* and juvenile leader prawn *Penaeus monodon*, whereas the highest levels of zinc were found in the hepatopancreas of the animals. Zinc concentrations of the tissues from *Nephrops* were very similar between male and female animals. Cuadras et al. (1981) indicated that there were no differences in concentrations of zinc in the organs studied between male and female hermit crab, *Dardanus arrisor*. White and Rainbow (1987) indicated that zinc concentrations decrease slightly with increasing dry weight of *Systellaspis debilis*. Rainbow and Abdennour (1989) found that total body zinc concentrations did not change significantly in the same species. In this study, except in the case of carapace of male animals, size did not affect zinc concentrations. Seasonal variations were found to be significant for the all tissues except the gill of the either sex and tail muscle of female animals. Seasonal variations of zinc could be due to moulting at different times of the year. Engel (1987) found significant variations in zinc concentrations of the haemolymph and digestive gland of the crab, *Callinectes sapidus* among moult, premoult and intermoult stages, with highest levels in premoult stage and lowest in soft crabs, and Engel and Brouwer (1987) found that zinc levels varied seasonally in relation to both moult and ambient temperature. However, Sivadasan and Nambisan (1988) could not find seasonal variations in zinc levels in the edible part of the prawn *Metapenaeus dobsoni*.

Iron concentrations in the tissues of male and female *Nephrops* were very different and enormous variations were found in the same sex. Males showed higher mean concentrations of iron in their carapace, hepatopancreas and tail muscle, while female animals showed higher mean concentrations of iron in their gill tissue. Ridout et al. (1989) found Fe concentrations to be between 13.7-80.9 $\mu g\ g^{-1}$ d.w. in a large variety of crustacean species from the North East Atlantic Ocean. Ober et al.

(1987) found Fe concentrations to be between 86-374 $\mu g\ g^{-1}$ d.w. in commercial decapod crustaceans from Chilean waters. Depledge (1989) found highest concentrations of iron in the gill tissue of *Carcinus maenas*. Present results showed that the highest iron levels were present in the gill tissue of *Nephrops* followed by carapace. Iron concentrations in the tissues of *Nephrops* showed great variation in male and female animals. White and Rainbow (1987) found Fe concentrations to be 38.1 $\mu g\ g^{-1}$ d.w. in *Systellaspis debilis*, indicating that iron concentrations decreased with increasing dry weights of the animals. The present study showed that size did not affect iron concentrations of the tissues, except for a negative relationship between iron levels in the tail muscle and carapace length of male animals.

This study showed that heavy metal concentrations in the tissues of *Nephrops norvegicus* in the Clyde Sea varied with season and size of male and female animals. Seasonal variations were a very important factor for all metals. This could be related mainly to the timing of moult, though variations in metal concentrations of sea water and diet may explain some of the variation. Although size was a significant factor affecting levels of most metals, it was a much more important factor for non-essential metals mercury and cadmium. Positive relationships between size and concentrations of mercury and cadmium could be interpreted as the result of continuous accumulation of these metals with no regulation. Differences in metal levels between the sexes might be related to differences in the metabolic activity, feeding and growth rates of the sexes. There was no evidence from this study that ambient levels of essential metals in the Clyde Sea area exceeded levels that can be regulated by *Nephrops* and thus essential metals are unlikely to present a toxic hazard to this population. Levels of non-essential metals were similar to those reported in several other studies of *Nephrops norvegicus* and other Crustacea and appear also to be unlikely to present a toxic hazard. However, it should be noted that there are

substantial variations in the metal levels in tissues due to season, size or sex, and this might have important implications for regulatory practice. These differences between seasons and sizes of animals also make comparisons between studies more difficult since data are often presented without reference to the size, sex and moult stage of animals sampled.

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CHAPTER 3

EFFECTS OF SEX AND SIZE ON THE ACCUMULATION AND TISSUE DISTRIBUTION OF HEAVY METALS FROM SEA WATER BY THE NORWAY LOBSTER *NEPHROPS NORVEGICUS*; TOXICITIES OF HEAVY METALS

3.1 INTRODUCTION

Marine animals naturally contain some heavy metals such as copper, zinc, iron and cobalt and accumulate them from sea water and food for normal functions of metabolism. Others, such as mercury, cadmium and lead have no biological function but also accumulate in marine animals. All heavy metals can be toxic in high environmental concentrations. Many areas of developed coastlines and estuaries are subject to anthropogenic inputs of heavy metals (Langston, 1990). Marine animals may accumulate heavy metals continuously from surrounded sea water, and tissue levels may rise to toxic levels for animals themselves or for humans who eat them . One of the best known heavy metal pollution incidents in the marine environment is the Minamata disaster, which caused many human deaths, impaired vision, loss of motor co-ordination, neurological abnormalities, hormonal and enzymatic disturbances (Salvatore et al. 1977). After the Minamata disaster, much attention has been focused on heavy metal pollution and many experiments have been carried out to examine the uptake and toxic effects of non-essential and essential metals.

The concentration of a metal which kills an aquatic organism depends both on the metal and on the organism. Generally speaking, mercury, silver and copper are the most toxic metals, followed by cadmium, zinc and lead. This order of toxicity is not rigid and varies among species (Bryan, 1971). There are also differences between different forms of the same metal in the effects on marine animals. For example, organic mercury has a greater accumulation rate, lower lethal concentrations and slower elimination than inorganic mercury (Ray and Tripp, 1976 ; Fowler et al., 1978 ; Riisgard and Famme, 1986). There are also different effects on metabolic processes in Crustacea (Kraus and Kraus, 1986; Kraus and Weis, 1988).

Accumulation of heavy metals, especially non-essential ones, is greatly dependent on concentration in the ambient water and the period that animals are exposed to that concentration, though there are other factors which affect accumulation such as temperature and salinity (Philips, 1980; Mance, 1987). Levels of essential metals such as copper and zinc can be regulated by decapod crustaceans at concentrations of dissolved copper and zinc below a threshold level. Accumulation of these metals only begins after the regulation mechanisms break down at metal concentrations above the threshold (Bryan, 1964 ; White and Rainbow, 1982 ; Rainbow, 1985 ; Bryan, 1986). By contrast, body levels of non-essential metals such as mercury, cadmium and lead cannot be regulated by Crustacea. They are accumulated by crustacea in proportion to the concentrations of these metals in water (Nimmo et al., 1977 ; Meadows and Erdem, 1982 ; Devineau and Amiard-Triquet, 1985 ; Riisgard and Famme, 1986 ; Krishnaja et al., 1987; Pastor et al., 1988). Crustaceans can develop enhanced tolerance to heavy metals by living in contaminated waters. They can survive in concentrations that animals from relatively clean environments cannot tolerate and may contain higher levels of metals in their tissues (Uma Devi, 1987 ; Uma Devi and Prabhakara Rao, 1989 ; Kraus et al., 1988).

Although there have been many studies on accumulation of heavy metals from sea water in crustacean species, no previous study has examined accumulation of metals from sea water by the Norway lobster, *Nephrops norvegicus*. Few studies have taken sex and size of animals into account in relation to metal accumulation and distribution among tissues. Fisheries for the Norway lobster, *Nephrops norvegicus* have increased over the last ten years and in economic terms *Nephrops* has now become the most important shellfish species in the U.K. as well as an important commercial species in many other countries (Howard, 1989), and so a better understanding of its sensitivity to heavy metal pollution is desirable. This study

reports on accumulation and tissue distributions of metals in animals exposed to sublethal concentrations of metals in sea water over a 30-day period. Studies were conducted in tissues such as carapace, hepatopancreas, gill, tail muscle, ovary and external eggs using metals such as organic mercury, inorganic mercury, cadmium, copper, lead and zinc. Size and sex of the animals were taken into account in expressing the tissue concentrations since metal concentrations may vary between sexes and with size (e.g. mercury) (Davies and McKie, 1983 ; Lima, 1984). Mortality studies were also carried out to determine toxicities of the metals to the animals.

3.2 MATERIALS AND METHODS

Nephrops norvegicus were caught on various dates from September 1990 to September 1991, from south of the Isle of Cumbrae in the Clyde Sea by trapping in creels. Sea surface temperatures at the times of collection were around 8-15 ^{o}C. The animals were transferred to a fibreglass holding tank which contained 10,000 litres of circulating sea water. The sea water which was used in all experiments was continuously circulated, oxygenated, passed through a biological filter to maintain levels of ammonia and nitrate within satisfactory limits, and cooled to approximately 10-12 ^{o}C. Chemical parameters of the seawater were routinely monitored each week by the aquarium technician. *Nephrops* to be used to measure control concentrations of the metals were caught by trawl or creel and frozen at -20 ^{o}C for later analyses alongside the experimental animals.

All experiments were conducted in 50 litre fibreglass tanks in a room at 17.5$\pm$1.5 ^{o}C and illuminated for 12 hours a day. *Nephrops* were kept in holding tanks for several days to ensure that any injured animals would not be used in experiments.

Each experimental tank was filled with 40 litres of filtered seawater with added metals. During the experimental treatments, the tanks were aerated using airstones attached to a compressed air supply. After all the conditions were set up, two healthy intermoult *Nephrops* were put into each tank. During the experiments, the water was changed completely every three days to maintain metal levels and to ensure that no changes in water quality occurred that might otherwise affect metal uptake. The animals were fed with fish (mackerel, *Scomber scombrus*) muscle once a week. Food was given on days that the water was due to be changed, and water was changed about three hours after feeding, so that any remaining food was cleared to avoid contaminating the water. The following analytical grade compounds of the metals were used to contaminate sea water; methylmercury (MeHg) CH_3HgCl, inorganic mercury (Hg) $HgCl_2$, cadmium (Cd) $CdCl_2$ 2 1/2 H_2O, copper (Cu) $Cu(NO_3)_2$ $3H_2O$, lead (Pb) $Pb(NO_3)_2$ and zinc (Zn) $ZnSO_4$ $7H_2O$. Fifteen replicate experimental tanks were used with two animals in each tank for MeHg uptake, 14 for Hg, 14 for Cd, 11 for Cu, 11 for Pb and 13 for Zn. Thus animals were exposed to only one of the study metals and not to interactions between metals.

Two *Nephrops* were also put in each of two tanks to use as control animals, while experiments were running. Their tanks were filled up to the same level with sea water. At the end of 30 days exposure, experimental and control animals were taken out, measured, killed, dissected and metal concentrations in their tissues were determined.

Dissection, digestion and metal determination of the tissues (carapace, hepatopancreas, gill, tail muscle, ovarium and external eggs) of male and female animals were carried out as explained in Chapter 2.

3.2.1 Experimental Details

Early during the 30-day experimental period, sea water solutions of the metals were sampled from the experimental tanks for analyses to establish whether there were any changes in dissolved metal levels between the day solutions were made and the third day in the tank. Since metal adsorption was expected to be most rapid at the start of the experiment it is likely that the reductions in metal concentrations in the seawater later during the experimental period will have been less than the reductions measured. Tanks were also set up with the experimental solution in for the same period and the same conditions but without animals present, and metal levels were also measured in these tanks, in order to assess the amount of metal loss by exchange with the atmosphere rather than as a result of uptake by the animals. Measurements of the metals were carried out directly for 100 μg Cd l^{-1}, 100 μg Zn l^{-1} and 100 μg Pb l^{-1}, but 10 μg Cu l^{-1} solutions were boiled to reduce volume. Both 10 μg mercury l^{-1} solutions were acidified with nitric acid and heated to 58 ^{o}C then 20 ml of solution was used to give a satisfactory reading (Table 3.1).

Metal concentrations in the carapace of experimental animals were determined to see if there was adsorption onto the carapace surface. Carapaces of control animals were removed and put into the same conditions as experimental animals. On the 3rd, 6th and 9th days two carapaces were taken and metal concentrations were measured. These values are given in Table 3.2.

3.2.2 STATISTICAL ANALYSIS OF DATA

Metal concentrations in the tissues of exposed animals were statistically analysed to investigate if there were differences between sexes and different sizes. Linear

regression analyses (e.g. Draper and Smith, 1981) were used to investigate the relationship between metal concentration (c) and sex (s) or carapace length (l). For each metal exposure, a 5-stage model was considered.

Model 1; $c = a \pm$ error.

Concentration does not depend on sex or size (carapace length).

Model 2; $c = a + b_s \pm$ error.

Concentration differs between sexes but not with size.

Model 3; $c = a_l + b \pm$ error.

Concentration varies linearly with carapace length but not with sex.

Model 4; $c = a_l + b_s \pm$ error.

Concentration varies linearly with size and differs between sexes.

Model 5; $c = a_l * b_s \pm$ error.

Concentration differs between sexes and varies with size but this is not a linear relationship. There is an interaction in concentration between size and sex.

In models above, a_l describes size dependent variations while b_s describes sex dependent variations in metal concentration of a tissue. The best description of models in tissue metal concentrations from the experiments was found in the similar way that was explained in Chapter 2.

Mean metal concentration of the tissues from the experiments and controls are shown in Table 3.3. If a metal concentration from the experiments shows significant

differences between the sexes, then they were shown in histograms giving the mean concentrations of metals and associated standard errors in the tissues of both males and females. In the case of size differences in metal accumulation, scatter graphs were drawn to show the relationship between size (carapace length) and metal concentrations in tissues. The results of the sublethal concentration of the both mercury experiments were compared with one way Anova. Copper and zinc concentrations of control animals were also compared with results from experimental animals using one way Anova.

3.3 RESULTS

Loss of the metals over three days in sublethal concentrations were not considerable, except for organic and inorganic mercury (Table 3.1). Adsorption of the metals onto the surface of the carapace occurred in sublethal concentrations, except for copper (Table 3.2). Concentrations of cadmium, zinc and lead in the dissected carapace at the end of nine days adsorption period were higher than carapace concentrations in 30 day-exposed live animals.

Table 3.1. Changes in dissolved metal concentrations of sea water during experiments between days when solutions were made and after 3 days. Concentrations are given as ppb ($\mu g\ l^{-1}$).

Metal	meHg		Hg		Cu		Cd		Zn		Pb	
Addition	10		10		10		100		100		100	
Days	0	3	0	3	0	3	0	3	0	3	0	3
With animals	16	5	18	10	11	12	119	123	157	150	106	90
No animal	23	14	20	12	16	16	106	105	150	152	108	101

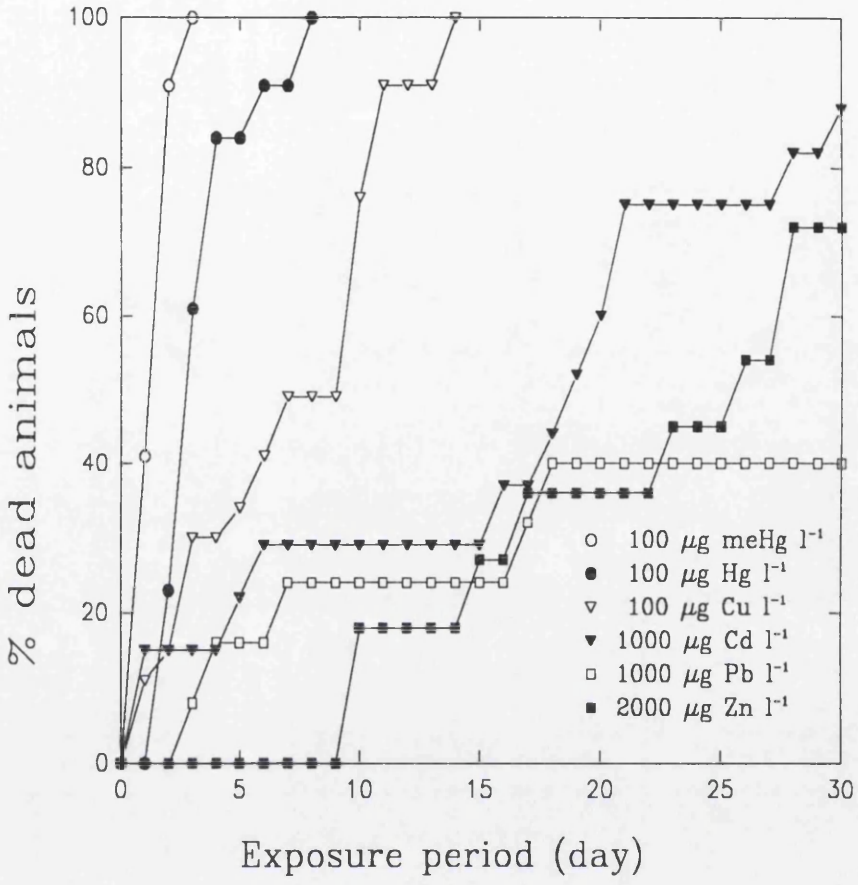
Table 3.2. Adsorption of the metals onto the surface of the carapace of *Nephrops norvegicus* in the sublethal concentrations of the metals. All data are given as μg g^{-1} dry weight.

Days	meHg	Hg	Cu	Cd	Zn	Pb
3	1.53	1.01	20.8	35.5	89.4	100.9
6	2.12	2.73	13.2	65.5	118.3	126.8
9	2.89	3.75	27.3	71.5	348.1	112.6

3.3.1 Uptake of Organic Mercury From Sea Water

A total of 30 *Nephrops* (13 males and 17 females) was used in an experiment in which they were exposed to 10 μg l^{-1} of methyl mercury chloride in sea water for a 30-day period. This concentration was found to be sublethal in the experimental conditions, though 100 μg l^{-1} methyl mercury killed 100 % of the lobsters in three days (Figure 3.1). Organic mercury was accumulated by the tissues of *Nephrops* in the sublethal concentration and increased the amount of total mercury in the tissues. The gill tissue accumulated the highest level of mercury (181 μg g^{-1}) (Table 3.3). Control animals had the highest concentrations of mercury in the gill and tail muscle (0.77 and 0.62 μg g^{-1}, respectively) (Table 3.4). Mean mercury concentrations in the gill, external eggs, ovary, carapace, hepatopancreas and tail muscle of treated animals increased 235, 160, 118, 62, 39 and 15 times over controls, respectively. Total tissue burdens of mercury in experimental animals were found to be predominantly in the tail muscle (38.9 μg Hg, 41 %) and gill (30.8 μg Hg, 32 %) (Tables 3.5 and 3.7), whereas the tail muscle had 80 % (20.4 μg Hg) of mercury in the control animals (Tables 3.6 and 3.7). There was a significant difference in mercury concentration of the hepatopancreas between male and female animals. Male animals (14.98 μg g^{-1}) had higher concentrations of mercury than female animals (9.40 μg g^{-1}) (Figure 3.4). A positive correlation was found between mercury levels in the hepatopancreas and tail muscle (Table 3.8).

Figure 3.1. Toxicity of metals to Nephrops norvegicus

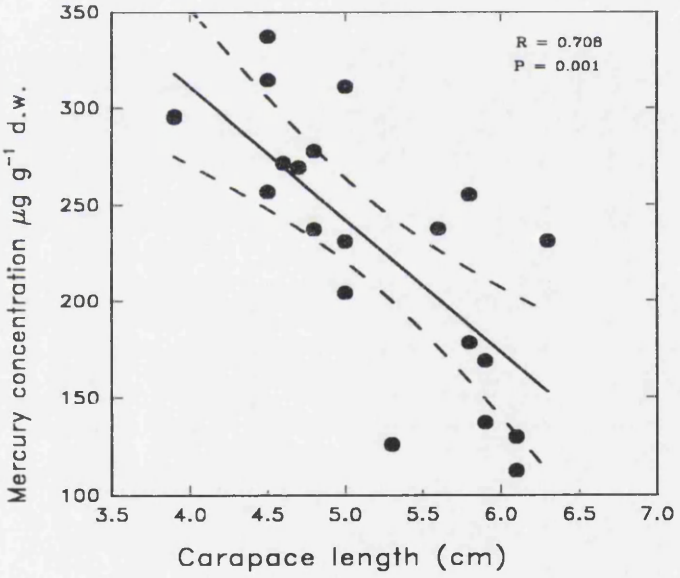


3.3.2 Uptake of Inorganic Mercury From Sea Water

A total of 27 *Nephrops* (12 males and 15 females) was used in an experiment in which they were exposed to a concentration of 10 μg l^{-1} of inorganic mercury over a 30-day period. As with organic mercury, this concentration was sublethal to the lobster in the experimental conditions though 100 μg l^{-1} of inorganic mercury killed 100 % of the animals in eight days (Figure 3.1). Inorganic mercury was accumulated by the tissues of *Nephrops* in the sublethal concentrations. Mean mercury concentrations of the gill, ovary, external eggs, carapace, hepatopancreas and tail

muscle increased 301, 81, 59, 46, 24, and 2.7 times over controls, respectively. The gill tissue accumulated the highest concentrations of mercury (232 $\mu g\ g^{-1}$) (Table 3.3), and held 63 % (42.2 μg Hg) of the tissue burden (Tables 3.5 and 3.7). There was a difference in mercury concentration of hepatopancreas between male and female animals; males (9.47 $\mu g\ g^{-1}$) had higher concentrations than females (5.48 $\mu g\ g^{-1}$) (Figure 3.3). Regression analysis showed that gill tissue concentration showed a relationship with size (Table 3.9). Large animals had lower concentrations of mercury in their gill tissue than smaller animals (Figure 3.2). No correlation was found between mercury levels in different tissues (Table 3.8).

Figure 3.2. The relationship between size and mercury in the gill of Nephrops norvegicus exposed to 10 $\mu g\ HgCl_2\ l^{-1}$ for 30 days

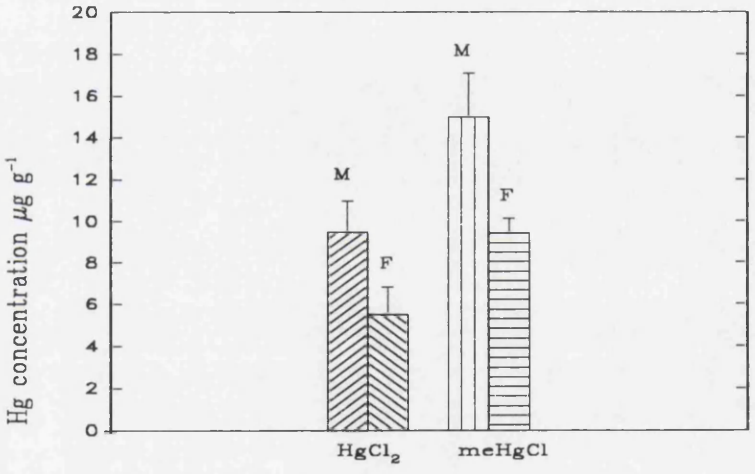


3.3.3 Comparisons of Organic and Inorganic Mercury Experiments

Table 3.10 shows the results of statistical comparison of the organic and inorganic mercury experiments in the sublethal concentration. Carapace, hepatopancreas, tail muscle and external eggs accumulated higher concentrations of mercury in the

organic mercury uptake experiment than in the inorganic mercury uptake experiment, while gill tissue had higher concentrations in the inorganic mercury uptake experiment. Ovarium mercury levels did not show any difference between the experiments. Total tissue burdens of mercury between the experiments were also different. Size of experimental animals affected mercury concentration of gill in the inorganic mercury uptake experiment. Both mercury experiments showed a similar difference between sexes in mercury concentrations of hepatopancreas (Figure 3.3). The toxicity studies showed that organic mercury was much more toxic to the animals than inorganic mercury (Figure 3.1).

Figure 3.3. Differences in hepatopancreas mercury concentrations of male and female Nephrops from the both mercury experiments

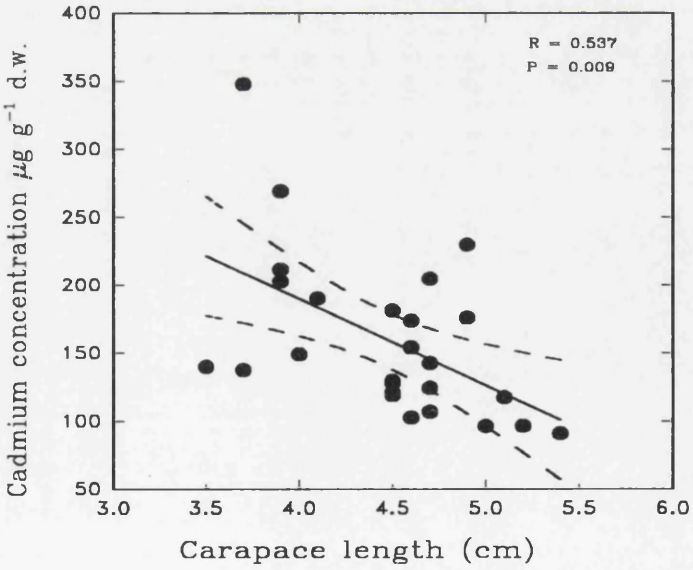


3.3.4 Uptake of Cadmium From Sea water

A total of 27 *Nephrops* (11 males and 16 females) was used in an experiment where they were exposed to a concentration of 100 $\mu g\ l^{-1}$ of cadmium in sea water over a 30-day period. This concentration of cadmium was found to be a sublethal dose in the experimental conditions, whereas 1000 $\mu g\ l^{-1}$ concentration was found to be lethal to 85 % of the animals in 30 days (Figure 3.1). Cadmium was accumulated by the tissues of *Nephrops* in the sublethal concentration. The hepatopancreas and

gill accumulated highest concentrations of cadmium, 224 and 161 $\mu g\ g^{-1}$ respectively (Table 3.3). The hepatopancreas had 85 % (289 μg Cd) of total body burden of cadmium in experimental animals (Table 3.5 and 3.7). Highest concentrations of cadmium were also found in the gill and hepatopancreas in control animals (13.11 and 10.34 $\mu g\ g^{-1}$, respectively) (Table 3.4). Mean cadmium concentrations in the hepatopancreas, gill, carapace, ovary, external eggs and tail muscle increased 21.6, 12.3, 7.20, 5.54, 4.42 and 1.77 times over controls, respectively. Cadmium burdens of the hepatopancreas and gill in controls were 55 % (17.3 μg Cd) and 30 % (9.34 μg Cd), respectively (Tables 3.6 and 3.7). Regression analysis showed that gill tissue cadmium had a negative relationship with carapace length (Figure 3.4), while carapace cadmium showed an interaction between sex and size (Table 3.9). There was no sex difference in relation to cadmium concentrations of the tissues (Table 3.9). Cadmium concentration in gill tissue showed correlations with cadmium in the carapace and hepatopancreas (Table 3.8).

Figure 3.4. The relationship between size and cadmium in the gill of Nephrops norvegicus exposed to 100 μg Cd l^{-1} for 30 days



3.3.5 Uptake of Copper From Sea Water

A total of 21 *Nephrops* (10 males and 11 females) was used in an experiment in which they were exposed to a concentration of 10 $\mu g\ l^{-1}$ copper over a 30-day period. This concentration was found to be sublethal in the experimental conditions, though 100 $\mu g\ l^{-1}$ copper killed 100 % of the animals in 14 days (Figure 3.1). Highest concentrations of copper were found in the hepatopancreas, gill and ovarium, 408, 381, and 393 $\mu g\ g^{-1}$ respectively (Table 3.3), while highest tissue burden of copper was found in the hepatopancreas (559 μg Cu, 65 %) in experimental animals (Table 3.5 and 3.7). The hepatopancreas contained 81 % burden of copper (906 μg Cu) in control animals (Table 3.4 and 3.7). The highest tissue concentrations of copper were found in the hepatopancreas and gill (607 and 249 $\mu g\ g^{-1}$, respectively) (Table 3.4). Regression analysis showed that there was no relationship with sex or size (Table 3.9). There was also no correlation among the tissues (Table 3.8). Anova results between controls and treated animals showed that 10 $\mu g\ l^{-1}$ copper addition to sea water for 30 days only increased concentrations of copper in the carapace, gill and ovarium. Mean level of hepatopancreas copper was lower in experimental animals, but this difference was not significant (Table 3.11).

3.3.6 Uptake of Zinc From Sea water

A total of 26 *Nephrops* (13 males and 14 females) was used in an experiment in which they were exposed to 100 $\mu g\ l^{-1}$ of zinc in sea water for a 30-day period. This concentration was found to be sublethal in the experimental conditions, though 2000 $\mu g\ l^{-1}$ zinc was toxic to 73 % of animals in 30 days (Figure 3.1). Highest concentrations of zinc were found in the hepatopancreas and gill tissue, 268 and 264 $\mu g\ g^{-1}$ respectively (Table 3.3), whereas highest tissue burdens of zinc were in the

hepatopancreas and tail muscle 55 % (427 μg Zn) and 31 % (242 μg Zn) respectively (Tables 3.5 and 3.7). Control animals showed the highest concentrations of zinc in the hepatopancreas (227 μg g^{-1} and gill (135 μg g^{-1}) (Table 3.4). However, the highest tissue burdens of zinc were in the hepatopancreas (50.5 %, 367 μg Zn) and tail muscle (40.9 %, 297 μg Zn) (Tables 3.4 and 3.7). There was no relationship with size and no differences were found between sexes (Table 3.9). Zinc in the hepatopancreas showed positive correlations with zinc in the carapace and tail muscle (Table 3.8). Anova test results showed that 100 μg l^{-1} zinc addition to sea water only increased levels of zinc in the carapace, hepatopancreas and gill (Table 3.11).

3.3.7 Uptake of Lead From Sea water

A total of 21 *Nephrops* (10 males and 11 females) was used in an experiment in which they were exposed to 100 μg l^{-1} lead in sea water for a 30-day period. This concentration was found to be sublethal in the experimental conditions, though 1000 μg l^{-1} lead was toxic to 42 % of animals in 30 days (Figure 3.1). Lead was accumulated by the tissues of *Nephrops* in the sublethal experiment. Highest concentrations of lead were found in the gill and external eggs 296 and 146 μg g^{-1} respectively (Table 3.3), while highest tissue burdens of lead were found in the carapace (42 %, 87.0 μg Pb) and hepatopancreas (25 %, 51.9 μg Pb) (Tables 3.5 and 3.7). Mean lead concentrations in the hepatopancreas, carapace, gill, ovary and tail muscle increased 19.8, 17.4, 17.1, 5.40 and 4.37 times over controls respectively. Control animals showed highest concentration of lead in the gill tissue 2.84 μg g^{-1}. Carapace had the highest tissue burden of lead (42.5 %, 3.83 μg Pb) (Tables 36 and 3.7). No significant sex or size related differences were found in concentrations of lead in the tissues (Table 3.9). Lead levels in the gill tissue and in the carapace showed a positive correlation (Table 3.8).

Table 3.3. Mean concentrations (μg g^{-1} d.w.) and standard deviations of metals (meHg, Hg, Cd, Cu, Zn and Pb) in the tissues of *Nephrops norvegicus* which lived in sublethal concentrations of the metals for 30 days.

Metal	CL	Carapace	Hepato.	Gill	Tail mus.	ovary	ex.eggs
No	30	30	30	30	30	6	6
meHg	4.9	5.59	11.72	181.2	9.32	8.25	20.83
sd	0.7	2.18	6.32	67.0	4.75	4.70	12.79
No	27	27	27	21	27	5	7
Hg	5.0	4.16	7.25	232.3	1.66	5.66	7.72
sd	0.7	1.31	4.43	67.5	0.54	4.94	1.16
No	27	27	27	27	27	10	3
Cd	4.4	11.51	224.3	161.0	3.08	13.64	12.08
sd	0.5	6.45	117.0	59.1	1.57	6.74	1.41
No	21	20	20	21	21	8	7
Cu	4.8	75.66	408.6	381.3	30.97	393.7	110.2
sd	0.6	41.35	360.4	93.4	7.19	154.4	33.9
No	26	26	26	26	26	11	10
Zn	4.9	44.15	267.8	263.8	53.27	63.46	179.2
sd	0.4	11.37	104.3	56.5	15.28	14.95	34.2
No	21	21	21	21	21	11	11
Pb	4.8	71.41	47.13	296.0	7.44	17.42	145.8
sd	0.5	42.05	55.39	238.6	4.14	10.98	96.2

Table 3.4. Mean concentrations (μg g^{-1} d.w.) of metals in control *Nephrops norvegicus*. Mean carapace length of samples was 4.9±0.8 cm (27 females and 14 males). Lead measurements are from 11 *Nephrops* (6 females and 5 males) CL = 4.9±0.7 cm.

Tissue	Carapace	Hepatop.	Gill	Tail mus.	Ovary	ex.egg
No	39	41	32	41	15	6
Hg	0.09	0.30	0.77	0.62	0.07	0.13
sd	0.05	0.17	0.34	0.29	0.04	0.12
Cd	1.60	10.34	13.11	1.74	2.46	2.73
sd	0.86	7.38	5.10	0.71	0.79	1.64
Cu	46.61	607.2	249.1	25.95	114.9	92.51
sd	21.88	257.3	101.8	9.03	25.7	20.91
Zn	25.95	227.2	135.3	61.55	100.9	120.2
sd	12.36	70.2	33.3	8.96	17.5	16.8
Pb	4.10	2.38	17.33	1.70	3.22	-
sd	3.63	0.99	8.90	2.07	1.26	-

Table 3.5. Total tissue burdens of the metals in experimental animals treated with sublethal concentrations of metals and standard deviations (). Amount of the metals in the tissues are given as µg metal in whole amount of wet tissues. Mean wet weight (W.W.) of the tissues are given as gram with their standard deviations ().

Metal	CL	Carapace	Hepatopancreas	Gill	Tail muscle
meHg	4.9 (0.7)	7.57 (3.05)	17.3 (11.0)	30.8 (12.6)	38.9 (18.9)
WW		3.10 (0.82)	4.4 (1.33)	1.60 (0.52)	20.7 (6.62)
Hg	5.0 (0.7)	5.65 (2.11)	11.4 (7.29)	42.2 (11.0)	7.65 (3.27)
WW		3.11 (0.81)	4.5 (1.42)	1.82 (0.62)	21.8 (7.02)
Cd	4.5 (0.5)	12.8 (4.90)	289.5 (139)	24.5 (11.1)	11.2 (4.96)
WW		2.61 (0.52)	3.81 (0.91)	1.43 (0.42)	16.9 (4.21)
Pb	4.8 (0.5)	87.0 (58.9)	51.9 (45.0)	41.0 (34.9)	24.9 (12.1)
WW		2.71 (0.82)	3.51 (1.12)	1.31 (0.52)	17.8 (5.81)
Cu	4.8 (0.6)	98.7 (51.0)	559.2 (475)	64.2 (22.4)	131.9 (42.7)
WW		2.81 (0.63)	4.41 (1.09)	1.61 (0.50)	20.3 (5.09)
Zn	4.9 (0.4)	59.5 (22.6)	427.6 (205)	47.8 (14.6)	241.9 (81.2)
WW		2.92 (0.59)	4.61 (0.79)	1.72 (0.42)	21.5 (3.52)

Table 3.6. Total tissue burdens of the metals in control animals and standard deviations (). Amount of the metals in the tissues are given as µg metal in whole amount of wet tissues. Mean wet weight (WW) of the tissues are also given as gram with their standard deviations (). Mean carapace length = 4.9±0.8 in 20 animals. Lead burdens were calculated in 11 animals (CL=4.9±0.7).

Metal	Carapace	Hepatopancreas	Gill	Tail muscle
WW	2.82 (0.91)	4.21 (1.33)	1.71 (0.62)	20.4 (6.32)
Hg	0.12 (0.07)	0.50 (0.25)	0.25 (0.25)	3.58 (2.50)
Cd	2.02 (1.06)	17.3 (18.3)	2.40 (1.65)	9.34 (3.30)
Cu	55.6 (34.4)	905.9 (443)	42.2 (24.5)	109.7 (54.3)
Zn	39.3 (22.5)	367.3 (146)	23.2 (9.72)	297.3 (110)
WW	2.51 (0.81)	3.41 (1.72)	1.74 (0.69)	18.9 (5.43)
Pb	3.83 (2.10)	3.22 (2.06)	2.84 (1.82)	3.07 (0.96)

Table 3.7. Metal burdens in tissues as a percentage of the total body burden (excluding ovary and external egg of females).

Treatment	Carapace %	Hepatopancreas %	Gill %	Tail muscle %
Exp.meHg	8.0	18.3	32.5	41.1
Exp.Hg	8.4	17.0	63.1	11.4
Control Hg	2.7	11.2	5.6	80.4
Exp.Cd	3.8	85.6	7.2	3.3
Control Cd	6.5	55.6	7.7	30.1
Exp.Pb	42.5	25.3	20.0	12.1
Control Pb	29.5	24.8	21.9	23.7
Exp.Cu	11.5	65.5	7.5	15.4
Control Cu	5.0	81.3	3.8	9.8
Exp.Zn	7.6	55.0	6.1	31.1
Control Zn	5.4	50.5	3.2	40.9

Table 3.8. Rank Correlation between metal levels in different tissues of *Nephrops* exposed to metals in sea water.

* = P values of 0.01
**= P values of 0.001
ns= not significant (P>0.05)

Experiment	Tissue	Hepato.	Gill	Tail muscle
10 $\mu g\ l^{-1}$ meHg	Carapace	ns	ns	ns
	Hepato.		ns	*
	Gill			ns
10 $\mu g\ l^{-1}$ Hg	Carapace	ns	ns	ns
	Hepato.		ns	ns
	Gill			ns
100 $\mu g\ l^{-1}$ Cd	Carapace	ns	**	ns
	Hepato.		**	ns
	Gill			ns
100 $\mu g\ l^{-1}$ Pb	Carapace	ns	**	ns
	Hepato.		ns	ns
	Gill			ns
10 $\mu g\ l^{-1}$ Cu	Carapace	ns	ns	ns
	Hepato.		ns	ns
	Gill			ns
100 $\mu g\ l^{-1}$ Zn	Carapace	**	ns	ns
	Hepato.		ns	*
	Gill			ns

Table 3.9. The following table shows the results of linear regression analyses. The best suitable model for the tissues on the metal accumulation and tissue concentrations of metals in each treatment are described in relation to effects of sex and size. CL = Carapace length (cm). Sex.CL = Interaction between sex and carapace length. ns = not significant ($P > 0.05$).

Experiment	Tissue	Sex	CL	Sex.CL	Model
10 $\mu g\ l^{-1}$ meHg	Carapace	ns	ns	ns	1
	Hepato.	0.016	ns	ns	2
	Gill	ns	ns	ns	1
	Tail	ns	ns	ns	1
10 $\mu g\ l^{-1}$ Hg	Carapace	ns	ns	ns	1
	Hepato.	0.022	ns	ns	2
	Gill	ns	0.001	ns	3
	Tail	ns	ns	ns	1
100 $\mu g\ l^{-1}$ Cd	Carapace	-	-	0.005	5
	Hepato.	ns	ns	ns	1
	Gill	ns	0.009	ns	3
	Tail	ns	ns	ns	1
100 $\mu g\ l^{-1}$ Pb	Carapace	ns	ns	ns	1
	Hepato.	ns	ns	ns	1
	Gill	ns	ns	ns	1
	Tail	ns	ns	ns	1
10 $\mu g\ l^{-1}$ Cu	Carapace	ns	ns	ns	1
	Hepato.	ns	ns	ns	1
	Gill	ns	ns	ns	1
	Tail	ns	ns	ns	1
100 $\mu g\ l^{-1}$ Zn	Carapace	ns	ns	ns	1
	Hepato.	ns	ns	ns	1
	Gill	ns	ns	ns	1
	Tail	ns	ns	ns	1

Table 3.10. Comparisons of organic and inorganic mercury experiment in the sublethal concentrations with of one way Anova. P values are given in following table. Mercury concentrations of the tissues are given in Table 3.3. ns = not significant (P>0.05).

Tissues	P value
Carapace length	ns
Carapace	0.005
Hepatopancreas	0.0001
Gill	0.008
Tail muscle	0.0001
Ovary	ns
Eggs	0.020

Table 3.11. Comparisons of control and experimental samples of copper and zinc with the one way Anova. The following table shows the tissues which had significant increases in concentrations of copper and zinc. Concentrations of copper and zinc in the treated animals and controls are given in Tables of 3.3 and 3.4 respectively.

Tissues	Copper experiment P value	Zinc experiment P value
Carapace length	ns	ns
Carapace	0.001	0.0001
Hepatopancreas	ns	0.018
Gill	0.001	0.0001
Tail muscle	ns	ns
Ovary	0.0001	ns
Eggs	ns	0.002

3.4 DISCUSSION

Both the mercury compounds accumulated in the tissues of *Nephrops norvegicus* after exposure to 10 μg Hg l^{-1} and tissue concentrations increased. There were differences in tissue concentrations of mercury in treated animals from both the mercury experiments. Organic mercury accumulated in higher levels than inorganic mercury in the tissues except for the gills. Mercury concentrations increased many fold after treatments. The gill tissue mercury concentrations increased 301 and 235 times over control animals in inorganic and organic mercury treatments respectively. Tail muscle mercury concentrations also increased 15 times in organic mercury treatment ,though this increase was low in inorganic mercury treatment. Organic and inorganic mercury also showed different toxicity namely organic mercury was more toxic to animals than inorganic mercury. Differences found in this study between organic and inorganic mercury were similar to those with some other studies in Crustacea (Ray and Tripp, 1976 ; Fowler et al., 1978 ; Riisgard and Famme, 1986 ; Kraus et al., 1988). Different toxicity levels of organic and inorganic mercury and different tissue concentrations from both the experiments could be due at least in part to the differences in accumulation rates. Eisler and Hennekey (1977) found that 125 μg l^{-1} $HgCl_2$ killed 100 % of crab *Pagurus longicarpus* in 7 days while 10 μg l^{-1} mercury was LC0, which is similar to the situation for *Nephrops norvegicus*. Larvae of American lobster, *Homarus americanus* seemed to be very sensitive to inorganic mercury since 97 % of the larvae died in 100 μg l^{-1} copper within 24 hours (Johnson and Gentile, 1979). Tail muscle mercury burdens of *Nephrops* in the organic mercury experiment were found to be 4 times higher than in the inorganic mercury experiment. Guarino et al. (1976) indicated that a major portion of tail muscle uptake of organic mercury from water occurs through the shell and/or chitin in the tail region. They supported the present results by showing the highest

percentage of mercury was in the tail muscle (50 %), followed by hepatopancreas (23 %) after exposing the lobster *Homarus americanus* to 100 $\mu g\ l^{-1}$ organic mercury for 6 days. Gill tissue tends to have the highest concentrations of mercury from all dissolved mercury experiments (Thurberg et al., 1977 ; Del Ramo et al., 1988 ; Brown et al., 1988). There were differences between male and female *Nephrops* from both mercury experiments, namely males accumulated higher mercury than females. My observations showed that male animals or smaller animals were more active than female animals or larger animals. Differences in mercury concentrations of the hepatopancreas between sexes could be due to activity of the animals. The negative relationship between size and mercury concentration of the gill tissue from the inorganic mercury experiment could also be due to higher activity of smaller animals.

Nephrops accumulated cadmium, and the highest concentrations of cadmium were found in the hepatopancreas and gill tissue. 85 % of cadmium was distributed to the hepatopancreas, while gill, carapace and tail muscle shared the remaining percent of the metal. These findings were similar to some earlier studies on crustaceans (Nimmo et al., 1977 ; Dethlefsen, 1979 ; Jennings and Rainbow, 1979 ; Wright and Brewer, 1979 ; Ray et al., 1981). Although cadmium increased many fold in the tissues of treated animals, this increase was not as high as mercury accumulation in most tissues. This could be due to high concentrations of cadmium in control animals than mercury concentrations of controls. The sex of the animals did not affect the tissue concentrations of cadmium, while size affected concentrations in the gill tissue. As with inorganic mercury, small animals had higher concentrations. Ahsanullah et al., (1981) indicated that small shrimp *Callianassa australiensis* accumulated higher concentrations of cadmium than medium or large-sized shrimps at all test concentrations. Toxicity of cadmium varied among different species of

crustaceans (Nimmo et al., 1977 ; Eisler and Hennekey, 1977). Pesch and Stewart (1980) found that 1000 μg l^{-1} Cd killed 50 % of *Palaemonetes pugio* and *Pagurus longicarpus* in 21 and 23 days, respectively, while 120 μg l^{-1} Cd was not a lethal dose in a 30-day period, which is similar to the results for *Nephrops*.

Nephrops norvegicus seems to be very sensitive to copper, like *Homarus americanus*. McLeese (1974) found similar sensitivity for the American lobster killing 50 % of the animals in less than one week in 100 μg l^{-1} copper concentration. But some other crustaceans seem to be more resistant to copper (White and Rainbow, 1982 ; Saliba and Krzyz 1976 ; Rainbow, 1985 ; Zia and Alikhan, 1989). 10 μg l^{-1} copper failed to increase the concentrations in the hepatopancreas and the external eggs, while the other tissues showed significant increases in their copper concentrations. Zia and Alikhan (1989) found that the gill and exoskeleton of *Cambarus bartoni* showed increasing copper concentrations with increasing levels in water, though the hepatopancreas, gut and muscle did not show the same trend. They indicated that digestive gut and abdominal muscle are not considered to be specific physiological sites for the storage of copper. Rainbow (1985) could not find any increase in copper concentrations of the tissues or whole body from the crab, *Carcinus maenas* until exposed to more than 100 μg l^{-1}. White and Rainbow (1982) indicated that body concentrations of copper were regulated until 100 μg l^{-1} in 21 days by *Palaemon elegans*, but after this concentration copper was accumulated and tissue concentrations were increased. Rainbow and White (1989) also indicated that 100 μg l^{-1} copper could not increase the concentrations of copper in shrimp, *Palaemon elegans*. It is interesting to note that the concentration which would kill 100 % of *Nephrops* in two weeks does not affect the survival or increase the copper concentrations of this animal. No sex or size related difference was found in relation to tissue concentrations of copper from this study. Hilmy et al.

(1988) also could not find any sex difference in tissue concentrations of copper in the crab *Portunus pelagicus*.

As happened for *Nephrops*, toxicity of zinc occurs at much higher concentrations than copper (Eisler and Hennekey, 1977 ; Ahsanullah et al., 1981 ; Uma Devi, 1987 ; Uma Devi and Prabhakara Rao, 1989). Exposure to high concentration of zinc in sea water increased the concentrations of zinc in the carapace, hepatopancreas and gill, while levels in the tail muscle and external eggs were not increased significantly. Some other studies on Crustacea also support the present result by indicating that sublethal zinc concentrations increased tissue concentration at least in the gill and hepatopancreas (Bryan, 1964 ; Bryan and Hummerstone,1986 ; Waidwood et al., 1987 ; Uma Devi and Prabhakara Rao, 1989). However, White and Rainbow (1982) in *Palaemon elegans*, Rainbow (1985) in *Carcinus maenas* and Nugegoda and Rainbow (1989) in *Palaemon elegans* could not find any increase in zinc concentrations of tissues or whole body. Present results indicated that there were no size and sex dependent differences in zinc concentrations of the tissues. Hilmy et al. (1988) also indicated that there was not any sex related difference in relation to zinc accumulation in crab, *Portunus pelagicus*. Nugegoda and Rainbow (1989) also indicated that sex or size of individual does not affect the rate of zinc uptake of *Palaemon elegans*.

Lead accumulated in the tissues of *Nephrops norvegicus* after treatment with 100 μg Pb l^{-1} and highest concentrations were found in the hepatopancreas and external eggs. Although lead concentrations of treated animals increased over controls, this increase was not as high as mercury in the tissues. Like cadmium, lead levels in controls were higher than levels of mercury. Therefore, lower accumulation rate of lead could also be related to high levels of lead in controls. Lead concentrations did

not show any relationship with size and no difference was found between sexes. Amiard et al. (1987) indicated that lead was accumulated by 5 species of crustacean in proportion to the metal concentration in sea water. Lead was the only metal that carapace had large part of tissue burdens. But this could be adsorption of the metal onto carapace (Table 3.2). As happened in present study, Pastor et al. (1988) also found that lead concentrations were in highest in the gill and antennal gland, followed by hepatopancreas and muscle in *Procambarus clarkii.*

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CHAPTER 4

ACCUMULATION OF HEAVY METALS FROM A FOOD SOURCE AND COMPARATIVE ROUTES OF MERCURY AND CADMIUM ACCUMULATION AND TISSUE DISTRIBUTION FROM FOOD SOURCE AND SEA WATER BY THE NORWAY LOBSTER, *NEPHROPS NORVEGICUS*

4.1. INTRODUCTION

The major uptake and accumulation routes of metals by marine animals have been generally recognised to be uptake from sea water and from food, though uptake from sediment can also be an important route, especially for deposit-feeding animals (Bryan and Langston, 1992). Crustaceans require trace metals, such as copper, zinc and iron for their metabolism and accumulate them by both routes. Although some heavy metals, such as mercury and cadmium are known to be non-essential, they are also accumulated by marine animals from both routes.

It is well known that heavy metals accumulate in tissues of different marine animals, from small invertebrates to large vertebrates. Alliot and Frenet-Priron (1990) indicated that heavy metal concentrations in sea water during different periods of the year show relationships with concentrations in shrimps. Once metals are accumulated in one member of the marine food-chain, they can be transferred through trophic levels. Therefore, metal accumulation may be higher in later levels of the food-chain than in earlier levels. Berk and Colwell (1981) showed that mercury can be transferred by the microbial food-chain. Sarkka et al. (1978) also indicated that mercury concentrations increase through upper levels of the food-chain. Riisgard and Famme (1986) in a shrimp and Pentreath (1976) in a fish showed that mercury was accumulated from food in relation to exposure regime. Cadmium is also transferred in the food-chain (Nimmo et al., 1977), and the dominant route of cadmium accumulation in crustaceans can be uptake from food sources as long as the previous trophic level has the ability to accumulate cadmium to such extent as to make it more available to the consumer than by direct uptake from sea water (Jennings and Rainbow, 1979 ; Davies et al., 1981).

Heavy metal concentrations in the tissues of marine animals are normally more concentrated than those of surrounding sea water. Therefore, heavy metal transfer through food-chain can be very important if heavy metals occur in high levels. The most dramatic incidents of heavy metal pollution have occurred by consumption of contaminated foods by man. One of the most famous examples of this situation is the Minamata disaster which caused many human deaths and severe permanent disabilities, as well as ruining marine life in the environment (Clark, 1989). The reason in the Minamata disaster was mercury contaminated sea food consumed by local people. Another example of heavy metal poisoning in food is the itia itia disease caused by cadmium. This disease also caused human fatalities and disabilities (Clark, 1989). In the Minamata disaster, mercury concentrations in some of the marine food-chain were 5 $\mu g\ g^{-1}$ in plankton, 10-39 $\mu g\ g^{-1}$ (d.w.) in bivalves and 10-55 $\mu g\ g^{-1}$ (d.w.) in fish, mostly methylated (Clark, 1989). Clark (1989) also indicated that the bivalve, *Pecten novaezetlandiae* can accumulate cadmium up to 2000 $\mu g\ g^{-1}$ (d.w.) and similarly 1900 $\mu g\ g^{-1}$ (d.w.) was found in the oceanic squid, *Symplectoteuthis oualaniensis*. Limpets, *Patella vulgata* and dog whelks, *Nucella lapillus* also acquire high concentrations of cadmium. Crabs, *Cancer irroratus* and lobster, *Homarus americanus* contained very high levels of cadmium in their tissues from Belledune Harbour (Canada) which was contaminated by cadmium (Ray et al., 1980 ; Uthe et al., 1980). Levels of cadmium were so high that fishing was banned in certain areas of the Harbour (Riley, 1980).

The most important factor affecting metal bioavailability depends first on the free ion concentrations of metals in sea water (Sunda et al., 1978 ; Luoma, 1983). Decapod crustaceans are known to be able to regulate concentrations of essential metals up to a threshold concentration when they are exposed to metals dissolved in sea water. Net accumulation of these metals begins after these threshold levels are exceeded

(Bryan, 1967 ; White and Rainbow, 1982 ; Rainbow, 1985 ; Rainbow and White, 1989). However, there is no evidence that non-essential metals can be regulated by Crustaceans. Non-essential metals are accumulated in proportion to environmental concentration without any regulation which means tissue concentrations increase with increases of exposure time and concentration in sea water (Nimmo et al., 1977 ; Jennings and Rainbow, 1979 ; Meadows and Erdem, 1982; Devineau and Amiard-Triquet, 1985 ; Riisgard and Famme, 1986 ; Krishnaja et al., 1987). Mercury and cadmium have no known role in biological systems. In addition to being present at low natural background concentrations, they are released by anthropogenic activities such as from chlor-alkali plants, the use of fungicides, pesticides, antifouling preparations, mining and smelting facilities (Campbell et al., 1986 ; Mance, 1987 ; Langston, 1990). They can be accumulated in tissues of marine animals in very high levels and transferred to higher trophic levels. Dallinger and Kautzky (1985) indicated that since the metal concentrations of sea water are much lower than those in marine animals, the absorption through the gills may be of secondary importance compared with the supply through the food.

The high concentrations of metals in the food of most marine animals, relative to water, intuitively suggests that food should be an important vector of metal uptake, especially those metals (e.g. mercury) which accumulates over lifetime and show positive relationships with size or age of marine animals. However, the importance of metal uptake from food has proven to be less effective than uptake from water (Luoma, 1983). Three general approaches have been employed : 1. Experimental separation of the food and water vectors and comparison of their importance; 2. Use of mass balance models in combination with experimental studies of uptake; 3. Comparisons of tissue distributions observed in laboratory studies with observations of animals in nature (Luoma, 1983).

Nephrops norvegicus is a very important commercial species in U.K. and most other European Countries (Howard, 1989). The Clyde Sea receives anthropogenic inputs of pollutants including heavy metals (Mackay, 1972 ; Steel et al., 1973 ; Mackay, 1986 ; Clark and Davies, 1989). Although crustaceans take up heavy metals directly from sea water and food, it is useful to describe which is the more important route for concentration of a particular metal in a tissue. There has been no previous study of accumulation of heavy metals from both routes by the Norway lobster, *Nephrops norvegicus*. The present study deals with the accumulation and distribution of heavy metals (Hg, Cd, Cu, Zn and Fe) from a food source in tissues of *Nephrops norvegicus*, and compares tissue distributions of mercury and cadmium among the gill, hepatopancreas and tail muscle after exposing the animals to the metals dissolved in sea water and feeding with a food source which contains high concentrations of mercury and cadmium. Results are also compared with metal distribution among these tissues in animals from the Clyde Sea Area.

4.2 MATERIALS AND METHODS

Capture and maintenance of the Norway lobsters, *Nephrops norvegicus* were the same as explained in Chapter 2 and 3.

All feeding experiments were conducted in fibre glass tanks with circulating sea water held in a room where sea water temperature was at $10\pm0.5^{o}C$. The experimental room was illuminated with six fluorescent lamps for 12L : 12D lighting regime. Animals were allowed to acclimate for at least one week in experimental conditions before experiments. Healthy intermoult animals were chosen and placed individually into separate experimental tanks. Because the amount of food consumed per week by *Nephrops* in captivity was small, it was necessary to feed animals on

food containing high concentrations of metals in order to cause a measurable increase in tissue concentrations of metals in the experimental animals. Initially, it had been intended to feed animals with mussels, *Mytilus edilus* contaminated with metals after living in contaminated sea water. However, different tissues of mussels accumulated different concentrations of metals and after exposing mussels to metals in sea water there were also big differences in metal concentrations of the same tissue from different animals. This would create difficulty in calculation of metal assimilation by each animal. *Nephrops norvegicus* are scavenging animals that will feed on a wide range of foods and a number of possible foods were considered. Fish meat was ruled out as metal concentrations were too low. Seabirds may accumulate very high concentrations of metals, especially mercury and cadmium in their liver and kidney. From material available to me I chose liver tissue from a wandering albatross *Diomedea exulans* as a convenient food with high concentrations of metals. I used one albatross' liver and different parts of liver showed the same concentrations of metals, so it gave confidence that metals were homogeneously distributed in the liver. Therefore, each food given to animals contained the same levels of metals. One other advantage of albatross' liver is the naturally very high concentrations of cadmium and especially of mercury. The albatross is a top predator in the marine food-chain. It obtains its food entirely at sea, feeding predominantly on squid and fish.

Nephrops were fed twice a week with a sample of wandering albatross' *Diomedea exulans* liver which contained metals in the following concentrations:

Total mercury = 163.0 $\mu g\ g^{-1}$ wet weight

Organic mercury = 3.72 $\mu g\ g^{-1}$ wet weight

Cadmium = 13.2 $\mu g\ g^{-1}$ wet weight

Copper = 77.6 $\mu g\ g^{-1}$ wet weight

Zinc = 47.8 $\mu g\ g^{-1}$ wet weight

Iron = 365.9 $\mu g\ g^{-1}$ wet weight

Before experiments, the liver was cut into small pieces and frozen in small plastic bags. When feeding time came, one of the plastic bags was taken out from the freezer and thawed. The liver pieces were weighed to the nearest 1 mg using a Precisa 300MC (Metagram Instrument Ltd., Aspley Guise, Buckinghamshire) top-pan balance. Thus each animal was given a known amount of food between 250 and 800 mg. In first feeding days, less food (about 300 mg) was given to the animals to let them become accustomed to the liver. Then the amount was gradually increased (up to 700 mg) if an animal ate the liver. In this way, most of the animals consumed the food served within 1-2 hours, though a few times food was not completely finished within 8 hours. Any food remaining after 8 hours was taken out and put into an oven which was set to 60 ^{o}C. Drying was conducted for at least six days to ensure that the liver remains achieved a constant dry weight. By this way, wet weight of remaining food and eaten food could be calculated by transforming the dry weight to wet weight as it was not possible to obtain a reliable wet weight of the liver after 8 hours in sea water. Concentrations of metals in the liver up to 24 hours in sea water did not vary from the initial concentrations. Therefore, leaching of metals from the liver during a few hours feeding time can be ignored. The sea water in the experimental tanks was oxygenated by air stones and water circulation. During feeding, the outflows of the tanks were closed by fine-mesh nets to prevent any possible loss of food. After feeding the nets were removed. Animals were taken out two days after the last feed to give time for digestion of the last meal, since within one day of last feeding pieces of the liver could still be found in the stomach of animals.

A total of 26 *Nephrops norvegicus* were fed with albatross' liver for up to 50 days (between 22 and 50 days). 27, 24 and 24 *Nephrops* were exposed to sublethal concentrations of methyl mercury (10 $\mu g\ l^{-1}$), mercuric chloride (10 $\mu g\ l^{-1}$) and cadmium (100 $\mu g\ l^{-1}$) respectively for 30 days. Two control *Nephrops* were also put in two tanks while experiments were running and fed mackerel muscle only. A further 27 *Nephrops* were caught from the same area in the Clyde Sea when the experiments were running to be used as a second control group.

All metal uptake experiments from sea water were conducted as explained in Chapter 3. During experiments, control animals were fed with fish (mackerel, *Scomber scombrus*) muscle once a week. Metal levels in the macherel muscle were Cd < 0.01 $\mu g\ g^{-1}$, Hg 0.1 $\mu g\ g^{-1}$ (95 % organic), wet weight.

Dissection, digestion and metal analysis were carried out as explained in Chapter 2.

4.3 STATISTICAL ANALYSIS OF DATA

The amounts of food given to the animals were different since food was served to individuals depending on their appetite and feeding period. Therefore, total weight of liver consumed by each animal was different, namely animals with great appetite and longer feeding period consumed higher amount of liver than animals with little appetite and shorter exposure period. Data from the feeding experiment were investigated for increases in metal concentrations of the tissues such as carapace, hepatopancreas, gill and tail muscle in controls and in animals fed with the albatross' liver. Before any statistical analysis, data were plotted on graphs to examine distribution for normality using Minitab 8.2 statistical package programs. Data not normally distributed were transformed by $\log_{10}$ or square root. Concentrations of

metals in the tissues of controls (Table 4.1) and fed animals (Table 4.2) were compared with one way Anova. Results of this comparison are given in Table 4.2. Metal concentrations in the tissues of the animals were also investigated in relation to feeding rate (food g/day) by linear regression analysis. Only mercury and cadmium concentrations showed increases in at least some tissues, while copper, zinc and iron levels did not increase significantly with feeding rate, so further studies were carried out with mercury and cadmium only. Comparisons of mercury and cadmium accumulation among controls, fed animals and animals exposed to sublethal concentrations of organic mercury, inorganic mercury and cadmium were investigated. Comparisons were done using triangular diagrams among the hepatopancreas, gill and tail muscle. Total tissue burdens of mercury and cadmium were calculated in whole wet tissues and their relative total percentages among three tissues, hepatopancreas, gill and tail muscle. Total percentages of these tissue burdens of mercury and cadmium from the three treatments were plotted onto triangular diagrams. Therefore, data in the triangular diagrams represent the percent values of the total tissue burdens of the metals. Tissue burdens were first statistically compared among all the treatments with one way Anova. Significant differences ($P<0.05$) were reanalysed with one way Anova between paired treatments. Assimilation rates of cadmium, organic and inorganic mercury were also calculated. First, total amounts of metals (as μg metal) taken by each animal were calculated by multiplying total eaten food and liver concentration of metals (A). Then, mean tissue burdens of metals in fed animals were subtracted from control burdens of metals for each tissue. Thus, net assimilated amounts (as μg metal) of metals were found (B). Finally, assimilation rates of metals were calculated by dividing B to A for each tissue.

4.4 RESULTS

4.4.1 Control Animals

Concentrations of metals in control *Nephrops norvegicus* are shown in Table 4.1. Highest concentrations of mercury were in the gill (0.777 $\mu g\ g^{-1}$) and tail muscle (0.580 $\mu g\ g^{-1}$), while highest concentrations of cadmium were in the gill (13.05 $\mu g\ g^{-1}$) and the hepatopancreas (11.76 $\mu g\ g^{-1}$). Copper was in highest concentrations in the hepatopancreas (594.0 $\mu g\ g^{-1}$) and gill (255.5 $\mu g\ g^{-1}$). Zinc was also in highest concentrations in the hepatopancreas (219.0 $\mu g\ g^{-1}$) and gill (160.1 $\mu g\ g^{-1}$). The highest iron concentration was in the gill (715.0 $\mu g\ g^{-1}$).

Table 4.1. Mean concentrations (µg g^{-1} d.w.) of metals in the tissues of control *Nephrops norvegicus*. Data are from 14 males and 13 females which CL (carapace length) are 4.9±0.8 and 5.0±0.8 cm, respectively.

Metal	Carapace	Hepatopancreas	Gill	Tail muscle
Hg	0.099	0.262	0.777	0.580
sd	0.062	0.146	0.307	0.329
Cd	1.523	11.76	13.05	0.591
sd	0.949	6.67	3.88	0.475
Cu	36.20	594.0	255.5	22.93
sd	20.41	266.1	96.0	10.40
Zn	29.50	219.0	160.1	60.49
sd	13.22	59.6	35.5	6.95
Fe	99.89	134.0	715.0	9.85
sd	95.00	84.3	734.0	10.70

Total tissue burdens of mercury and cadmium with their relative percentages are shown in Table 4.6. Highest tissue burden of mercury was in the tail muscle (2.64 µg Hg, 80.5 %), while the hepatopancreas and gill shared most of the remainder.

Highest tissue burden of cadmium was in the hepatopancreas (16.6 μg Cd, 74.1 %) while the gill (12.6 %) and tail muscle (13.3 %) shared about equally.

4.4.2 Feeding experiment

Concentrations of metals in the tissues of *Nephrops norvegicus* after feeding with the liver are given in Table 4.2. Highest concentrations of mercury were found in the hepatopancreas (4.96 μg g^{-1}) and gill (3.87 μg g^{-1}). Cadmium were also highest in the hepatopancreas (28.4 μg g^{-1}) and gill (13.55 μg g^{-1}). Copper and zinc levels showed the same trend being highest in the hepatopancreas (863 μg Cu $g^{-1}$and 227 μg Zn g^{-1}) and gill (347 μg Cu g^{-1} and 179 μg Zn g^{-1}). However, highest concentrations of iron were in the gill (619 μg g^{-1}), while the hepatopancreas and gill held similar concentrations (Table 4.2). Results of one way Anova between metal concentrations of the tissues between controls and fed animals are also given in Table 4.2 and shows that mercury concentrations increased in all tissues of the animals very significantly ($P<0.0001$) after feeding with the liver. Cadmium concentrations were also increased after feeding with the liver but only in the hepatopancreas ($P<0.0001$). Copper concentrations were also increased in the hepatopancreas ($P=0.005$), gill ($P=0.002$) and tail muscle ($P=0.01$) of fed animals but the carapace copper concentrations did not increase. Concentrations of zinc and iron did not show any significant increase after feeding in any tissues ($P>0.05$).

Linear regression analysis showed that mercury concentrations of all tissues except for carapace showed positive relationship with feeding rate (Table 4.3). Relationships between feeding rate and mercury concentrations of the tissues are shown in Figures 4.1 to 4.4. Cadmium in the hepatopancreas and carapace also showed positive relationships with feeding rate. These relationships are shown in Figures 4.5 to 4.8.

Copper, zinc and iron concentrations in all tissues did not show any increase in relation to feeding rate. These relationships are shown in Figures 4.9 to 4.20. Therefore, later investigations were focused on mercury and cadmium. Total tissue burdens of mercury were highest in the hepatopancreas (6.37 μg Hg g^{-1}, 52.8 %) and tail muscle (4.34 μg Hg g^{-1}, 41.4 %), while the bulk of cadmium burdens were in the hepatopancreas (36.8 μg Cd g^{-1} , 88.4 %) (Table 4.6). Feeding rates (gram food/day) of male and female *Nephrops* were also statistically compared. Male animals (0.15$\pm$0.041) showed higher feeding rates (P=0.04) than female animals (0.11$\pm$0.031).

Table 4.2. Mean concentrations (µg g^{-1} d.w.) of metals in the tissues of *Nephrops norvegicus* after feeding with the liver. Data are from 13 males and 13 females which CL are 4.8±0.4 and 4.9±0.5 cm, respectively. Results of one way Anova between metal concentrations of controls and fed animals are also given in the following table indicating P values. ns = P>0.05.

Metal	Carapace	Hepatopancreas	Gill	Tail muscle
Hg	0.669	4.96	3.87	1.14
sd	0.246	3.22	1.38	0.42
P	0.0001	0.0001	0.0001	0.0001
Cd	1.216	28.40	13.55	0.665
sd	0.556	13.31	4.67	0.272
P	ns	0.0001	ns	ns
Cu	45.97	863.0	346.8	30.53
sd	20.53	395.0	95.9	9.93
P	ns	0.005	0.002	0.01
Zn	35.81	227.3	179.3	57.45
sd	10.02	96.3	42.8	5.63
P	ns	ns	ns	ns
Fe	110.0	125.9	462.0	7.63
sd	85.7	62.8	570.8	4.45
P	ns	ns	ns	ns

Figure 4.1. The relationship between feeding rate and mercury concentrations in the carapace

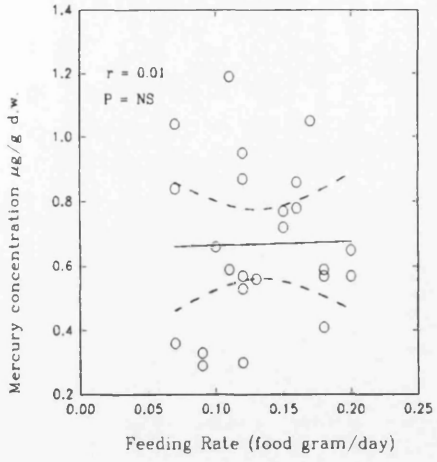

Figure 4.2. The relationship between feeding rate and mercury concentrations in the hepatopancreas

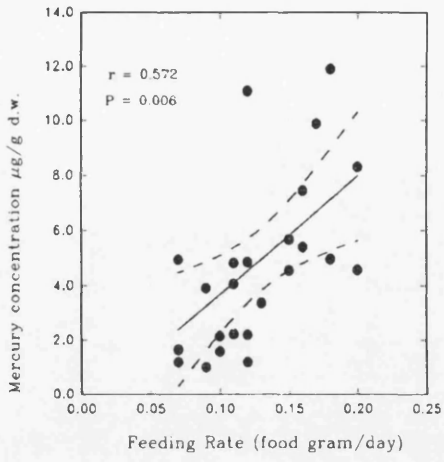

Figure 4.3. The relationship between feeding rate and mercury concentrations in the gill

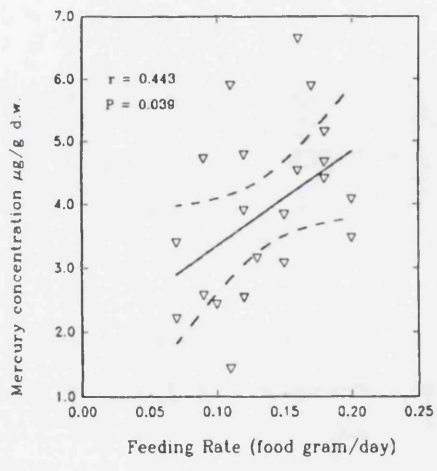

Figure 4.4. The relationship between feeding rate and mercury concentrations in the tail muscle

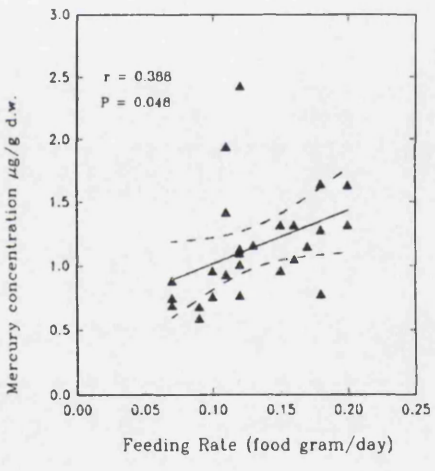

Figure 4.5. The relationship between feeding rate and cadmium concentrations in the carapace

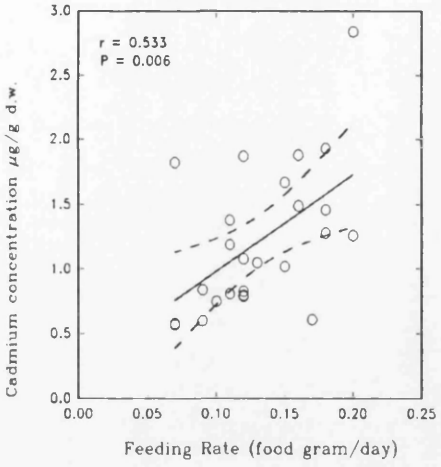

Figure 4.6. The relationship between feeding rate and cadmium concentrations in the hepatopancreas

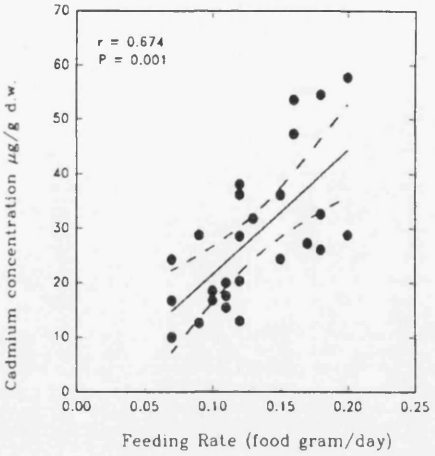

Figure 4.7. The relationship between feeding rate and cadmium concentrations in the gill

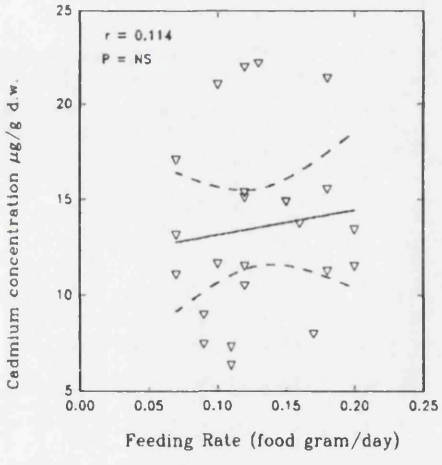

Figure 4.8. The relationship between feeding rate and cadmium concentrations in the tail muscle

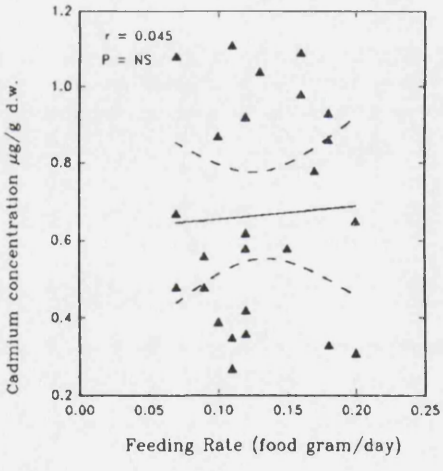

Figure 4.9. The relationship between feeding rate and copper concentrations in the carapace

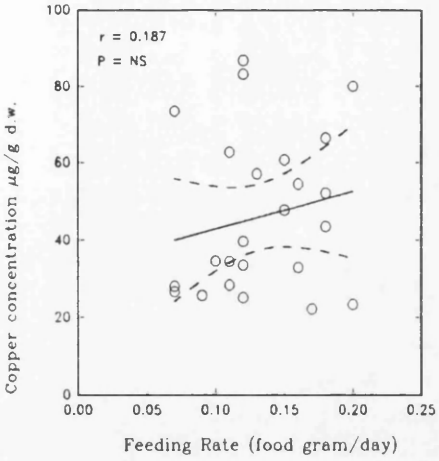

Figure 4.10. The relationship between feeding rate and copper concentrations in the hepatopancreas

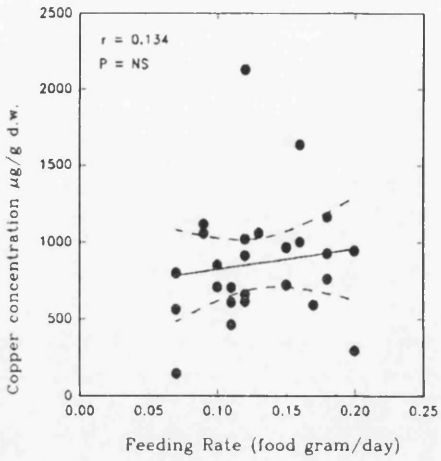

Figure 4.11. The relationship between feeding rate and copper concentrations in the gill

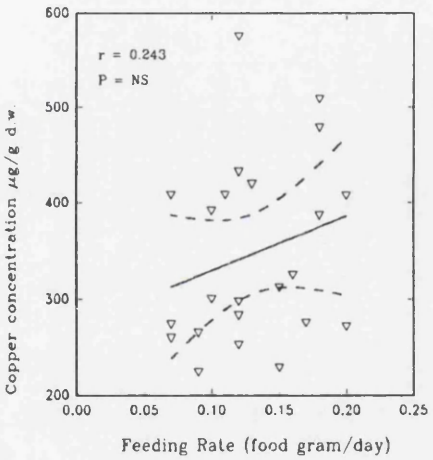

Figure 4.12. The relationship between feeding rate and copper concentrations in the tail muscle

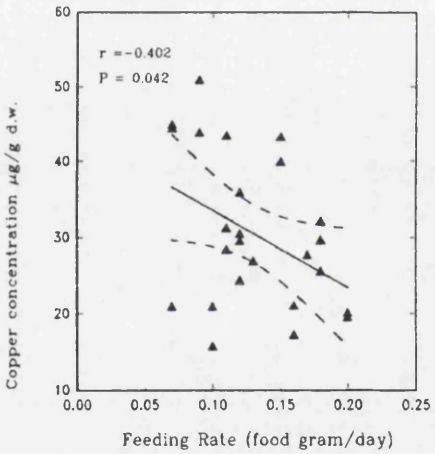

Figure 4.13. The relationship between feeding rate and zinc concentrations in the carapace

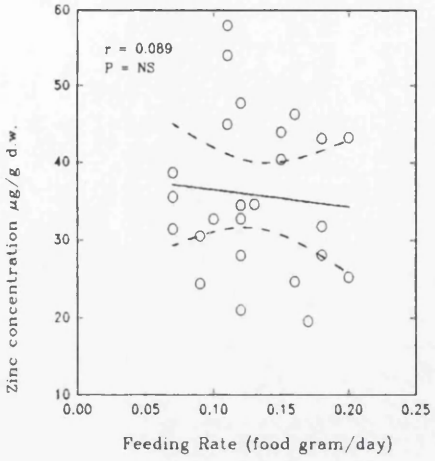

Figure 4.14. The relationship between feeding rate and zinc concentrations in the hepatopancreas

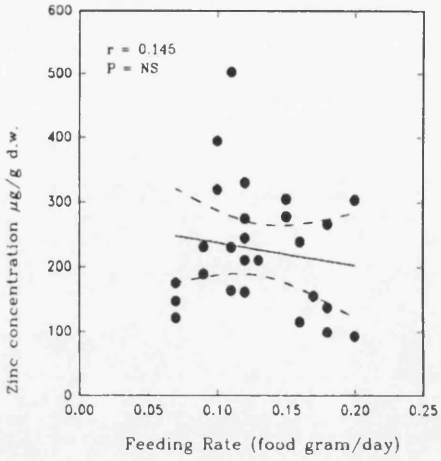

Figure 4.15. The relationship between feeding rate and zinc concentrations in the gill

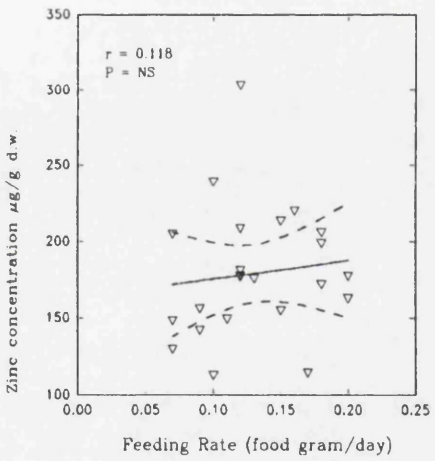

Figure 4.16. The relationship between feeding rate and zinc concentrations in the tail muscle

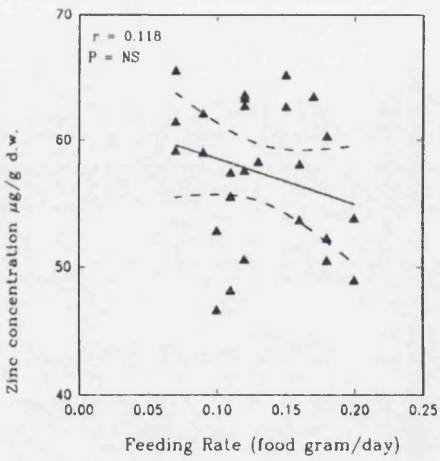

Figure 4.17. The relationship between feeding rate and iron concentrations in the caropace

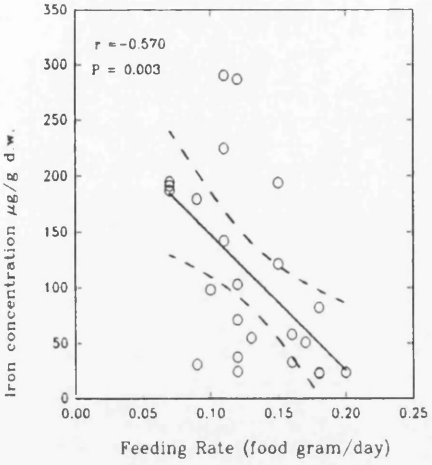

Figure 4.18. The relationship between feeding rate and iron concentrations in the hepatopancreose

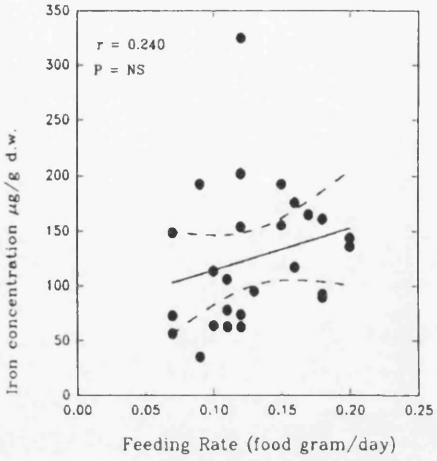

Figure 4.19. The relationship between feeding rate and iron concentrations in the gill

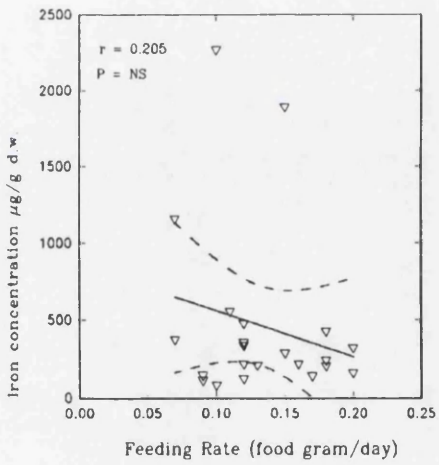

Figure 4.20. The relationship between feeding rate and iron concentrations in the tail muscle

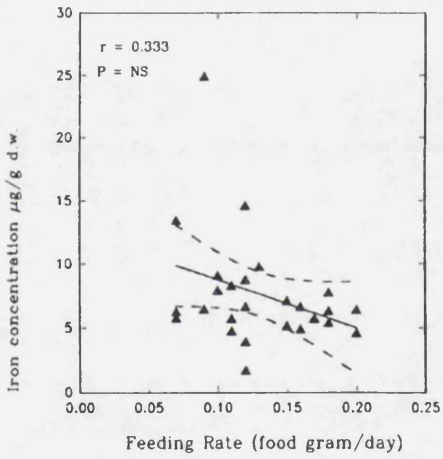

Table 4.3. Relationships between feeding rate (g wet weight liver / per day) and metal concentrations (μg g^{-1} d.w.) of the tissues after feeding with the liver. These relationships are shown in Figures of 4.1 to 4.20.

Metal	Tissue	Regression equation	DF	P
Mercury	Carapace	$y = 0.652+0.124^*$ $R^2=0.001$	22	ns
	Hepatopancreas	$y =-0.624+42.99^*$ $R^2=0.278$	24	0.006
	Gill	$y = 1.851+14.94^*$ $R^2=0.196$	20	0.039
	Tail muscle	$y = 0.599+4.164^*$ $R^2=0.151$	24	0.048
Cadmium	Carapace	$y = 0.238+7.454^*$ $R^2=0.284$	23	0.006
	Hepatopancreas	$y =-1.111+227.0^*$ $R^2=0.454$	24	0.0001
	Gill	$y = 11.87+12.98^*$ $R^2=0.013$	22	ns
	Tail muscle	$y = 0.622+0.331^*$ $R^2=0.002$	24	ns
Copper	Carapace	$y = 33.32+96.38^*$ $R^2=0.035$	23	ns
	Hepatopancreas	$y = 688.1+1349^*$ $R^2=0.018$	24	ns
	Gill	$y = 273.0+564.0^*$ $R^2=0.059$	21	ns
	Tail muscle	$y = 43.68-101.1^*$ $R^2=0.162$	24	0.042
Zinc	Carapace	$y = 38.74-22.15^*$ $R^2=0.008$	23	ns
	Hepatopancreas	$y = 272.7-350.0^*$ $R^2=0.021$	24	ns
	Gill	$y = 163.4+121.4^*$ $R^2=0.014$	21	ns
	Tail muscle	$y = 62.15+36.19^*$ $R^2=0.064$	24	ns
Iron	Carapace	$y = 271.0-1227^*$ $R^2=0.325$	23	0.003
	Hepatopancreas	$y = 76.22+382.0^*$ $R^2=0.058$	24	ns
	Gill	$y = 853.7-2941^*$ $R^2=0.042$	20	ns
	Tail muscle	$y = 12.50-37.51^*$ $R^2=0.111$	24	ns

4.4.3 Assimilation of metals from food

Assimilation of organic mercury, inorganic mercury and cadmium are given in Table 4.4. This table shows that there were differences in the percent of ingested organic and inorganic mercury deposited into the hepatopancreas and tail muscle. Of the total ingested, 4.9 % of the organic mercury was deposited into the hepatopancreas and 6.2 % into the tail muscle. However, inorganic mercury was deposited 0.61 % into the hepatopancreas and 0.05 % into the tail muscle. Very little (about 0.05 %) of

total ingested mercury was deposited into the gill tissue. Cadmium assimilation from food also varied between tissues (Table 4.4). The gill tissue and tail muscle did not receive much of the cadmium from food (0.00 % and 0.22 % respectively). However, 32.5 % of the cadmium ingested was deposited into the hepatopancreas.

Table 4.4. Assimilation of mercury and cadmium from the food.

AME = Amounts of metals in eaten food (as µg metal). ASA = Assimilated amount of metals (as µg metal) by tissues. AS = Assimilation of metals (as % of ingested) by tissues. H = Hepatopancreas, G = Gill, T = Tail muscle.
Gill assimilation for mercury is calculated as total mercury assimilation.

	Organic mercury		Inorganic mercury		Tot Hg	Cadmium		
AME	20.84		814.6		835.5	62.24		
	H	T	H	T	G	H	T	G
ASA	1.02	1.29	4.99	0.41	0.49	20.2	0.14	0.00
AS %	4.9	6.2	0.61	0.05	0.06	32.5	0.22	0.00

4.4.4 Exposure to mercury and cadmium dissolved in sea water

Concentrations of mercury and cadmium in the tissues of *Nephrops* after exposure to 100 μg Cd l^{-1} and 10 μg l^{-1} organic and inorganic mercury in sea water are given in Table 4.5. Highest concentrations of mercury from the organic and inorganic mercury treatments were in the gill (190 and 232 μg g^{-1}, respectively), while highest concentrations of cadmium were in the hepatopancreas (231 μg g^{-1}) and gill (162 μg g^{-1}). Mercury and cadmium concentrations in the tissues were increased many fold. The gill tissue had the bulk of mercury burdens in inorganic mercury treatment (41.8 μg Hg g^{-1}, 68 %), whereas in the organic mercury treatment the tail muscle (37.0 μg Hg g^{-1}, 43 %) and gill (37.1 μg Hg g^{-1}, 38 %) shared almost equally highest

mercury burdens. The hepatopancreas contained similar levels of mercury burdens from both mercury treatments. Cadmium burdens, however, were mostly in the hepatopancreas (303.9 μg Cd g^{-1}, 89 %) (Table 4.6).

Table 4.5. Mean metal concentrations (µg g^{-1} d.w.) and standard deviations of the tissues after exposing *Nephrops* to 10 µg l^{-1} organic and inorganic mercury and 100 µg l^{-1} cadmium for 30 days.

Carapace length of experimental animals are as follows : organic mercury experiment ; 13 males CL = 4.6±0.6 cm and 14 females CL = 5.0±0.7 cm, inorganic mercury experiment ; 10 males CL = 4.8±0.6 cm and 11 females CL = 5.1±0.7 cm, cadmium experiment 14 males CL = 4.6±0.7 cm and 11 females CL = 4.7±0.4 cm.

Exposure	Hepatopancreas	Gill	Tail muscle
10 µg meHg l^{-1}	11.90 ± 6.51	190.1 ± 64.5	9.12 ± 4.82
10 µg Hg l^{-1}	7.16 ± 4.48	232.3 ± 67.5	1.71 ± 0.58
100 µg Cd l^{-1}	230.7 ± 116.3	162.3 ± 60.0	2.89 ± 1.35

4.4.5 Comparisons of mercury and cadmium distributions among tissues

Comparisons of cadmium and mercury distributions among the hepatopancreas, gill and tail muscle of controls, fed animals and animals exposed to both mercury and cadmium in sea water were investigated in relation to percentages of total tissue burdens. The distributions of metals among the tissues are shown in Figures 4.21 to 4.23. Statistical comparisons of the percent distributions of metals among the tissues were also carried out and given in Table 4.7. Mercury distributions between control and fed animals were significantly different in the hepatopancreas ($P<0.0001$) and in the tail muscle ($P<0.0001$), while the gill did not show any difference between the two treatments.

Table 4.6. Total tissue burdens and standard deviations (μg metal in whole wet tissues) of mercury and cadmium in the tissues of control animals, animals fed with the liver and animals exposed to metals in sea water.

Treatment		Hepatopancreas	Gill	Tail muscle
Control	Hg	0.36 ± 0.18	0.13 ± 0.07	2.64 ± 1.80
	Hg %	14.6	4.8	80.6
	Cd	16.6 ± 12.1	2.19 ± 1.15	2.48 ± 2.27
	Cd %	74.1	12.6	13.3
Feeding	Hg	6.37 ± 3.90	0.62 ± 0.24	4.34 ± 1.47
	Hg %	52.8	5.8	41.4
	Cd	36.8 ± 17.4	2.10 ± 1.02	2.62 ± 1.17
	Cd %	87.2	5.6	7.2
Exposure	Hg	11.5 ± 6.9	41.8 ± 19.9	8.31 ± 3.29
	Hg %	17.8	68.5	13.7
	meHg	17.1 ± 10.8	31.9 ± 12.5	37.0 ± 17.3
	meHg %	19.6	37.8	42.6
	Cd	303.9 ± 131.7	24.3 ± 6.3	10.9 ± 5.0
	Cd %	88.4	8.1	3.5

As can be seen in Figure 4.21 mercury in controls was mainly distributed to tail muscle whereas in fed animals (Figure 4.22) this distribution was moved towards the hepatopancreas corner. A comparison of mercury distribution between controls and inorganic mercury exposure in sea water showed that the gill and tail muscle showed significant differences ($P<0.0001$), while the hepatopancreas did not show any difference. As can be seen in Figure 4.23 mercury from the inorganic mercury experiment was mainly in the gill. Distributions of mercury from the organic mercury treatment were significantly different from that in controls for the hepatopancreas ($P=0.036$), gill and tail muscle ($P<0.0001$). Mercury was mainly distributed between gill and tail muscle (Figure 4.23) from the organic mercury experiment, though the percentage in the hepatopancreas was also higher than the controls. Comparisons of the treatments between themselves showed also significant differences in tissue distribution of mercury. Tissue distribution of mercury between

Table 4.7. Results of oneway Anova between total tissues burdens (%) among different treatments. These results also represent significancy of data shown in the triangular diagrams. There was no differences in carapace length of groups compared in following table (P>0.05). MC = mercuric chloride exposure in sea water, MMC = methyl mercuric chloride exposure in sea water, ns = not significant (P>0.05).

Treatments	Hepatopancreas	Gill	Tail muscle
Mercury			
Control & Feeding	0.0001	ns	0.0001
Control & MC exposure	ns	0.0001	0.0001
Control & MMC exposure	0.036	0.0001	0.0001
Feeding & MC exposure	0.0001	0.0001	0.0001
Feeding & MMC exposure	0.0001	0.0001	ns
MC & MMC exposure	ns	0.0001	0.0001
Control & Feeding & MC	0.0001	0.0001	0.0001
Control & Feeding & MMC	0.0001	0.0001	0.0001
Cadmium			
Control & Feeding	0.0001	0.0001	0.006
Control & Cd exposure	0.0001	0.002	0.0001
Feeding & Cd exposure	ns	0.001	0.0001
Control & Feeding & Cd ex.	0.0001	0.0001	0.0001

the feeding experiment and the inorganic mercury experiment were very significantly different in all tissues ($P<0.0001$) (Table 4.7). This was also true for the inorganic mercury experiment ($P<0.0001$), except for tail muscle where there was no difference in mercury distribution between the feeding experiment and the organic mercury treatment. Except for the hepatopancreas mercury distribution, there were also very significant differences between both mercury experiments in the gill and tail muscle ($P<0.0001$). Although main cadmium distributions were in the hepatopancreas from the all treatments (Figure 4.21-4.23), there were differences in cadmium burdens between controls and fed animals in the tail muscle ($P=0.006$) and in the hepatopancreas and gill ($P<0.0001$) (Table 4.7). These differences were also found in cadmium distribution between controls and Cd exposure in sea water in the gill ($P=0.002$) and in the hepatopancreas and tail muscle ($P<0.0001$). Comparisons of tissue distribution between fed animals and animals exposed to cadmium in sea water showed that except for the hepatopancreas ($P>0.05$), significant differences were found in the gill ($P=0.001$) and in the tail muscle ($P<0.0001$) (Table 4.7).

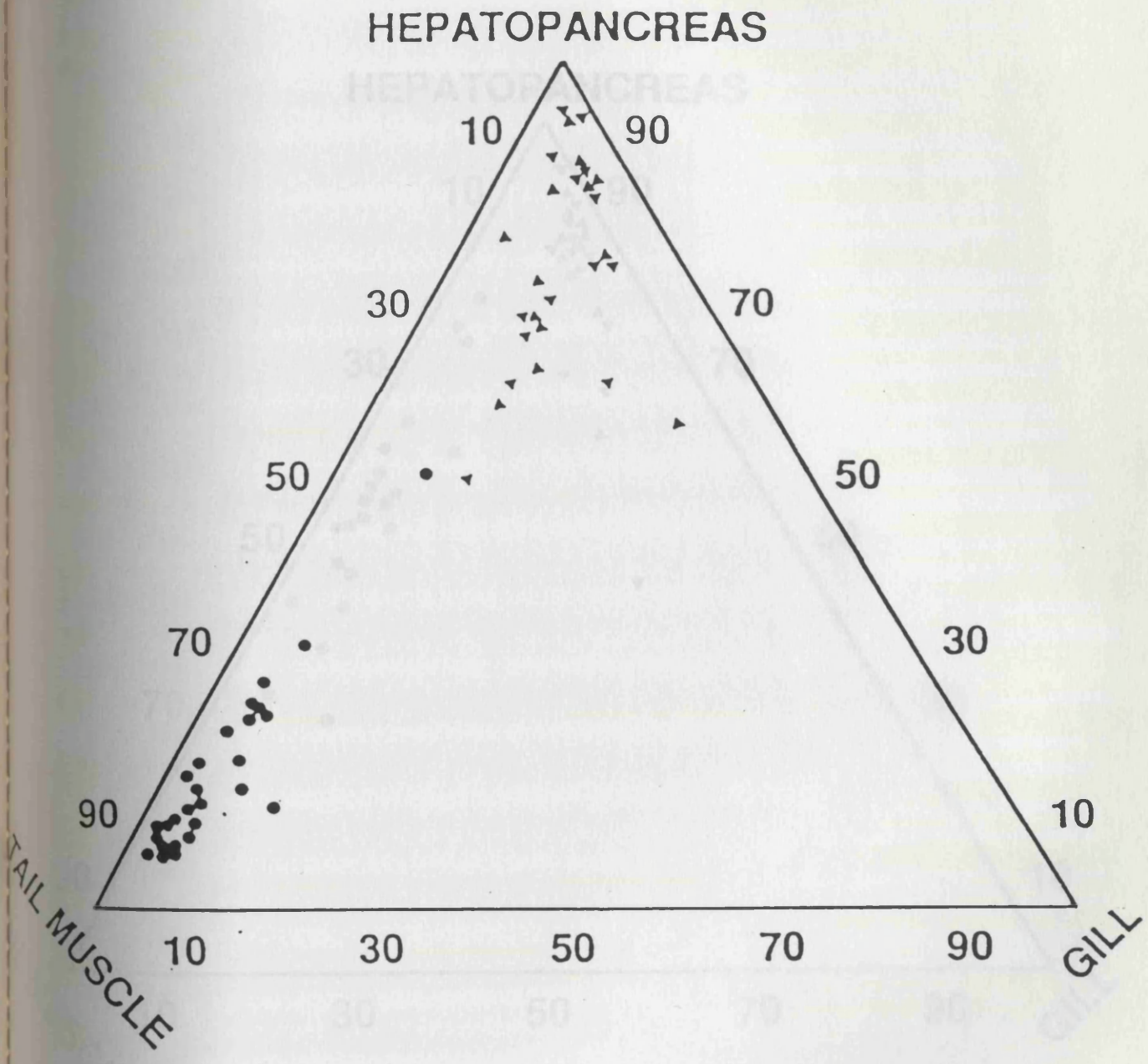

Figure 4.21. Triangular diagram representing percentages of total mercury and cadmium burdens in the hepatopancreas, gill and tail muscle of individual control *Nephrops norvegicus*. Circles for mercury, triangles for cadmium.

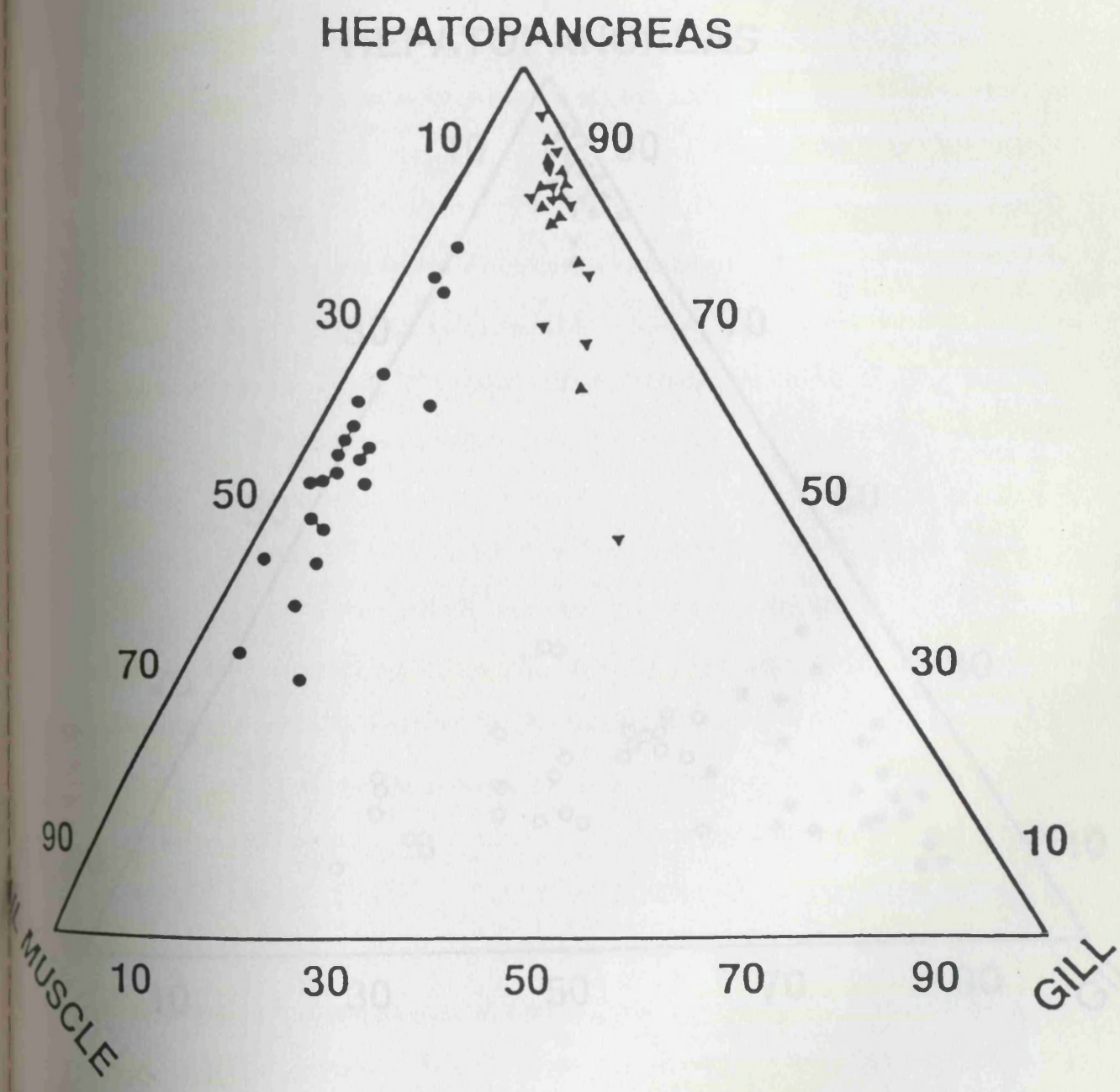

Figure 4.22. Triangular diagram representing percentages of total mercury and cadmium burdens in the hepatopancreas, gill and tail muscle of individual *Nephrops norvegicus* in the feeding experiment. Circles for mercury, triangles for cadmium.

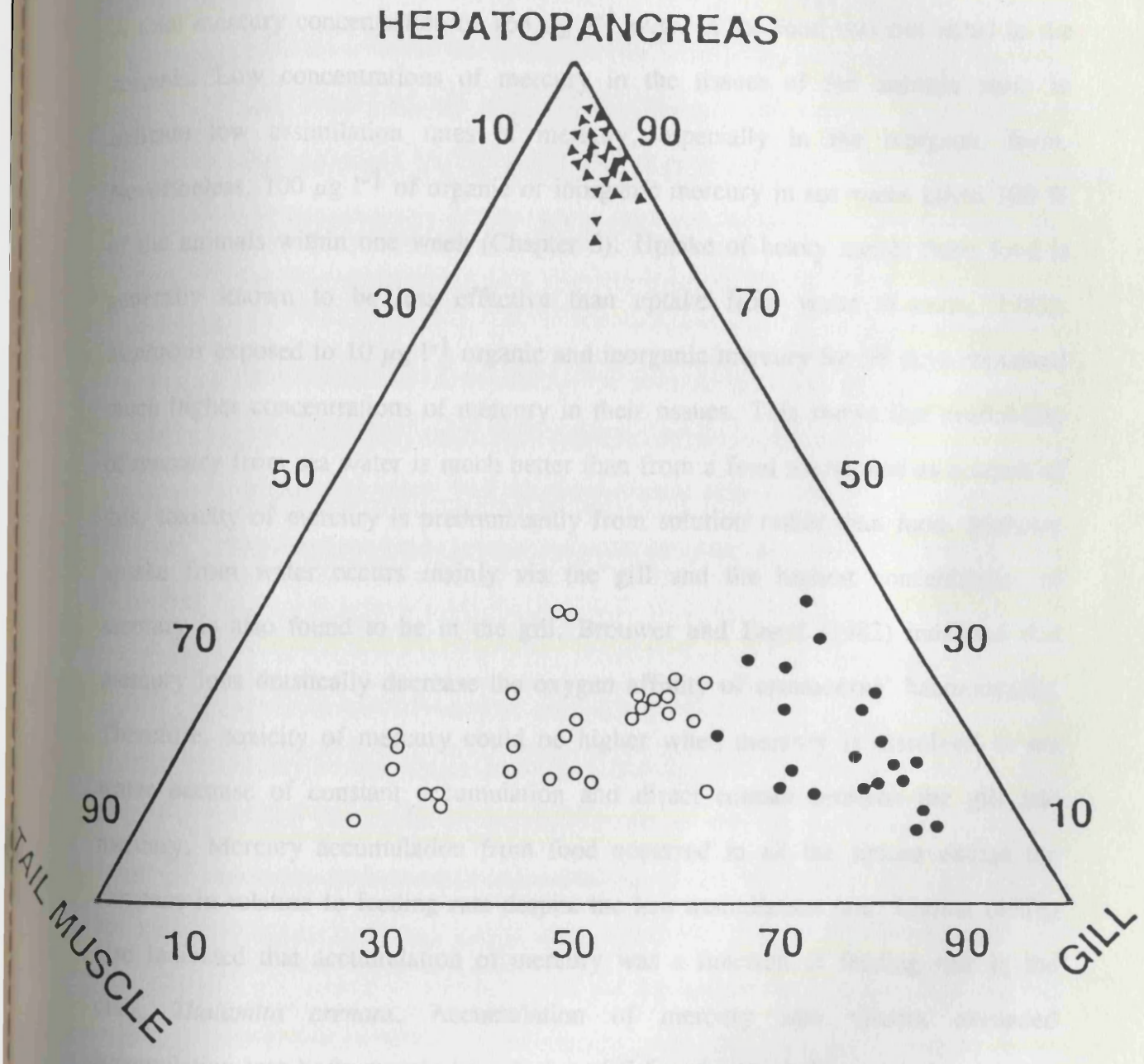

Figure 4.23. Triangular diagram representing percentages of total mercury and cadmium burdens in the hepatopancreas, gill and tail muscle of individual *Nephrops norvegicus* in the experiments uptake from seawater. Closed circles for inorganic mercury, open circles for organic mercury and triangles for cadmium.

4.5 DISCUSSION

A total mercury concentration of 163 $\mu g\ g^{-1}$ w.w. in the food was not lethal to the animals. Low concentrations of mercury in the tissues of fed animals seem to indicate low assimilation rates of mercury, especially in the inorganic form. Nevertheless, 100 $\mu g\ l^{-1}$ of organic or inorganic mercury in sea water killed 100 % of the animals within one week (Chapter 3). Uptake of heavy metals from food is generally known to be less effective than uptake from water (Luoma, 1983). *Nephrops* exposed to 10 $\mu g\ l^{-1}$ organic and inorganic mercury for 30 days contained much higher concentrations of mercury in their tissues. This shows that availability of mercury from sea water is much better than from a food source and as a result of this, toxicity of mercury is predominantly from solution rather than food. Mercury uptake from water occurs mainly via the gill and the highest concentration of mercury is also found to be in the gill. Brouwer and Engel (1982) indicated that mercury ions drastically decrease the oxygen affinity of crustaceans' haemocyanin. Therefore, toxicity of mercury could be higher when mercury is dissolved in sea water because of constant accumulation and direct contact between the gill and mercury. Mercury accumulation from food occurred in all the tissues except the carapace in relation to feeding rate despite the low assimilation rate. Luoma (1976) also indicated that accumulation of mercury was a function of feeding rate in the crab, *Thalamita crenata*. Accumulation of mercury into viscera exceeded accumulation into body muscle by a factor of 7.5 and into chela muscle by a factor of 21. In the present study, low tissue concentrations of mercury in experimentally fed *Nephrops* despite high levels of mercury in the food could be due to the fact that 98 % of the mercury in the food was inorganic form. Results showed that assimilation of organic mercury was 8 and 124 times higher in the hepatopancreas and tail muscle respectively than inorganic mercury. Riisgard and Famme (1986) also indicated that organic mercury in food is accumulated to a greater degree than

inorganic mercury. They also supported the present results by indicating that concentrations of mercury in *Crangon crangon* fed contaminated mussels were increased with increasing in feeding period. Several other studies also indicated that organic mercury has a higher accumulation rate than inorganic mercury from water (Ray and Tripp, 1976 ; Fowler et al., 1978 ; Riisgard and Famme, 1986; Kraus et al., 1988). Guarino et al., (1976) indicated that once mercury is absorbed, whether administered intravascularly, in the water, or via food, the hepatopancreas and muscle are the dominant storage sites of absorbed mercury in the lobster, *Homarus americanus*. The triangular diagrams in the present study showed that mercury is distributed in different ways depending on source. Mercury burdens in control *Nephrops* are found mainly in the tail muscle. After feeding with food containing high mercury concentrations, the hepatopancreas also showed high burdens of mercury and mercury was mainly distributed between the tail muscle and hepatopancreas. Gill mercury burden was high only when animals were exposed to elevated levels of mercury in sea water. Distribution of mercury in the tissues of animals among controls, fed animals and animals exposed to the both organic and inorganic mercury in sea water were significantly different in most comparisons (Table 4.7). From the results of assimilation studies and the triangular diagrams, it might be concluded that mercury concentrations of the hepatopancreas were mainly derived from the food, though uptake from water was also an important route. For the gill the main source of mercury was via the uptake from water. For the tail muscle mercury concentrations, accumulation from food was an important route, although accumulation also occurred from sea water, and was much more pronounced when mercury was in an organic form.

Although the cadmium concentration of the food was not as high as that of mercury, concentrations in the hepatopancreas increased significantly and this increase showed a positive relationship with feeding rate. Assimilation of cadmium by the

hepatopancreas was also very high when compared to assimilation by the gill and tail muscle. Concentrations of cadmium in the gill, carapace and tail muscle were not significantly different between control and fed animals. Overnell and Trewhella (1979) and Davies et al. (1981) also found that gill concentrations of the crab *Cancer pagurus* were not affected from feeding with cadmium contaminated food, whereas hepatopancreas concentrations were increased significantly in fed animals. Jennings and Rainbow (1979 a and b) showed that cadmium was accumulated by *Artemia salina* and *Cancer pagurus* from food sources and indicated that the food chain can be major source of cadmium as long as the previous trophic level has the ability to accumulate the metal. However, accumulation of cadmium from water was found to be a more important route than food in an experiment carried out with the freshwater Isopod *Asellus aquaticus* (Van Hattum et al., 1989). Cadmium was taken up linearly by the digestive gland of the juvenile American lobster, *Homarus americanus* from food, but much lower accumulation occurred in the muscle tissue (Chou et al., 1987). Cadmium accumulation from sea water occurred in the all tissues of *Nephrops* predominantly in the hepatopancreas and gills. Several studies on crustaceans have also indicated that the hepatopancreas and gill were the major accumulation site of cadmium (Dethlefsen, 1977: Wright and Brewer, 1979 ; Overnell and Trewhella, 1979 ; Ray et al., 1981 ; Davies et al., 1981). Accumulation and toxicity of cadmium were found to be dependent on free cadmium ions in the grass shrimp, *Palaemonetes pugio* (Sunda et al., 1978). Distributions of cadmium among the tissues of *Nephrops* from the treatments seem very similar. The hepatopancreas always had the bulk of the cadmium. However, all the treatments showed significantly different cadmium distributions among the tissues, except in the hepatopancreas between fed animals and water exposed animals (Table 4.7). From these results, it might be concluded that the dominant accumulation route of cadmium in the gill is via uptake from water, whereas for the hepatopancreas both accumulation routes are important. The tail muscle seems not to be affected clearly

from either accumulation route.

Concentrations of the essential metals, zinc and iron did not show any increase after feeding with the liver, while mean copper concentrations increased in all tissues except for carapace. However, these increases of copper did not show any positive relationship with feeding rate in any tissue. Therefore, copper increases in the tissues could not be related to the food. In general, food may not be a significant route in accumulation of these metals or concentrations of metals in the liver may be too low to increase the basal tissue concentrations, as a consequence of the homeostatic regulation of tissue concentrations of essential metals.

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CHAPTER 5

CHARACTERIZATION OF GILL ATPASES AND THE EFFECTS OF *IN VIVO* EXPOSURE TO CADMIUM, COPPER AND ZINC ON THE ACTIVITIES OF ATPASES IN THE GILL OF THE NORWAY LOBSTER, *NEPHROPS NORVEGICUS*

5.1 INTRODUCTION

The marine environment is the sink for heavy metals produced by anthropogenic and natural activities. It is well known that heavy metals are accumulated from sea water by marine animals and are toxic at high levels (Eisler and Hennekey, 1977 ; Ahsanullah et al., 1981 ; Krishnaja et al., 1987 ; Rainbow and White, 1989). Environmental Quality Standards (EQS) levels were set in the UK to determine the maximum acceptable levels of heavy metals in the marine environment. In estuaries and coastal waters these are ($\mu g\ l^{-1}$) : Hg $<$ 0.5 and $<$ 0.3, Cd $<$ 5.0 and $<$ 2.5, Cu $<$ 5.0 and $<$ 5.0, Zn $<$ 40.0 and $<$ 40.0 (McLusky, 1989).

The gills of marine animals are crucial for functions such as respiration, osmotic and ionic regulation and excretion. The branchial epithelium is a tissue where both active and passive exchange occurs between the animal and the environment (Schmidt-Nielsen, 1990). Branchial Na,K-ATPase is found in the membrane of gill epithelial cells and plays a central role in whole body ion regulation (Neufeld et al., 1980 ; Towle, 1981). Skou (1957) first described an ATPase from the nerve membrane of the crab *Carcinus maenas* that was stimulated by Na^+ and K^+. The role of this ATPase was proposed to be in active transport of monovalent cations, the plasma membrane sodium pump (Skou, 1957 and 1960). To maintain cytoplasmic concentrations of Na^+ below and K^+ above those in the cellular fluid in animals, active transport of these cations against their electrochemical gradients is dependent on metabolic energy which is generally in the form of ATP (Adenosine Triphosphate) (Robinson and Flasner, 1979 ; Towle, 1984). The Na^+ / K^+ pump under physiological conditions affects the efflux of Na^+ across the plasma membrane coupled to the influx of K^+ the active transport driven by the intracellular hydrolysis of ATP to ADP and Pi. The ratio of Na^+ to K^+ transport is usually 3

Na^+ / 2 K^+ / 1 ATP, though there are some different ratios of this exchange as in squid giant axons where there are ratios of Na^+ / K^+ as high as 4:1 (Robinson and Flashner, 1979). The overall reaction catalysed by membrane bound Na,K-ATPase can be shown as;

$$3\ Na^+\ (in) + 2\ K^+\ (out) + ATP \xrightarrow{H_2O + Mg^{++}} 3\ Na^+\ (out) + 2\ K^+\ (in) + ADP + Pi$$

The pump can also run under different electrochemical gradients. For example, uncoupled Na^+ efflux occurs in the absence of both extracellular Na^+/ K^+. The ratio of transport of Na^+ is 2-3 Na^+ / 1 ATP. This efflux does not occur against a concentration gradient, as extracellular Na^+ is added the rate of Na^+ efflux falls. Na^+ / Na^+ exchange also occurs in the absence of extracellular K^+, but the presence of both extracellular and intracellular Na^+. The pump effects a 1:1 exchange of Na^+, across the membrane with no net transport of Na^+. K^+ / K^+ exchange occurs when in the presence of both intracellular and extracellular K^+, the pump affects a 1:1 exchange of K^+ across the membrane with no net transport. Na,K-ATPase consists of two units that are α (catalytic) and β (glycoprotein). Molecular weights of different units and the whole functional complex have been studied and reviewed by Cantley (1981). Equilibrium sedimentation is one of the methods to determine molecular weights of the enzyme and its units. Studies carried out with this method showed that α (catalytic) and β (glycoprotein) units have molecular weights of 106,000 and 37,000 respectively, while whole functional ATPase has a molecular weight of 380,000 (Cantley, 1981). There are several inhibitors of Na,K-ATPase. **Ouabain** is a cardioactive steroid which is water soluble and is the most frequently used inhibitor of the ATPase in experimental studies because (1) by virtue of its specificity, it not only defines the enzyme and pump, with a generally accepted ratio of one ouabain binding site per functional pump or enzyme complex, (2) it binds to the extracellular surface of the enzyme and thus

provides a marker, in disrupted preparations, for that surface, (3) the interaction with the enzyme affords insights into the reaction process. Oligomycin and Vanadate are other inhibitors of Na,K-ATPase. Therefore, vanadate-free ATP should be used when Na,K-ATPase activity is studied (Silva et al., 1977 ; Robinson and Flashner, 1979 ; Towle, 1984).

Mg-ATPase has been shown to comprise an oligomycin sensitive fraction which is thought to be the mitochondrial Mg-ATPase involved in the oxidative phosphorylation pathway and a non-specific, oligomycin insensitive residual ATPase which is thought to be found mainly in the endoplasmic reticulum (Boyer et al., 1977). The process of oxidative phosphorylation occurs in the inner membranes of mitochondria in animals and other eukaryotes. It is now firmly established that the aerobic oxidation of one molecule of NADH through the mitochondrial respiratory chain can give rise to the synthesis of three molecules of ATP from ADP and Pi (Ernster, 1977). Oligomycin sensitive Mg-ATPase (os-Mg-ATPase) is actively involve in this processes and therefore is a crucial ATPase for oxidative phosphorylation (Boyer et al., 1977). Oligomycin insensitive Mg-ATPase (ois-Mg-ATPase), however, has no proven specific role in metabolism. **Oligomycin** is an antibiotic from various streptomycetes and inhibits the transfer of high-energy phosphate to ADP. Therefore, it also inhibits electron transfer coupled to phosphorylation (McGilvery and Goldstein, 1983). Oligomycin is widely used as experimental tool for discriminating between the two different reactions such as discrimination of Mg-ATPase.

Heavy metals have been reported to have inhibitory effects on gill ATPases in marine animals (Bouguegneau, 1976 ; Kuhnert and Kuhnert, 1976 ; Tucker and Matte, 1980 ; Stagg and Shuttleworth, 1982 ; Haya et al., 1983 ; Verma et al., 1983 ; Lauren

and McDonald, 1987). Generally, *in vitro* exposure to metals causes a decrease in ATPase activity but the *in vivo* effects are not so clear and possibly relate to homeostatic mechanisms causing compensatory alterations in the rate of the enzyme as well as to direct effects of the metals (Stagg and Shuttleworth, 1982). Metals may alter functions or activities of enzymes by binding to their active sites including imidazole, histidyl, carboxyl and especially sulfhydryl chains. Conformational changes can still occur when metals bind away from the active site (Ulmer, 1970). Effects of metals on the activity of ATPases can vary widely depending on the metal. Britten and Blank (1973) showed that Na,K-ATPase activity was inhibited in the rabbit kidney *in vitro* by metals in the order; Hg > Ag > Cu > Cd > Zn > Pb. Metals also affect the isolated mitochondria and it is obvious that mitochondrial activities containing metal-reactive ligands and located on the outer surface of the inner membrane will be the first targets for low concentrations of toxic heavy metal ions (Brierley, 1977). Thus, in isolated mitochondria, the substrate and phosphate transporters and certain portions of the electron transport system are often found to be sensitive to metal ions (Brierley, 1977). Few studies have been carried out on the effects of heavy metals on ATPase activity in crustaceans.

The aim of this study were ; a) to characterise the optimum working conditions of the gill ATPases such as oligomycin sensitive and insensitive Mg-ATPase and Na,K-ATPase in the gill of *Nephrops norvegicus*, b) to describe some of the biological parameters which could affect the activity of the ATPases such as size and sex of the animal and histological conditions of the gill, and c) to determine the effects of different concentrations of cadmium, copper and zinc in combination on the activity of the ATPases. Exposure concentrations were chosen to be representative of contamination in the marine environment (Nolting, 1986 ; Peerzada and Ryan, 1987 ; Balls and Toppings, 1987) and the lowest concentrations of the metals used were

lower than the environmental quality standards. Furthermore since metals rarely occur in the environment alone an important aspect of this study was that the animals were exposed to a mixture of the three metals selected for study.

5.2 MATERIALS AND METHODS

All *Nephrops norvegicus* used in this study were caught by trawling on the same day (11.09.1991) and same location south of the Isle of Cumbrae, Clyde Sea area, Scotland. The animals were transferred to the laboratory, kept in running seawater for two weeks in 500 l holding tanks containing 50 animals each.

5.2.1 HEAVY METAL EXPOSURE

Nephrops were exposed to sublethal concentrations of a mixture of cadmium, copper and zinc in a static exposure system over an 18-day period. Concentrations were chosen as follows:

0- Controls (no metal addition).

1- 1, 1 and 8 ppb ($\mu g\ l^{-1}$) Cd, Cu and Zn respectively.

2- 5, 5 and 40 ppb Cd, Cu and Zn respectively.

3- 25, 25 and 200 ppb Cd, Cu and Zn respectively.

The metal concentrations were prepared using 40 litres of sea water in each tank and the metal salts were mixed very well before use. $CdCl_2$ 2 1/2 H_2O, $Cu(NO_3)2$ and $ZnSO_4$ $7H_2O$ were used as chemical forms of the metals (BDH Chemicals Ltd. Poole, England; G.P.R. Chadwell Heath, Essex, England; Riedel-de Haen Ag Seelze, Hannover, Germany).

5.2.2 EXPERIMENTAL PROCEDURE

Male *Nephrops* were studied in all the treatments. A set of female animals was also studied but only in the control and the highest concentration treatment. All animals' claws were bound with rubber bands to prevent fighting between individuals. For each treatment 8 animals in 3 replicate tanks were used except for the control female group where only a single tank was used. Healthy intermoult *Nephrops* were chosen after a two week holding period. They were put into each 50 liters fibreglass tank containing 40 liters of seawater (control) and metal-added seawater tanks. During the 18 days of the experimental period, the aquaria were aerated using air stones attached to a compressed air supply system and experimental room was illuminated with six fluorescent lamps in a 12L : 12D regime. Animals were not fed during this period and the seawater temperature was kept at 19.5 ± 0.65 ^{o}C (mean $\pm$ standard deviation of 18 days daily measurements). Seawater in both experimental and control tanks was changed every 3rd day to maintain metal levels and water quality. Concentrations of copper, cadmium and zinc were previously found to remain stable in seawater for up to three days (Chapter 3).

A second group of male *Nephrops* comprising different size groups, was caught from west of the Isle of Arran, in the Firth of Clyde and kept in running seawater for 2 days at the University Marine Biological Station, Millport. These animals were used to measure ATPase activity in relation to the size of the animals.

5.2.3 TISSUE PREPARATION FOR ASSAY

At the end of 18 days exposure period, all animals were killed by decapitation. They were quickly weighed to the nearest 1 mg and carapace length measured (from the

rear of the eye socket to the mid dorsal edge of the carapace) to nearest 1 mm. Animals were dissected on ice-cold clean plates. The gills were removed and put on ice-cold petri dishes. Dissected gills were placed immediately into plastic bags (small cut in one of the corners) and quick frozen in liquid nitrogen. All tissues were then transferred to a freezer (Series 100, Kelvinator Commercial Products Inc. Manitowoc, U.S.A) and kept at -70 ^{o}C until use. ATPase activity in the second group animals used to study the effects of size (carapace length) was measured immediately after killing.

5.2.4 PROCEDURE FOR ATPASE ACTIVITY

5.2.4.1 Homogenisation of gills

A homogenisation buffer was prepared with sucrose (250 mmol l^{-1}), imidazole (100 mmol l^{-1}) and EDTA (5 mmol l^{-1}) (Sigma Chemical Company, U.S.A). The pH of this solution was brought to 7.8 with HCl. Homogenisation of the gills was carried out in a cold room (4 ^{o}C). Before homogenisation, homogenisation buffer, glass homogenizers and Eppendorf tubes were pre-cooled by placing on ice.

Frozen gills were quickly weighed (approx. between 150-200 mg) to the nearest 1 mg using a Mettler AE240 balance. Frozen gills were transferred to the homogenizer on ice. They were homogenised in 2 ml of homogenisation buffer in a few minutes. Homogenates were transferred to Eppendorf tubes and centrifuged at 5000 G in a pre-cooled Eppendorf centrifuge for 1 minute (Eppendorf centrifuge 5415C). Supernatants were decanted to clean and cool Eppendorf tubes using a disposable pipette for each homogenate and placed on ice. ATPase assays were carried out with these homogenates within one hour.

5.2.4.2 Determination of ATPase Activity

ATPase activity was measured by determination of phosphate (Pi) liberated from the hydrolysis of the substrate adenosine triphosphate (ATP), ATP → ADP + Pi.

ATPase activity was measured at 37 ^{o}C with duplicate readings of each sample. Mg-ATPases were measured in the presence of Mg and ouabain for total Mg-ATPase activity and in the presence of Mg, ouabain and oligomycin for oligomycin insensitive Mg-ATPase activity. Differences between the two measurements gave the activity of oligomycin sensitive Mg-ATPase activity. Total ATPase activity was measured in the presence of Mg, Na and K with no inhibitor present in the buffer. Na,K-ATPase activity was measured in the presence of Mg, Na, K and ouabain. ATPase activity in this buffer was subtracted from the total ATPase activity to find the real Na,K-ATPase activity. Final assay concentrations of chemicals were as follows:

100 μmol ml^{-1} imidazole (Sigma U.S.A.), pH 7.4 with HCl
100 μmol ml^{-1} NaCl (B.D.H. England)
20 μmol ml^{-1} KCl (B.D.H.)
4 μmol ml^{-1} MgCl2.6H2O (B.D.H.)
6 μmol ml^{-1} ATP (Vanadium free, daily preparation) (Sigma)
1 μmol ml^{-1} Ouabain (Sigma)
0.3 μg ml^{-1} Oligomycin B (Sigma)

The following buffers were prepared using final assay concentrations of the chemicals. Double distilled water was used throughout this study.

Buffer 1 = Imidazole, Mg and ouabain (assay 1)

Buffer 2 = Imidazole, Mg, ouabain and oligomycin (assay 2)

Buffer 3 = Imidazole, Mg, Na and K (assay 3)

Buffer 4 = Imidazole, Mg, Na, K and ouabain (assay 4)

Blank 1 = ATP added buffers were used as first blank without homogenate present. Phosphate (Pi) concentration of this blank was measured at the end of 30 minutes incubation period at 37 ^{o}C to test for any breakdown of ATP without ATPases.

Blank 2 = Homogenate added buffers were used as second blank without ATP. Pi concentration of this blank was measured at time zero to find out Pi concentration which was not produced by enzymatic activity. There is very significant positive relationship ($P<0.0001$) between protein and phosphate concentrations in crude homogenate (Figure 5.3).

5.2.4.2.1 Assay

800 μl of buffers were thermoequilibrated in 6 ml disposable test tubes for 5 minutes at 37 ^{o}C using a thermostated water bath (Grant Instrument, Cambridge, England). 100 μl homogenate was added to each buffer tube, except blank, and 100 μl of ATP added to each tube to start the reaction. Tubes were vortexed with a spinmix and then shaken at 100 rev/min (Gallenkamp, England) for the duration of the incubation period. After 30 minutes, the reaction was stopped by adding 500 μl ice-cold distilled water and 3 ml of lubrol/ammonium molybdate (1:1) mixture.

5.2.5 DETERMINATION OF INORGANIC PHOSPHATE

Phosphate determination depends on the spectrophotometric determination of inorganic phosphate (Pi) after the formation of the soluble yellow complex of phosphomolylodic acid and lubrol (Atkinson et al., 1973). After the incubation period, the reaction was stopped by the addition of 3 ml of 1 % ammonium molybdate (B.D.H.) and 1 % lubrol in a 1:1 ratio to the same volumed (1500 μl) samples and standards. The tubes were left for 10 minutes at room temperature to allow the yellow colour to develop. Solutions then were transferred to cuvettes and the intensity of yellow colour read at 390 nm on a LKB Ultrospec II spectrophotometer. If there was any excess colour development or protein precipitation in samples with high ATPase activity which prevented accurate reading on the spectrophotometer, then the homogenate was diluted and the assay repeated.

Six standard solutions were prepared between 0.2 and 1.2 μmol Pi ml^{-1} using KH_2PO_4 (B.D.H.). A calibration curve obtained from standard readings in each run was used to calculate phosphate concentrations of samples. An example of a calibration curve is shown in Figure 5.1. Double distilled water was used as blank.

5.2.5.1 Calculation of Results

All pairs of duplicate readings (blanks, standards and samples) were averaged and blank values were subtracted from sample readings. Phosphate concentrations of samples were calculated using the regression line of standards. Phosphate concentration in 1500 μl of final samples after 30 minutes reaction were calculated as follows (μmol Pi/ml /hour); Pi concentration (μmol ml^{-1}) * 1.5 ml * 60 min./ 30 min.

This calculation was carried out for each specific activity after associated subtractions as follows;

Total ATPase activities = assay 3

Total Mg-ATPase activity = assay 1

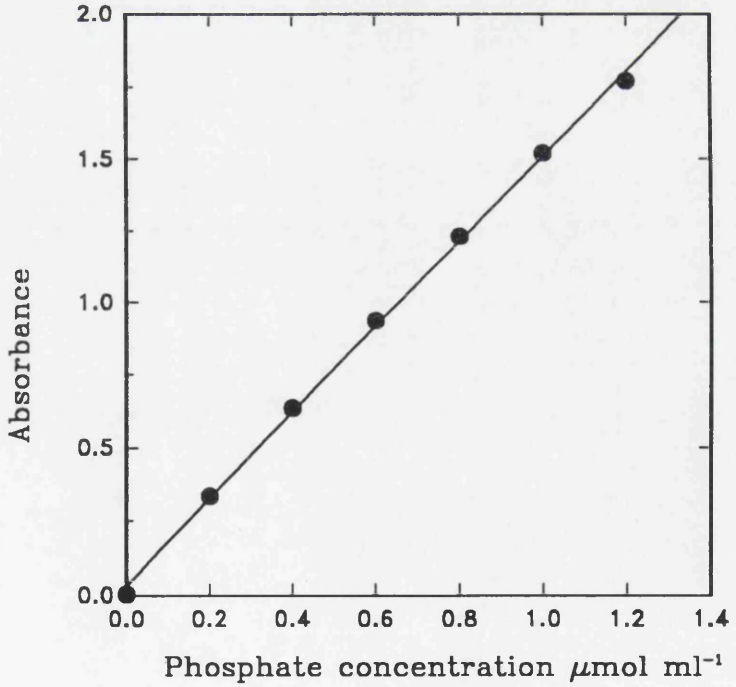
Na,K-ATPase activity = assay 3-4

Oligomycin sensitive Mg-ATPase activity = assay 1-2

Oligomycin insensitive Mg-ATPase activity = assay 2

Results of these calculations were converted to protein concentration of 100 μl of homogenate (mg protein/100 μl homogenate) to justify real phosphate amounts produced in 30 minutes by ATPases.

Figure 5.1. A typical relationship between phosphate concentration and absorbance



5.2.6 DETERMINATION OF PROTEIN IN CRUDE HOMOGENATE

Protein determinations in crude homogenate were conducted with the method of Bradford (1976) in microtitre plates (Biorad, Richmond, England) using gamma globulin as standard. This technique is a non-specific quantitative determination of protein using a dye-binding technique. The dye reagent, Coomasie brilliant blue G250, has an absorbance maximum at 465 nm which shifts to 595 nm when it binds to protein, thus the optical density of the assay, measured as absorbance at 595 nm, is proportional to protein concentration. Binding of the dye to the analyte protein(s) may occur at a slower rate than binding to the standard, so a time delay between addition of the dye and absorbance reading is necessary.

5.2.6.1 Preparation of Standards

Crude homogenates were diluted ten times with distilled water to be able to read accurately. 2 mg ml^{-1} of stock concentrations of standard was prepared as 20 ml using Biorad protein assay standard I (Bovine Plasma, Gamma Globulin Lyophilized) and aliquoted into Eppendorf tubes as 800 μl to store at -20 ^{o}C. In each protein assay, a 800 μl of standard was thawed. Five concentrations of standards were prepared between 0.2 and 1.0 mg ml^{-1} as 500 μl. Dilutions of standards were carried out with addition of distilled water and homogenisation buffer. Final assay concentrations of standards contained 10 % of homogenate buffer, because crude homogenates of samples were ten times diluted with distilled water.

Figure 5.2. A typical relationship between protein (gamma globulin) concentration and absorbance

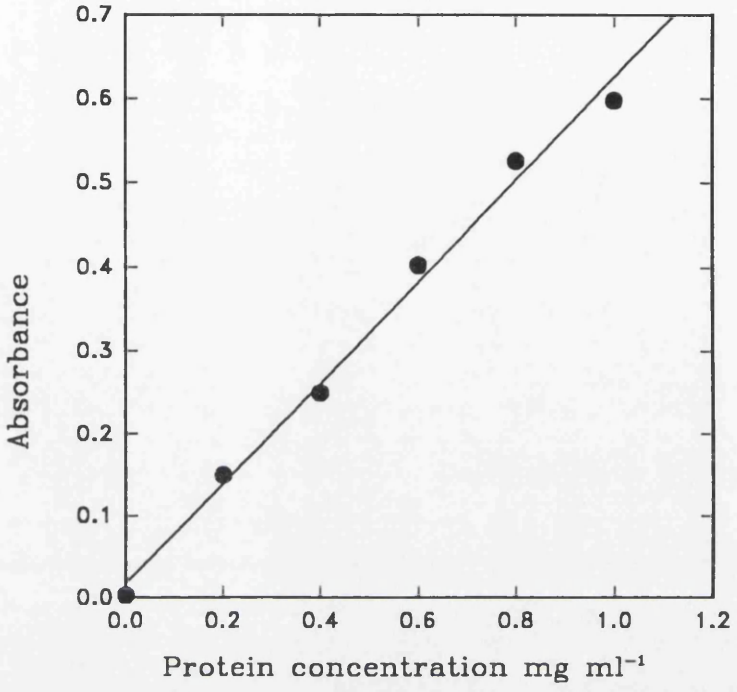
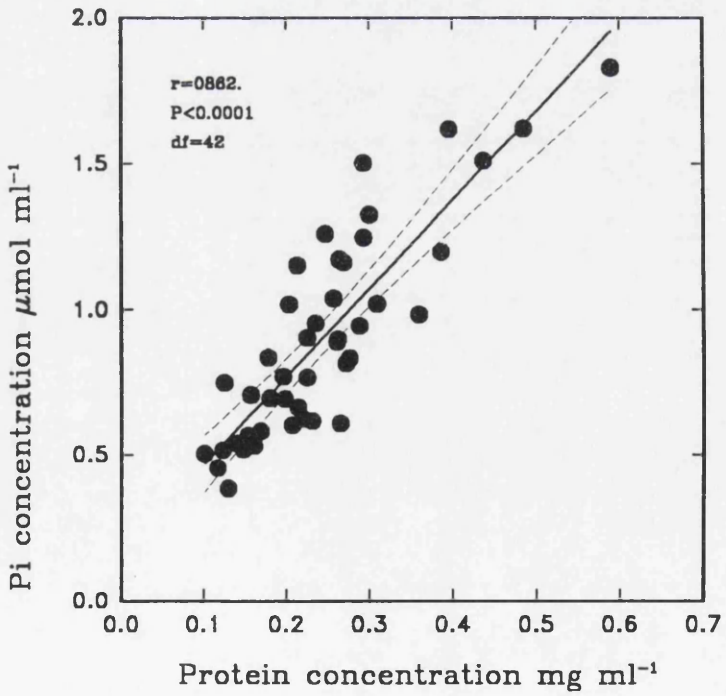


Figure 5.3. Relationship between protein and phosphate concentrations in gill crude homogenate



5.2.6.2 Assay for Protein Measurements

Dye agent (Biorad) was diluted one in four (1:4) with distilled water and filtered through a GF/F microfibre filter (Whatman) using a syringe (Millipore, Swinnex-25). Protein assays were conducted in disposable microplates using double distilled water as blank. Protein determination was carried out using 10 μl duplicates of blanks, standards and 10 times diluted homogenates. 200 μl dye agent and distilled water mixture (1:4) was added in each case and the microplate was left at room temperature for 15 minutes to allow development of blue colour. The intensity of blue colour was read on a spectrophotometer (Argus 300 microplate reader, Bio-Tek instrument Inc.). The instrument was connected to a computer (IBM) to produce the regression line for standards, absorbance and concentration values of samples. A typical calibration line of standards is shown in Figure 5.2. Samples which fell out with standard values were rediluted and remeasured. If samples showed a high coefficient of variation, then they were remeasured. From these calculations, the protein concentration of 100 μl crude homogenate was found for each case and this was used to divide Pi concentration produced in the reaction period. ATPase activity was finally expressed as μmol Pi / mg protein / hour.

5.2.7 STATISTICAL ANALYSES OF DATA

Statistical analysis of data was carried out with Unistat statistical package programs. Before any statistical analysis, all data were plotted on graphs and homogeneity of data were checked by Bartlett's homogeneity test. Data not normally distributed were $\log_{10}$ or square root transformed as appropriate. One way analysis of variance (Anova) was used to compare variables between controls and treatments. If a variable differed significantly ($P<0.05$) among treatments and controls in male animals,

these data were reanalysed between control and individual treatment to find out individual contribution of groups to the overall comparison. Linear regression analyses were carried out between branchial ATPase activity and metal concentrations.

5.3 RESULTS

5.3.1 Characterization of Gill ATPases in *Nephrops norvegicus*

Figure 5.4 shows the effects of Na and K on the activity of Na,K-ATPase. Maximal activity occurred between 20 and 40 mmol K l^{-1} at the range of Na concentrations used (Figure 5.4a). The effect of Na was much more marked (Figure 5.4b) than K (Figure 5.4a) and maximum ATPase activity was found at a Na concentration of 100 mmol l^{-1} at all potassium concentrations used. Figure 5.5 shows the effect of Mg ions on the activity of Mg-ATPase. Maximal Mg-ATPase activity occurred at a concentration of 4 mmol Mg l^{-1}. All the ATPases studied in the gill had optimum ATP concentrations of 6 mmol l^{-1} (Figure 5.6a and 5.6b). Inhibition of Na,K-ATPase at different concentrations of ouabain is shown in Figure 5.7 and shows that 100 % inhibition of Na,K-ATPase occurred at a concentration of 1 μmol ouabain ml^{-1}, with an IC50 (the concentration which inhibits 50 % of the enzyme activity) value at 35 μmol ouabain l^{-1}. Likewise, the effects of oligomycin on the activity of Mg-ATPase is shown in Figure 5.8 and shows that 0.3 μg oligomycin ml^{-1} completely inhibited 100 % of oligomycin sensitive Mg-ATPase, with an IC50 value of 0.022 μg ml^{-1}. Effects of temperature on the activities of the ATPases are shown in Figure 5.9. This figure shows that all the ATPases had optimum activity at or near 37 ^{o}C. Oligomycin sensitive Mg-ATPase and Na,K-ATPase seemed to be sensitive to temperature as the activities of these ATPases were inhibited completely when

temperature was below 5 ^{o}C and above 55 ^{o}C. However, the activity of oligomycin insensitive Mg-ATPase was not totally inhibited at 5 ^{o}C and interestingly almost 50 % of the ATPase was active when temperature reached 70 ^{o}C (Figure 5.9). Effects of freezing at -70 ^{o}C on the activities of the ATPases were also investigated. For this, gill filaments from five animals were homogenised and mixed very well. ATPase activities in this homogenate were measured on the same day of homogenisation, after one month and two months storage at -70 ^{o}C. Results are given in Table 5.1. This table shows that in general, all ATPase activities were reduced after storage at -70 ^{o}C after one month and even more so after two months, especially in the activities of Na,K-ATPase and oligomycin sensitive Mg-ATPase. Oligomycin insensitive Mg-ATPase activity ,however, was not affected by freezing.

Table 5.1. Reduction of ATPase activities (µmol Pi/mg protein/hour) in crude homogenate of the gill after storage at -70 ^{o}C.

	Fresh	One month	% loss	Two months	% loss
Total ATPase	1.993	1.614	19.0	1.550	22.0
Total Mg-ATPase	1.638	1.382	15.0	1.360	17.0
Ois-Mg-ATPase	1.037	1.003	3.3	0.970	6.4
Os-Mg-ATPase	0.601	0.412	31.4	0.357	40.6
Na,K-ATPase	0.465	0.305	34.4	0.256	44.9

Figure 5.4a. Effect of K ions on Na,K–ATPase activity in different Na concentrations in the gill of Nephrops norvegicus

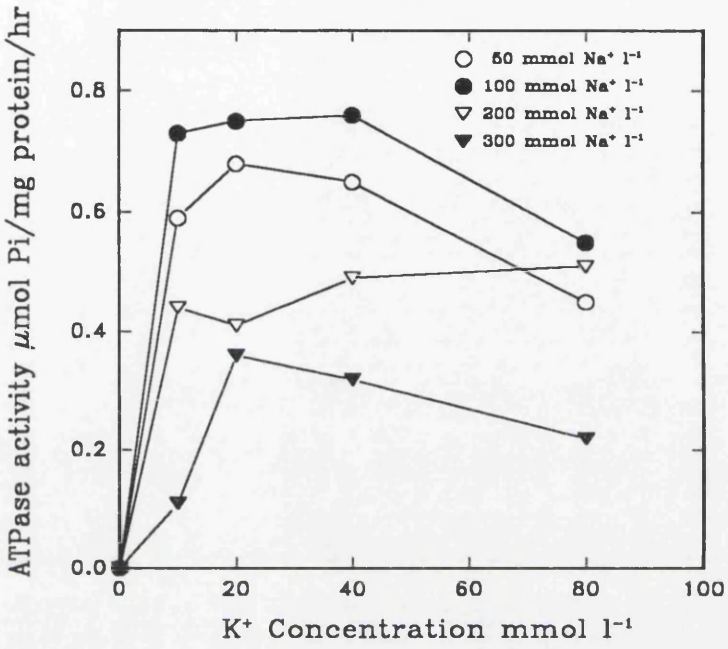


Figure 5.4b. Effect of Na ions on Na,K–ATPase activity in different K concentrations in the gill of Nephrops norvegicus

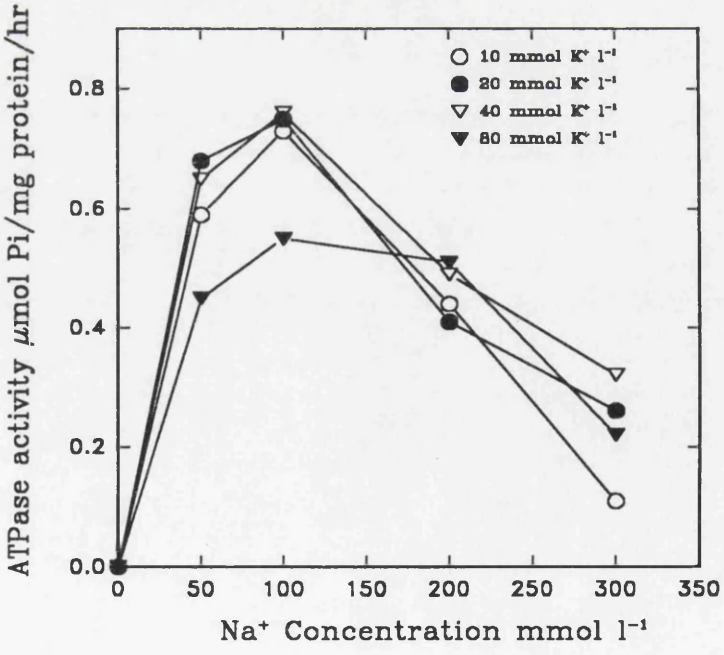


Figure 5.5. Effect of Mg ions on Mg–ATPase activity in the gill of Nephrops norvegicus

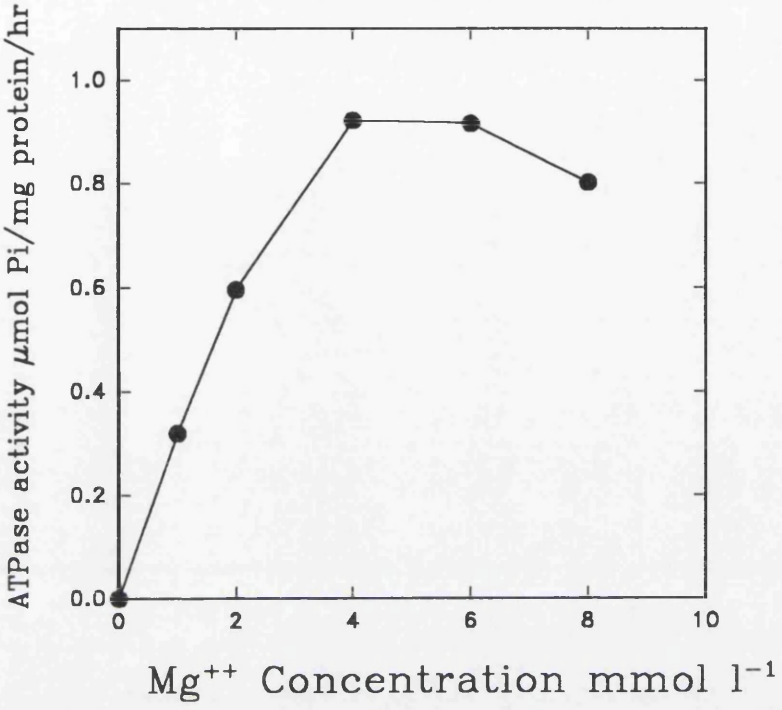


Figure 5.6a. Effect of ATP on total–ATPase activity in the gill Nephrops norvegicus

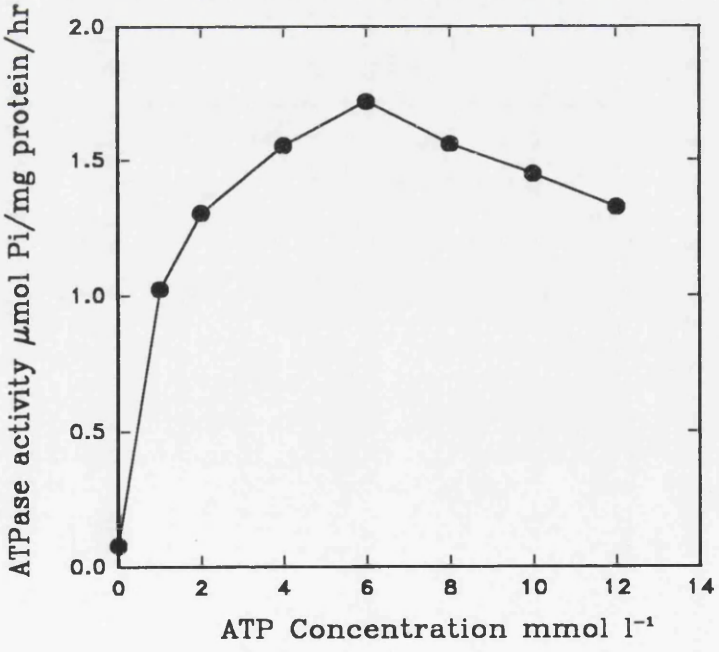


Figure 5.6b. Effect of ATP on different ATPase activities in the gill Nephrops norvegicus

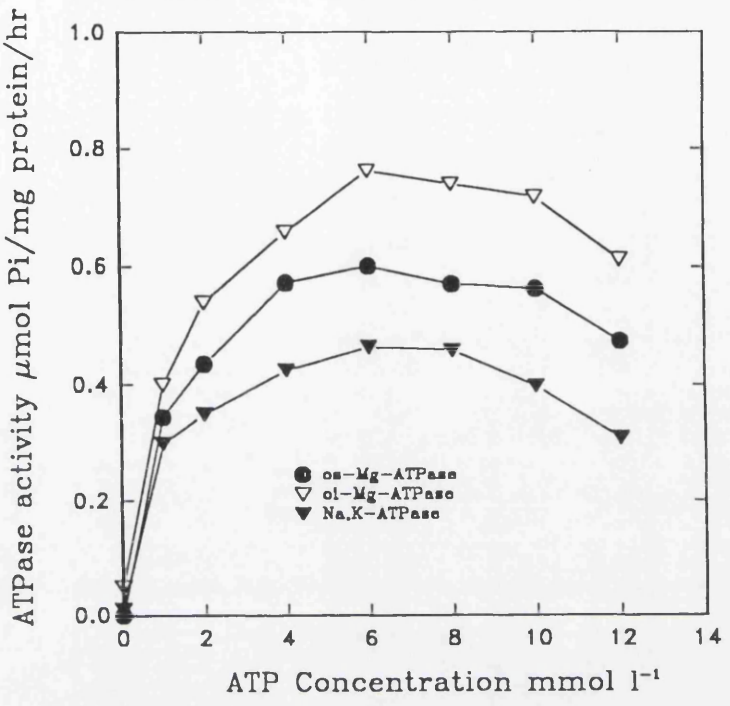


Figure 5.7. Effect of ouabain on Na,K-ATPase activity in the gill of Nephrops norvegicus

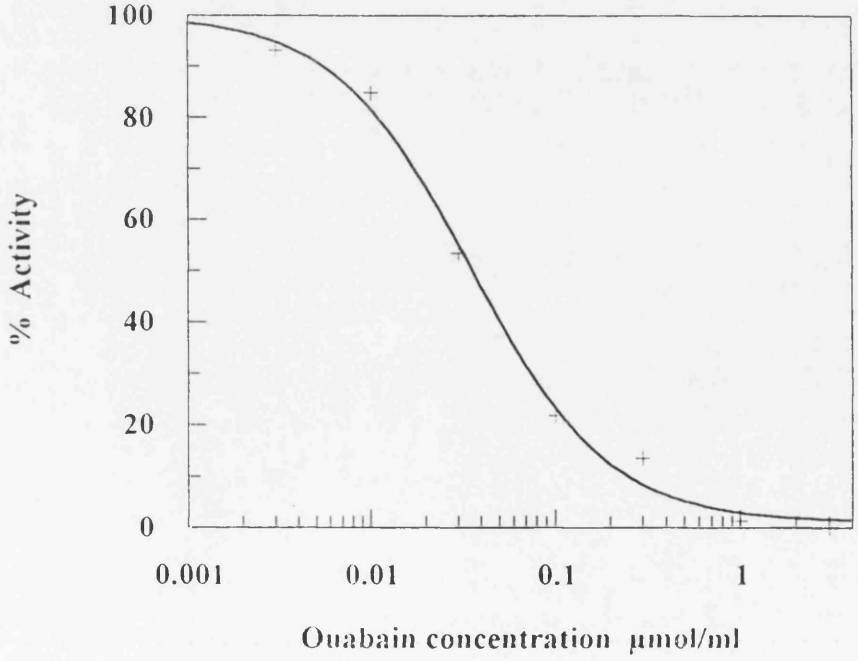


Figure 5.8. Effect of oligomycin on oligomycin sensitive Mg-ATPase activity in the gill of Nephrops norvegicus

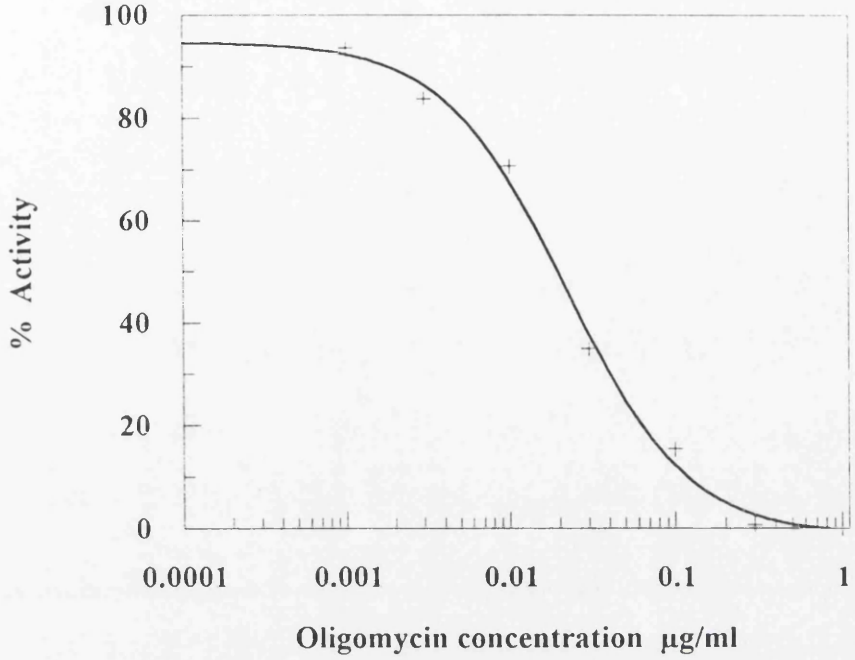
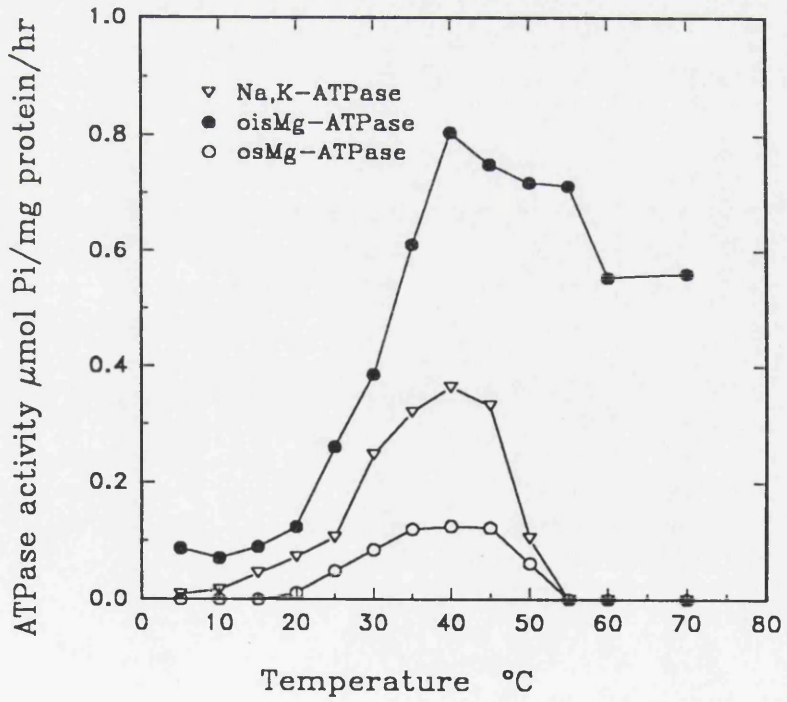


Figure 5.9.Effects of temperature on the activity of ATPases in the gill of Nephrops norvegicus



5.3.2 Effects of size on the activity of ATPases

Carapace length of male animals was significant related to ATPase activity. Regression analyses between ATPase activity and carapace length showed that neither total Mg-ATPase (Figure 5.10) nor its oligomycin insensitive (Figure 5.11) component showed any significant relationship with size of male animals ($P > 0.05$). However, the oligomycin sensitive Mg-ATPase (Figure 5.12) and Na,K-ATPase (Figure 5.13) activities showed significant negative relationships with carapace length ($r = -0.481$, $P < 0.05$) and ($r = -0.603$, $P < 0.01$), respectively.

Figure 5.10. The relationship between size (CL) and total Mg-ATPase activity in the gill of Nephrops norvegicus

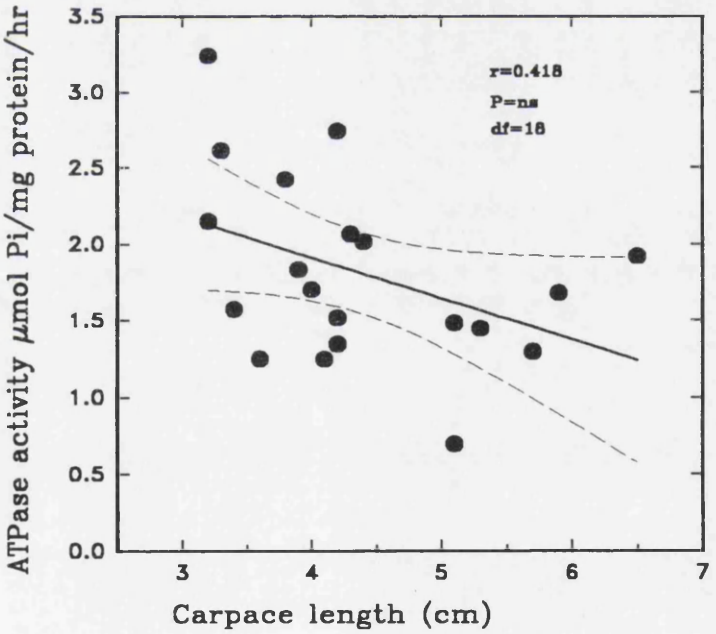


Figure 5.11. The relationship between size (CL) and oligomycin insensitive Mg–ATPase activity in the gill of Nephrops norvegicus

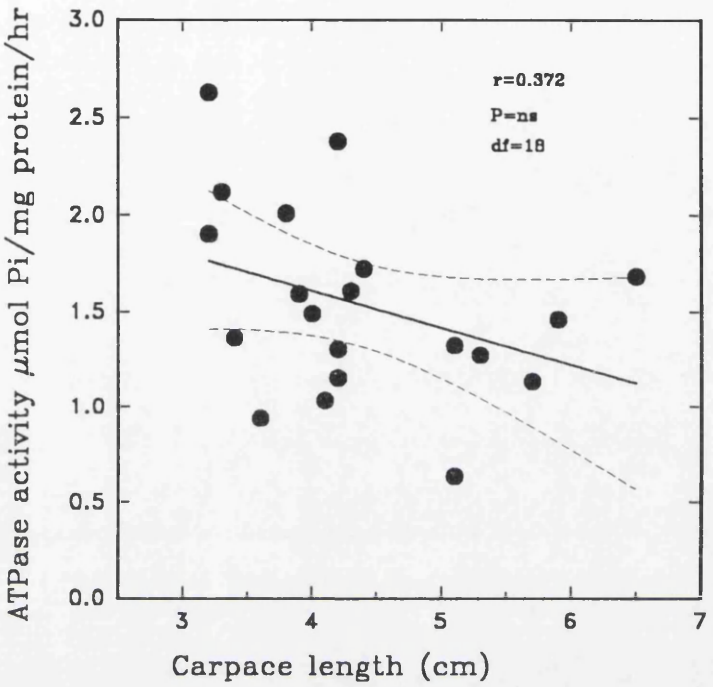


Figure 5.12.The relationship between size (CL) and oligomycin sensitive Mg–ATPase activity in the gill of Nephrops norvegicus

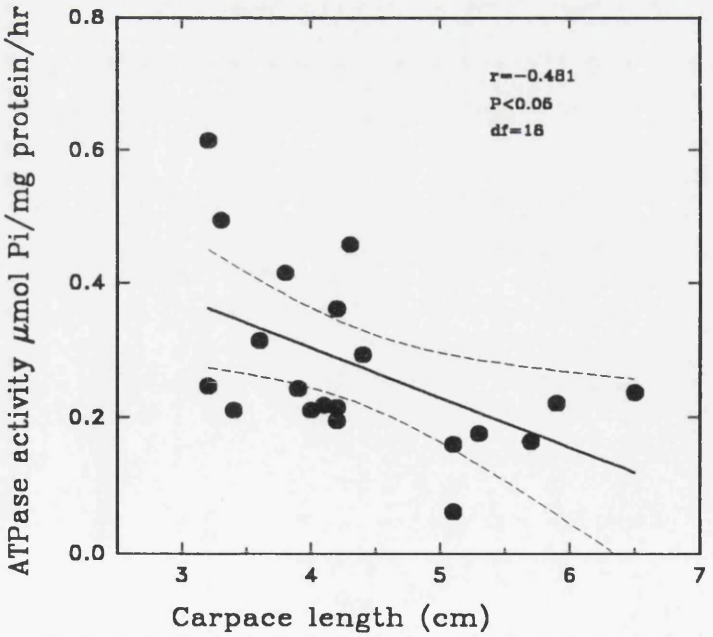
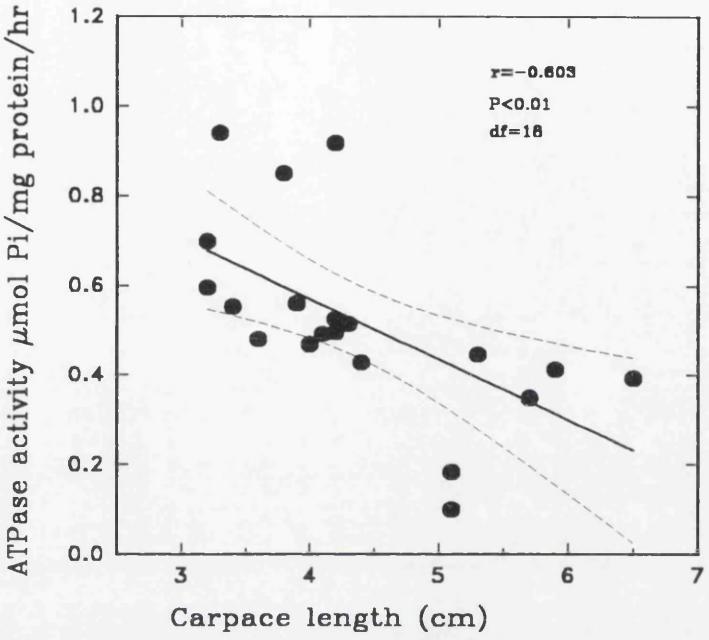


Figure 5.13. The relationship between size (CL) and Na,K–ATPase activity in the gill of Nephrops norvegicus



5.3.3 The effects of metals on metal accumulation and ATPase activities

Carapace lengths and weights of male and female *Nephrops norvegicus* used for this study are given in Table 5.2. This table shows that there was no significant difference in size between the groups ($P < 0.05$).

Table 5.2. Mean values and standard errors of carapace length (CL) of experimental animals. Results of one way Anova are also given in the following table.

	M A L E		A N I M A L S			FEMALE	ANIMALS	
	Control	Treat1	Treat2	Treat3	P	Treat3	Control	P
No	15	19	15	19		19	8	
CL	3.48	3.68	3.72	3.65	ns	3.56	3.68	ns
se	0.06	0.10	0.09	0.08		0.06	0.13	

The effects of heavy metal exposure on the activities of gill ATPases in male and female *Nephrops norvegicus* are shown in Figures 5.14-5.18 with results of one way Anova. In male *Nephrops* exposed to copper, zinc and cadmium the activity of branchial Na,K-ATPase was inhibited significantly ($P<0.01$) (Figure 5.18). Total Mg-ATPase and its oligomycin-insensitive and sensitive components in the gill from male animals were unaffected by the exposure regime (Figure 5.15-5.17). There was also no significant inhibition of total ATPase activity. In female *Nephrops* all the ATPases measured in the gills were affected by exposure to the metals, except for total Mg-ATPase which its activity was significantly ($P<0.05$) inhibited in the highest exposure regime (Figures 5.14-5.18). Significant results in male animals from overall comparison with one way Anova were reanalysed between control and treatments. Results of these analyses are given in Table 5.3. Results showed that Na,K-ATPase activity was significantly lower in the medium and the highest treatments ($P<0.01$ and $P<0.05$, respectively), though in the lowest treatment there was no difference from control. The same results were also found between the treatments as there was significant inhibition in the medium and the highest treatments compared to the lowest treatment (Table 5.3). There was no difference between the medium and the highest treatments.

Table 5.3. Results of comparison of Na,K-ATPase activity in male animals with one way Anova. Significant P values are given in the following table. ns = not significant ($P<0.05$).

	Control	Treatment1	Treatment2
Treat1	ns		
Treat2	0.006	0.005	
Treat3	0.022	0.026	ns

Male and female animals were also compared for levels of some variables and the activity of ATPases in controls and in the highest treatments (Table 5.4). Results showed that there was no significant difference in carapace length of male and female animals in both controls and in the highest treatments. Activities of the ATPases were not also significantly different between male and female animals, except for Na,K-ATPase activity for which there was a significant ($P<0.05$) difference between groups of control animals, namely males had higher Na,K-ATPase activity than females.

Table 5.4. Result of comparisons of some variables and ATPase activities between controls and the highest treatments of male and female animals. CL = Carapace length, W = Weight, Tr3 M&F= Male and female animals in the treatment 3, C M&F = Control males and females.

	CL	tot-ATP	tot-Mg	Na,K-ATP	ois-Mg-ATP	os-Mg-ATP
C M&F	ns	ns	ns	0.021	ns	ns
Tr3 M&F	ns	ns	ns	ns	ns	ns

Metal concentrations in the gills of male and female animals were described elsewhere (Chapter 6). Cadmium showed a significant increase in concentrations in the gill in relation to exposure concentrations in both male and female animals ($P<0.001$). Copper and zinc also showed significant increases ($P<0.05$) in male animals but not in females. Regression analyses were carried out to analyse the relationship between ATPase activity and metal concentrations for each metal treatment and ATPase activity. Results of these analyses showed that Na,K-ATPase activity had a negative relationship with copper ($P=0.005$) in male animals. However, activity of Na,K-ATPase in females showed positive relationships with zinc ($P=0.009$) and cadmium ($P=0.032$). There was no other significant relationship between any of the ATPase activities and metal concentrations in male and female animals.

Figure 5.14. The effects of metal exposure on total ATPase activity in the gill of Nephrops norvegicus

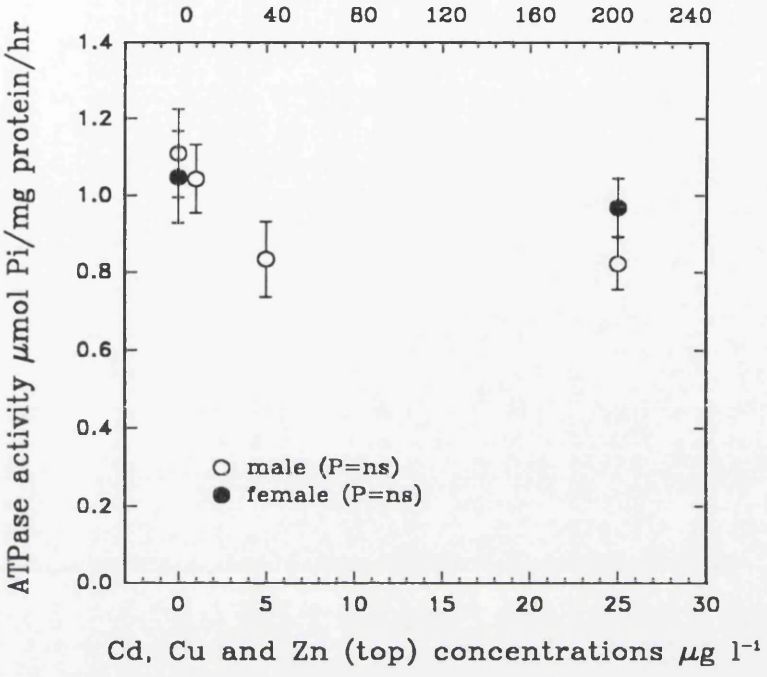


Figure 5.15. The effects of metal exposure on total Mg–ATPase activity in the gill of Nephrops norvegicus

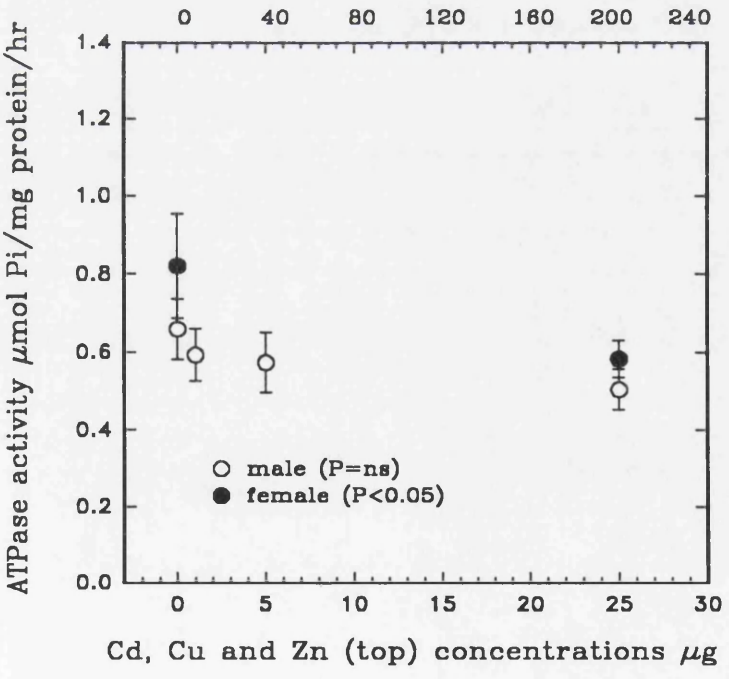


Figure 5.16. The effects of metal exposure on oligomycin insensitive Mg–ATPase activity in the gill of Nephrops norvegicus

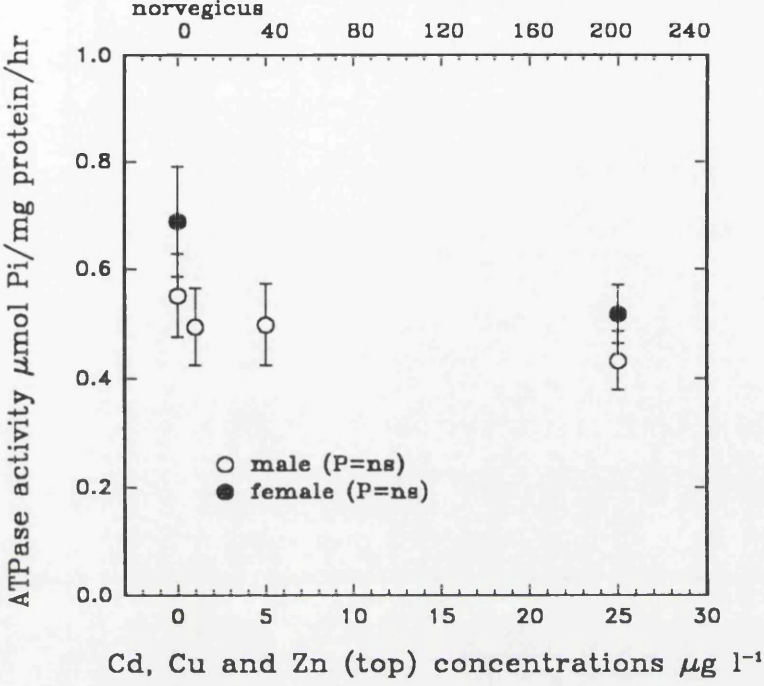


Figure 5.17. The effects of metal exposure on oligomycin sensitive Mg–ATPase activity in the gill of Nephrops norvegicus

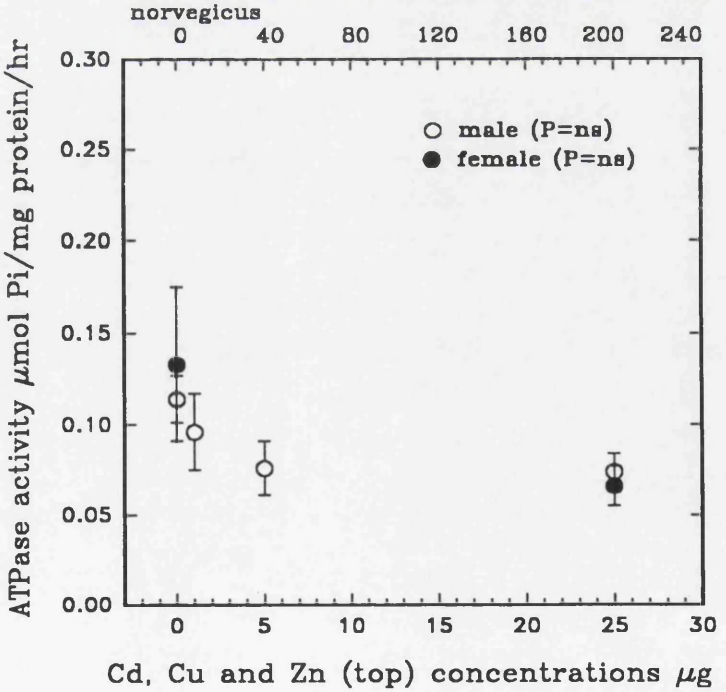


Figure 5.18. The effects of metal exposure on Na,K–ATPase activity in the gill of Nephrops norvegicus

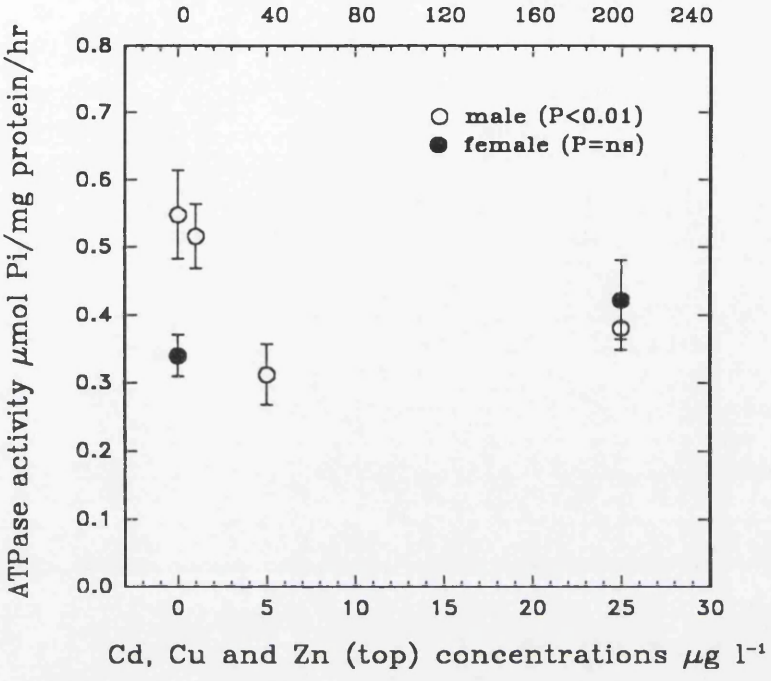


Figure 5.19. The relationship between copper and Na,K–ATPase activity in the gill of male Nephrops norvegicus

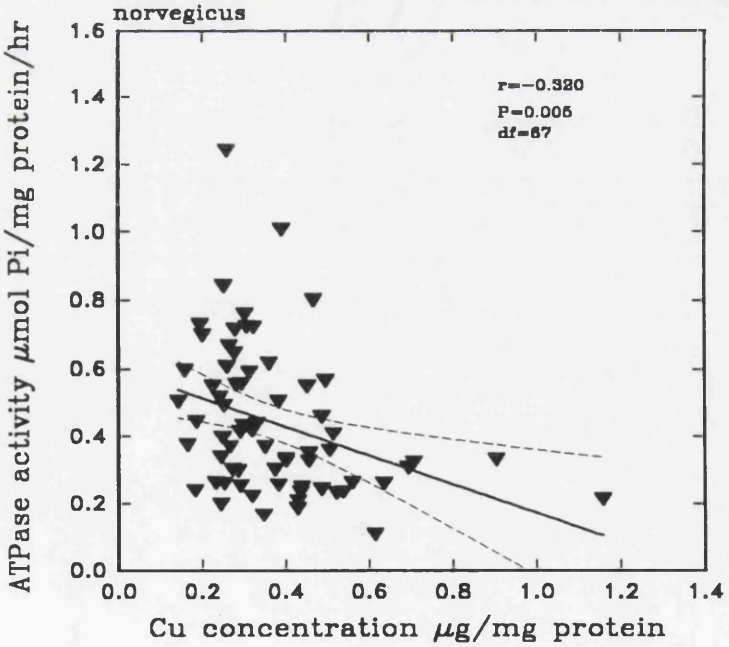


Figure 5.20. The relationship between zinc and Na,K–ATPase activity in the gill of female Nephrops norvegicus

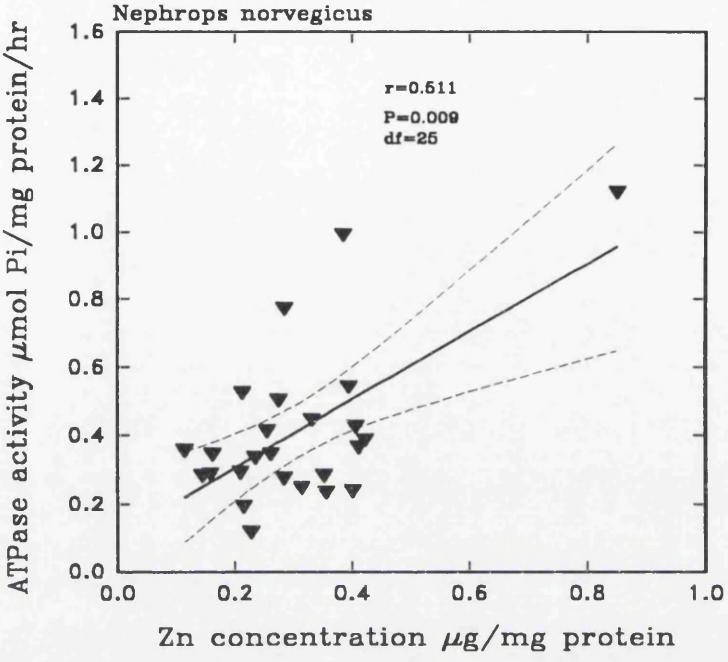
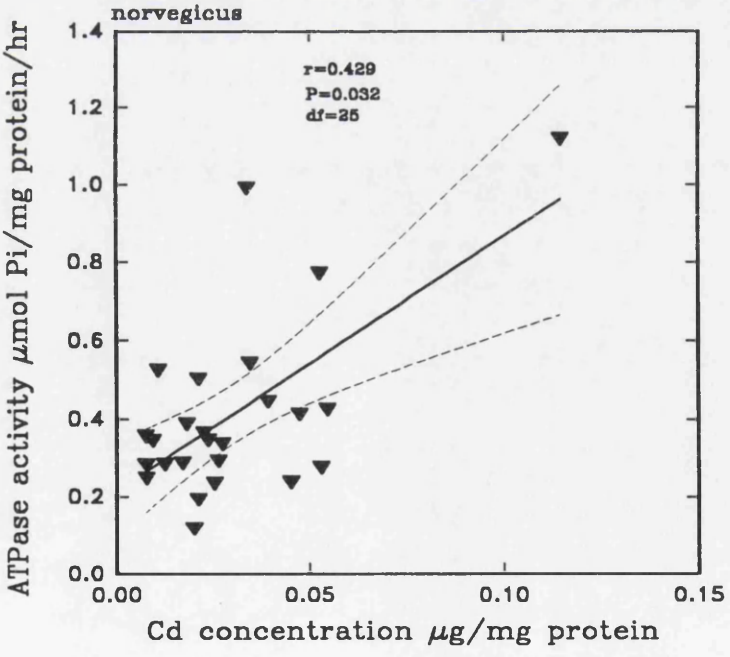


Figure 5.21. The relationship between cadmium and Na,K–ATPase activity in the gill of female Nephrops norvegicus



5.4 DISCUSSION

5.4.1 Characterization of the gill ATPases in Nephrops norvegicus

The optimum concentrations of Na^+ and K^+ in this study were found to be 100 and 20 mmol l^{-1} respectively (Figure 5.4a and 5.4b) which are similar to those reported in other studies carried out with decapod crustaceans (Towle et al., 1976 ; Horiuhi, 1977 ; Silva et al., 1977 ; Jowett et al., 1978 and 1981 ; Neufeld et al., 1980 ; Pequeux et al., 1984 ; Stern et al., 1984 ; Holliday, 1985 ; Trausch et al., 1989 ; Proverbio et al., 1990). The marked inhibition of ATPase activity by higher concentrations of Na^+ shown in this study was also shown in other decapod crustaceans (Horiuchi, 1977 ; Pequeux et al., 1984 ; Wanson et al., 1984 ; Harris and Bayliss, 1988). K^+ ion was also found to cause less inhibition at higher levels than Na^+ ions in decapod crustaceans (Hotiuchi, 1977 ; Wanson et al., 1984 ; Pequeux et al., 1984 ; Ventrella et al., 1990) as with *Nephrops*. The Mg^{2+} ion concentration used for the activities of Mg-ATPase is commonly in the range of 4-5 mmol/l in crustaceans (Tucker and Matte, 1979 ; Siebers et al., 1982 ; Haya et al., 1983 ; Pequeux et al., 1984 ; Harris and Bayliss, 1988 ; Torreblanca et al., 1989), although some studies on crustaceans used lower levels of Mg ion (Horiuchi, 1977 ; Wanson et al., 1984 ; Proverbio et al., 1990) or higher levels (Holliday, 1985 ; Towle and Kays, 1986). 6 mmol l^{-1} ATP was found to be optimal for all the ATPases studied in the gill and no marked inhibition occurred with increasing ATP levels (Figure 5.6a and 5.6b). Similar ATP concentrations were also used to study gill ATPases in other decapod crustaceans (Silva et al., 1977 ; Siebers et al., 1982 ; Haya et al., 1983 ; Pequeux et al., 1984 ; Wanson et al., 1984 ; Holliday, 1985 ; Dehn et al., 1985 ; Torreblanca et al., 1989). Similar paths of ATP in *Nephrops* was also shown in other decapod crustaceans (Stern et al., 1984 ; Wanson et al., 1984 ; Pequeux et al., 1984 ; Holliday et al., 1985). Ouabain completely inhibited Na,K-

ATPase at a concentration of 1 mmol l^{-1} (Figure 5.7). Similar sensitivity to ouabain has also been reported for other decapod crustaceans (Desaiah et al., 1972 ; Towle et al., 1976 ; Jowett et al., 1978 and 1981 ; Tucker, 1979 ; Siebers et al., 1982 ; Wanson et al., 1984 ; Holliday, 1985 ; Proverbio et al., 1990), although considerable variations in sensitivity have been reported (Silva et al., 1977 ; Horiuchi, 1977 ; Haya et al., 1983 ; Pequeux et al., 1984 ; Harris and Bayliss, 1988 ; Torreblanca et al., 1989 ; Trausch et al., 1989). For example, Harris and Bayliss (1988) could not detect Na,K-ATPase activity at a ouabain concentration of 10 mmol l^{-1} in a number of marine decapod crustaceans including *Nephrops*. This level of ouabain is 10 times higher than the present level and this study reports high levels of Na,K-ATPase activity in *Nephrops*. Oligomycin sensitive Mg-ATPase activity has not been studied widely and no data were found on the sensitivity of Mg-ATPase to oligomycin in decapod crustaceans. 0.3 μg ml^{-1} of oligomycin B caused 100 % inhibition with an IC50 value of 0.022 μg ml^{-1} (Figure 5.8). Desaiah et al. (1972) found the optimum inhibition at oligomycin (oligomycin A 15 %, and B 85 %) concentration of 0.03 μg ml^{-1} for the tissues of fish *Lepomis machrochirus*. Temperature of the incubation environment has been found to be an important factor for ATPase activity. All the ATPases studied had maximum activity at around 40 ^{o}C (Figure 5.9). Na,K-ATPase and oligomycin sensitive Mg-ATPase seemed to be much more heat sensitive than oligomycin insensitive Mg-ATPase. Although activities of Na,K-ATPase and oligomycin sensitive Mg-ATPase were zero at 5 ^{o}C and at 55 ^{o}C, oligomycin insensitive Mg-ATPase was still active at 5 ^{o}C and interestingly, approximately 50 % of the ATPase was active at an incubation temperature of 70 ^{o}C. Horiuchi (1977), Stern et al. (1984) and Holliday (1985) were also found maximum Na,K-ATPase activity at incubation temperatures of close to 40 ^{o}C in decapod crustaceans. However, there was no information from the literature on the effects of temperature on the two components of Mg-ATPase.

5.4.2 Variability of gill ATPases in decapod crustaceans

Activities of different ATPases ranged widely in the same group treatments. This suggests the importance of using large numbers to have more accurate measurement of ATPase activity. Tucker and Matte (1980) also found considerable variability in the activity of Na,K-ATPase in rock crabs, *Cancer irroratus*. The present results show that the sex of experimental animals is an important factor to consider, as male and female *Nephrops* had different degrees of inhibition by metals, and control males showed higher activity of Na,K-ATPase than in females. Neufeld et al. (1980) found that the effects of acclimation to different salinities caused different levels of Na,K-ATPase activity between male and female blue crabs, *Callinectes sapidus*. The condition of gill tissue is also an important factor to take into account ; for example it was observed that black spotted gills from three individuals (necrosis thought to be related to exposure to anoxic sediments) showed very high ATPase activities, having total ATPase activities of 3.498, 11.907 and 5.734 μmol Pi/mg protein/hr, respectively. Actually most of these activities were from Mg-ATPase, having activities of 1.865, 9.925 and 3.862 μmol Pi/mg protein/hr, respectively. Size of male animals was also found to be a significant factor (Figures 5.10-5.13) in determining ATPase activity. The activities of Na,K-ATPase and oligomycin sensitive Mg-ATPase were higher in smaller animals. Oligomycin sensitive Mg-ATPase is found in mitochondria and is involved in oxidative phosphorylation in the respiratory chain (Boyer et al., 1977). Laboratory observations have shown that smaller *Nephrops* are more active than larger animals and accumulate more mercury and cadmium in their gills from seawater, but not copper and zinc. This was related to higher activity of small animals than larger animals (Chapter 3). Animals which have high physical activity obviously would have higher ATP consumption as a result of high metabolic activity. These animals might also need more oligomycin

sensitive Mg-ATPase or turnover of this ATPase would be increased to give a higher oxidative phosphorylation rate. Higher activity of small animals could also increase efflux and influx of Na and K ions across membranes. Metabolic transfer of Na and K ions across the gill membrane may also be influenced by surface area of the gill. There is, however, no study found from literature on the effects of size on the ATPase activity. In decapod crustaceans, gill filaments can show differences in the activity of ATPases between anterior and posterior positions (Neufeld et al., 1980 ; Wanson et al., 1984 ; Holliday, 1985 ; Towle and Kay, 1986). In the present study all gills from one side were used to overcome these differences between the gill filaments. It is also well known that adaptation of marine decapod crustaceans to lower salinities causes an increase in Na,K-ATPase activity (Neufeld et al., 1980 ; Holliday, 1985 ; Towle and Kay, 1986). Adaptation to lower salinities may also show differences between different gill filaments and with sex in the blue crab, *Callinectes sapidus* (Neufeld et al., 1980). ATPase activity can differ among different developmental stages of crustaceans. Bouaricha et al. (1991) found that Na,K-ATPase activity differed among larvae, post larvae and adults of the shrimp *Penaeus japonicus* being lowest in larvae and highest in adults. When comparing ATPase activity from different studies, one should consider measurement time of the ATPase activity after killing animals. In this study, it was shown that ATPase activity was reduced after storage even at -70 ^{o}C, especially the activities of Na,K-ATPase and oligomycin sensitive Mg-ATPase.

5.4.3 Effects of in vivo metal exposure on the activity of gill ATPases

Natural concentrations of heavy metals in the tissues of *Nephrops norvegicus* have been found to be affected by sex, size and season and accumulation of metals from seawater could be affected by sex and size of the animals (Chapters 2 and 3). Male

and female animals were separated and the same size groups of the animals were used for the present study.

When considering the effects of individual metals on the activity of gill ATPases *in vivo* considerable variations have been reported. Tucker (1979) found that exposure of *Homarus americanus* to 6 ppb cadmium for 30 days *in vivo* did not alter Na,K-ATPase activity, while it enhanced Mg-ATPase activity. Haya et al. (1983) showed that in the lobster, *Homarus americanus* exposed to a high zinc concentration for 96 h *in vivo* significantly inhibited the activity of Na,K-ATPase. Interestingly the study also showed that effects were irreversible and activity failed to recover to control levels after 168 h depuration in normal seawater. Torreblanca et al. (1989) indicated that 1 ppm Cd *in vivo* did not show any clear effects on ATPase activities in the gill of freshwater crayfish *Procambarus clarkii*. Mg-ATPase was inhibited by cadmium. The present results, using combinations of Cu, Cd and Zn showed that Na,K-ATPase activities were significantly reduced by metals in male animals but not in females. Although the activity of oligomycin-sensitive and insensitive Mg-ATPase were inhibited by metals, these were not significant at 0.05 level in male or female animals. However, total Mg-ATPase activity became significantly inhibited in female animals at the highest exposure regime.

Interestingly, in male animals in which Na,K-ATPase activity was inhibited there were correlation dependent increases in levels of copper, zinc and cadmium following exposure to all three metals whereas in females there was no change in ATPase activity and cadmium levels in the gill increased but not those of copper and zinc. Comparisons of the relationship between Na,K-ATPase activity and levels of metals in the gill of individual male animals showed that there was a significant negative correlation with copper but not with cadmium and zinc. It is therefore

tempting to speculate that the inhibition of Na,K-ATPase in gills from male *Nephrops* is related to the accumulation of copper in the tissues and that no inhibition is observed in females because they are able to regulate copper and zinc in the face of environmental exposure. Regulation of essential metals has been shown for other decapod crustaceans (Bryan, 1964 ; White and Rainbow, 1982 ; Rainbow, 1985 ; Rainbow and White, 1989). They indicated that essential metals can be regulated by decapod crustaceans up to a threshold. Net accumulation of these metals begins after threshold levels are exceeded.

Studies on the effects of individual metals on the activity of ATPases in fish showed similar results to those from crustaceans. Stagg and Shuttleworth (1982) found significant inhibitions of Na,K-ATPase and Mg-ATPase in copper exposed flounder (seawater adapted) *Platichthys flesus in vitro*. They indicated that *in vivo* exposure to copper did not alter the activities of the enzymes. Watson and Benson (1987) exposed teleost fish *Lepomis macrochirus* and *Pimephales promelas in vivo* to different cadmium concentrations. They found that 1 μg Cd l^{-1} stimulated activity of Na,K-ATPase, while inhibitions were observed at higher exposures (10 and 100 μg Cd l^{-1}). Stagg and Shuttleworth (1982) indicated that *in vitro* exposures to metals, in general, causes a decrease in ATPase activity but *in vivo* effects are not so clear and possibly relate to some physiological alterations on the enzyme characteristics as well as direct effects of the metals. This is also true for the present study as inhibition of ATPase activity by metals in the gill of *Nephrops norvegicus* still remains unclear and needs more study. Especially, it is essential to examine the turnover rate of ATPases under normal and metal-exposed conditions to obtain a better understanding of the activities of ATPases.

5.5 REFERENCES

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CHAPTER 6

THE INDUCTION OF METALLOTHIONEIN IN THE TISSUES OF THE NORWAY LOBSTER, *NEPHROPS NORVEGICUS* AFTER EXPOSURE TO CADMIUM, COPPER AND ZINC

6.1 INTRODUCTION

Metallothioneins (MTs) are ubiquitous, heat stable, low molecular weight proteins that are characterized by an unusually high cysteine content (22 to 33 %) and a selective capacity to bind heavy metal ions such as mercury, cadmium, copper and zinc. MT was first discovered as a cadmium-binding protein in equine renal cortex by Margoshes and Vallee (1957) and this was further investigated and called 'metallothionein' by Kägi and Vallee (1960). Subsequently, MTs were found in different groups of living organisms such as prokaryotes, invertebrates and vertebrates including 80 species in the aquatic environment (Roesijadi, 1992). Metallothioneins occur in many tissues of mammals including liver, kidneys, pancreas, intestine, brain, bone marrow, and reproductive organs and they show close relationships with metal ions and metal metabolism (Bremner and Beattie, 1990).

6.1.1 Physicochemical Properties of Metallothionein

The mammalian form of metallothionein has ;
a single polypeptide chain consisting of 61 amino acid residues,
molecular weight of approximately 6000-7000 dalton
high cysteine content (33% ; 20 of 61 amino acid residue)
absence of disulfide bonds
absence of aromatic amino acids
Metal-binding capacity of 7 g-atoms/per mole protein
cytosolic localisation
heat stability and polymorphic configuration (Kägi and Schäffer, 1988 ; Klaassen and Lehman-McKeeman, 1989).

Although these characterization of metallothionein are basic to metallothioneins, there are also different forms of metallothioneins in animals. Metallothioneins were divided into three classes: MT I, MT II and MT III. Class I includes mammalian MTs and polypeptides from other phyla with related primary structure. Class II comprises MTs displaying no or only very distant correspondence to the mammalian forms, e.g., MTs from sea urchins, yeasts, and certain prokaryotes. Class III groups polypeptides containing γ- glutamylcysteinyl units (Kägi and Schäffer, 1988). All class I and II MTs characterized have been found to be single-chain proteins. Mammalian forms contain 61 to 62 amino acid residues; chicken MT and sea urchin MTs contain 63 and 64 residues, respectively. The shortest chain MT was found in a fungus *Neurospora crassa* having 25 amino acid residues. Class III MTs are often oligometric structures made up of two or more polypeptide chains of variable length (Kägi and Schäffer, 1988). Olafson et al. (1979 b) indicated that MTs show marked similarities on comparisons of molecular weight, U.V. absorption spectra, isoelectric points and amino acid composition in different groups of living organism. For example, MTs from the decapod crustacean *Scylla serrata* have been shown to be remarkably similar to mammalian MTs (Lerch et al., 1982 ; Otvos et al., 1982). Brouwer et al. (1989) also isolated copper metallothioneins MT I, MT II, and MT III from the American lobster, *Homarus americanus*. They indicated that MT I group proteins related to equine renal cortex metallothioneins and this group MTs can not transfer copper to copper-depleted apohaemocyanin. CuMT II belongs to the same class as CuMT I, but CuMT III cannot. They strongly suggested that the different forms of MT have different biological functions. The most conspicuous feature of all forms is, besides the abundance of Cysteine totalling up to one-third of all residues, the frequent occurrence of cys-x-cys tripeptide sequence, where x is an amino acid residue other than cysteine (Kägi and Schäffer, 1988). There are also heavy metal-binding proteins which are not metallothioneins. These non-metallothionein

cadmium, and zinc binding proteins have been characterized and found to show one or more differences than mammalian MTs (Stone and Overnell, 1985 ; Baer and Thomas, 1990). These non-metallothionein low molecular weight proteins usually differ from metallothioneins by having aromatic amino acids and low cysteine contents (George, 1990).

6.1.2 Induction of Metallothionein

Metallothionein production can be induced by many chemical and physical factors. Most important inducers of MTs, however, are heavy metals including cadmium, copper, zinc, mercury, gold, and bismuth (Bremner and Beattie, 1990), though the most effective inducers are cadmium and zinc (Klaassen and Lehman-McKeeman, 1989). Induction of metallothioneins by heavy metals in tissues of animals including mammals, fish and crustaceans occurs after exposure to heavy metals (Roesijadi, 1982 ; Otvos et al., 1982 ; Bonham and Gedamu, 1984 ; Engel and Brouwer, 1986 ; Kägi and Schäffer, 1988 ; Klaassen and Lehman-McKeeman, 1989 ; Hogstrand and Haux, 1989 ; Howard and Hacker, 1990 ; Bremner and Beattie, 1990 ; Roesijadi, 1992). The rate of MT synthesis closely parallels the production of metallothionein mRNA (Bonhams and Gedamu, 1984), and a high rate of transcription can be detected within one hour of stimulation by metals. The mRNA levels reach a maximum at about 6-8 hours after exposure to an inducer, although maximal levels of MT occur after 1-2 days (Bremner and Beattie, 1990). Jones et al. (1988) showed that induction of metallothionein by toxic heavy metal ions depends on electronic configurations of the metals. For example, induction of MTs was found to be prominent by ions with electronic configurations of $(n-1)d^8$, $(n-1)d^9$, $(n-1)d^{10}$ and ns^2- $(n-1)d^{10}$. These electronic configurations are also those of both the softest and many of the most toxic metal ions. They indicated that the relative ability of toxic

heavy metals to induce metallothionein is correlated with their softness parameters. Stress can also affect the induction of MTs in animals, though it is not clear if stress stimulates synthesis of MTs independently and directly, or its influence is translated by cellular mediators. It could be argued that all known inducers, including heavy metals, are stress factors and may elicit a general stress response in addition to any specific effect (Karin, 1985 ; Bremner and Beattie, 1990). Many of the stress inducers also raise circulating levels of glucocorticoids, which stimulate metallothionein synthesis (Karin, 1985 ; Bremner and Beattie, 1990). Steroid hormones such as estrogens and progesterone can also induce metallothionein synthesis. Bacterial infection was also shown to induces a marked increase in hepatic metallothionein levels (Sobocinski et al., 1978 ; Karin, 1985). Induction of cadmium-binding proteins increased when the grass shrimp *Palaemonetes pugio* was exposed to cadmium at higher temperature and lower salinity than control exposure (Howard and Hacker, 1990). In this case, high temperature and low salinity are stress factors, and also factors which increase uptake of cadmium from seawater. Other stressful conditions such as very cold or hot environment, strenuous exercise, burns and X-radiation could also increase levels of metallothioneins (Klaassen and Lehman-McKeeman, 1989).

6.1.3 Role(s) of Metallothionein

Although the role(s) of MTs in living organisms are not completely clear, it is now known that induction of MT is closely related to exposure to metals such as cadmium, copper and zinc and therefore one of the possible role of MTs could be a detoxification mechanism in animals against toxic effects of heavy metals. Metallothionein was discovered as a cadmium-binding protein, and speculations began after that as to its role in the detoxification of cadmium and other heavy

metals. Much evidence supported this assumption. Experimental studies in marine animals have showed that exposure of marine animals to heavy metals either by water and food or by injection increased the level of MTs (Olafson et al., (c) 1979 ; Otvos et al., 1982 ; Bonham and Gedamu, 1984 ; Engel and Brouwer, 1986 ; Hogstrand and Haux, 1989 ; Howard and Hacker, 1990). Once animals have been exposed to elevated levels of metals such as Cd, Cu and Zn, thionein synthesis is stimulated by these metals and levels of thionein increase markedly and metals bind to thionein by the cluster of thiolate bonds (Kägi and Schäffer, 1988). Bonhams and Gedamu (1984) also showed that exposure of fish to heavy metals causes increases in the induction of mRNA which indicates the induction of metallothioneins by heavy metals. When metals become part of metallothioneins, they are no longer toxic to animals. Thus, MTs are efficient detoxification mechanisms for metals which will be important in adaptation to elevated metal levels in the environment. Protection against toxic effects of cadmium has been found to be dependent on presynthesized metallothioneins (Kito et al., 1982 ; Goering and Klaassen, 1984). However, binding of heavy metals by MTs may not be enough for protection against heavy metal toxicity, when uptake rates of these metals exceed the induction rate of MTs in tissues of marine animals. Perhaps, toxicity of metals begins only after the rate of metal uptake exceeds the induction of metallothionein.

In contrast, metallothioneins are naturally present in animals serving as storage forms for the essential trace metals copper and zinc and play roles in both the extracellular (homeostatic) and intracellular control of zinc and copper metabolisms. Karin (1985) indicated that production of MTs against heavy metal toxicity is unlikely to be the primary function of MTs. First these metals ions are not present at high levels in most biotopes and probably do not exert a selection pressure significant enough to justify the existence of a special detoxification system. Second, if the role of MTs

was purely protective, one would expect to find these proteins only after exposure to toxic heavy metals. In fact, the basal level of expression of MT is relatively high (Karin, 1985). The intracellular concentrations of metals must be regulated for the maintenance of essential life processes. MTs could be the prime regulators of intercellular metal concentrations (George, 1982). Hamer (1986) also indicated that the synthesis of MTs is homeostatically regulated in cells and organisms exposed to heavy metals. There is evidence that MTs are actively involved in the production of haemocyanin and in zinc regulation in crustaceans (Brouwer et al., 1986 ; Engel, 1987 and Engel and Brouwer, 1987). In their model, MT-bound copper and zinc appear to be regulated at the cellular level for the synthesis of MTs such as haemocyanin (copper) and carbonic anhydrase (zinc), both of which are essential for normal growth and survival. Levels of MTs also showed close relation to copper and zinc metabolism during different developmental stages of crustaceans. Therefore, the basal levels of MT are considered to be involved in essential metal regulation, particularly regulation of copper and zinc as these metals are most often associated with basal MTs. For non-essential metals such as mercury and cadmium, however, binding by MT most likely represents a sequestration function associated with protection against toxicity of these metals (Roesijadi, 1992). Metallothionein was also suggested to be involved in the control of copper and zinc metabolism in mammals indicating that the synthesis of MTs enables the organism to adapt to changes in intracellular concentrations of zinc and copper and prevents adverse reactions with enzymes, membranes, or other molecules. It may also provide a buffering capacity that maintains intracellular steady-state kinetics for copper and zinc and ensures a supply of these metals for other metabolic functions (Klaassen and Lehman-McKeeman, 1989 ; Bremner and Beattie, 1990). George (1982) indicated that the composition of naturally occurring metallothioneins is variable and dependent upon the tissue of origin. For example, in the liver of newborn vertebrates

Cu-thionein predominates, while in the adults Zn-thionein is the major form and Cd-thionein accumulates with age. George (1982) also indicated that the metal-transporting proteins usually contain only one or two atoms per molecule (in common with metalloenzymes) and, subsequently, do not account for large accumulations of metals within the body. In contrast, metallothioneins have the capacity for binding large numbers of metal ions and may make a significant contribution to the total metal burdens.

Much attention has been paid to the occurrence and characterization of MTs in different species of marine animals, whereas quantification of MTs have gained less attention partly due to the difficulty of satisfactory measurement techniques of MTs. Differential pulse polarography (DPP) has been gaining importance for quantification of MTs in marine animals (Roch et al., 1982 ; Olsson and Haux, 1986 ; Bebianno and Langston, 1991 and 1992) after improvements of Brdicka's (1933) technique for the estimation of thiolic proteins described by Olafson and Sim (1979 a) and Thompson and Cosson (1984). DPP has also been shown to be a reliable quantification method of MTs in a study which compares DPP and radioimmuno-assay (Hogstrand and Haux 1992) and was found to show better correlation in relation to MT concentrations than that of cadmium saturation assay (Onasaka and Cherian, 1982).

The aim of this study is to investigate the induction of MTs and its relation to the metals cadmium, copper and zinc in different concentrations in the gill and hepatopancreas of male and female *Nephrops norvegicus*. Quantification of MTs was carried out by differential pulse polarography. Contamination of seawater by metals were representable in natural marine environment (Nolting, 1986 ; Peerzada and Ryan, 1987 ; Balls and Topping, 1987). Combinations of copper, cadmium and zinc

were used since they are rarely present alone in the environment. There is a need to develop methods to detect the presence of trace metals in the environment before any pathological damage occurs. The direct measurements of metal concentrations dissolved in water suffers from two drawbacks; firstly, metal levels may vary with time, secondly, toxicity may be greatly reduced by the concomitant release of complexing agents (Overnell et al., 1987). Metallothioneins could be used as an indicator of metal contamination in the aquatic environment (Olafson et al., (b) 1979 ; Roch et al., 1982 ; Haux and Förlin 1988 ; Hogstrand and Haux, 1990). The possibility of the use of MTs as indicators of cadmium, copper and zinc contamination will be discussed for the gill and hepatopancreas of *Nephrops norvegicus*.

6.2 MATERIALS AND METHODS

Animals used for ATPase assays in Chapter 5 were also used to measure metal and metallothionein concentrations in the gill and hepatopancreas. Therefore, sampling, and experimental procedure are the same as explained in Chapter 5 until tissue preparations.

6.2.1 TISSUE PREPARATION FOR METALLOTHIONEIN ANALYSES

6.2.1.1 HOMOGENIZATION OF THE TISSUES

Homogenization of the gills and hepatopancreas was carried out in a cold room with a constant temperature at approximately 4 ^{o}C. All equipment was washed prior to use in 10 % nitric acid (Analar) and rinsed with distilled water. Washing with 10 % nitric acid and rinsing with distilled water was also carried out between samples. Frozen gill samples were slightly thawed on ice to help separation of the filaments

and the arches on petri dishes. Between 100-350 mg of the filaments of the gills and the hepatopancreas were weighed using a Mettler AE240 5 place balance and put into homogenizers which were a hand driven glass homogenizer for the gill filaments and a silica glass mortar with motorized teflon pestle for the hepatopancreas. A 50 mM Tris buffer was prepared using 3.0275 g Trizma Base (Tris [hydroxymethyl] aminomethane, Sigma) in 500 ml double distilled water. The pH of this buffer was brought to 8 with concentrated HCl (PHM84 Research pH meter, Radiometer). 1500 μl of 50 mM Tris buffer was added to each sample and the tissues were homogenized with the homogenizer. 500 μl of crude homogenates were transferred into Eppendorf test tubes (acid washed) to determine total metal concentrations. 100 μl of crude homogenates were also transferred into Eppendorf tubes to determine total protein concentrations. Remaining homogenates were poured into other Eppendorf test tubes (acid washed) and centrifuged for 5 minutes at 10,000 g (Centrifuge 5415 C, Eppendorf). Supernatants were decanted and transferred to acid washed Eppendorf tubes to form the 'cystosol' fraction.

6.2.1.2 Preliminary experiment on the contents of heat-treated homogenate

A preliminary experiment was carried out to check if there were any high molecular weight heat-stable thiol proteins which could respond in the pulse polarography. For this study, heat treated homogenates of *Nephrops* gills were run on a gel filtration column (Pharmacia LKB Biotechnology, Hiload 16/60). The column was used in conjuction with a detector, and eluted with a 0.15 M Tris (buffered to pH 8 with HCl) and 0.05 M mercaptoethanol buffer, at a rate of 1 ml/min. The absorbance of the eluate was monitored at 254 and 280 nm and 2.5 ml fractions collected for analysis of copper, cadmium, zinc and metallothionein. A range of molecular weights standards such as albumin (66,000), carbonic anhydrase (29,000),

cytochrome C (12,400), apoprotinin (6.500) and adrenocorticoptropic hormone (4,567) was used as molecular weight markers to identify the approximate molecular weight of the fractions obtained. Two different homogenates of the gill were run on the column, firstly an unfiltered sample, then a sample which had been filtered using an ultrafiltration unit (Millipore, cellulose) with a nominal molecular weight of 30,000. The results of the gel filtration of the unfiltered sample showed a large protein peak eluting at 40-50 ml. This corresponds to molecular weights of more than 70,000, and is clearly not metallothionein (Figure 6.1). The subsequent peaks on the trace, however, correspond to molecular weights of less than 10,000, and will contain the metallothionein proteins. Analysis of the high molecular weight fractions for cadmium, copper, zinc and thiol proteins showed that the high molecular weight fractions contained trace metals and also heat stable thiol proteins which were not metallothionein. These results showed that if trace metal and metallothionein analyses were to be carried out on the untreated homogenate then erroneous results would occur. The gel filtration trace of the filtered sample showed that the high molecular weight protein peak was removed by filtration. It was therefore decided that the analyses of all *Nephrops* tissues should by carried out on filtered samples to enable investigation of the low molecular weight metallothionein fraction.

6.2.1.3 Preparation of Metallothionein Extract

The cytosol fractions were placed in a heating block (Thermostat 3401, Eppendorf) for 4 minutes at 95 ^{o}C, then centrifuged at 10,000 g for 5 minutes. 400 μl supernatant was decanted for each sample and transferred to an ultrafilter test tube (Millipore, Cellulose, 30,000 MW) and centrifuged for 30 minutes at 10,000 g. Concentrations of metallothionein were determined in this filtered fraction. These processes are summarised in Figure 6.2. All fractions were kept at -70 ^{o}C until use.

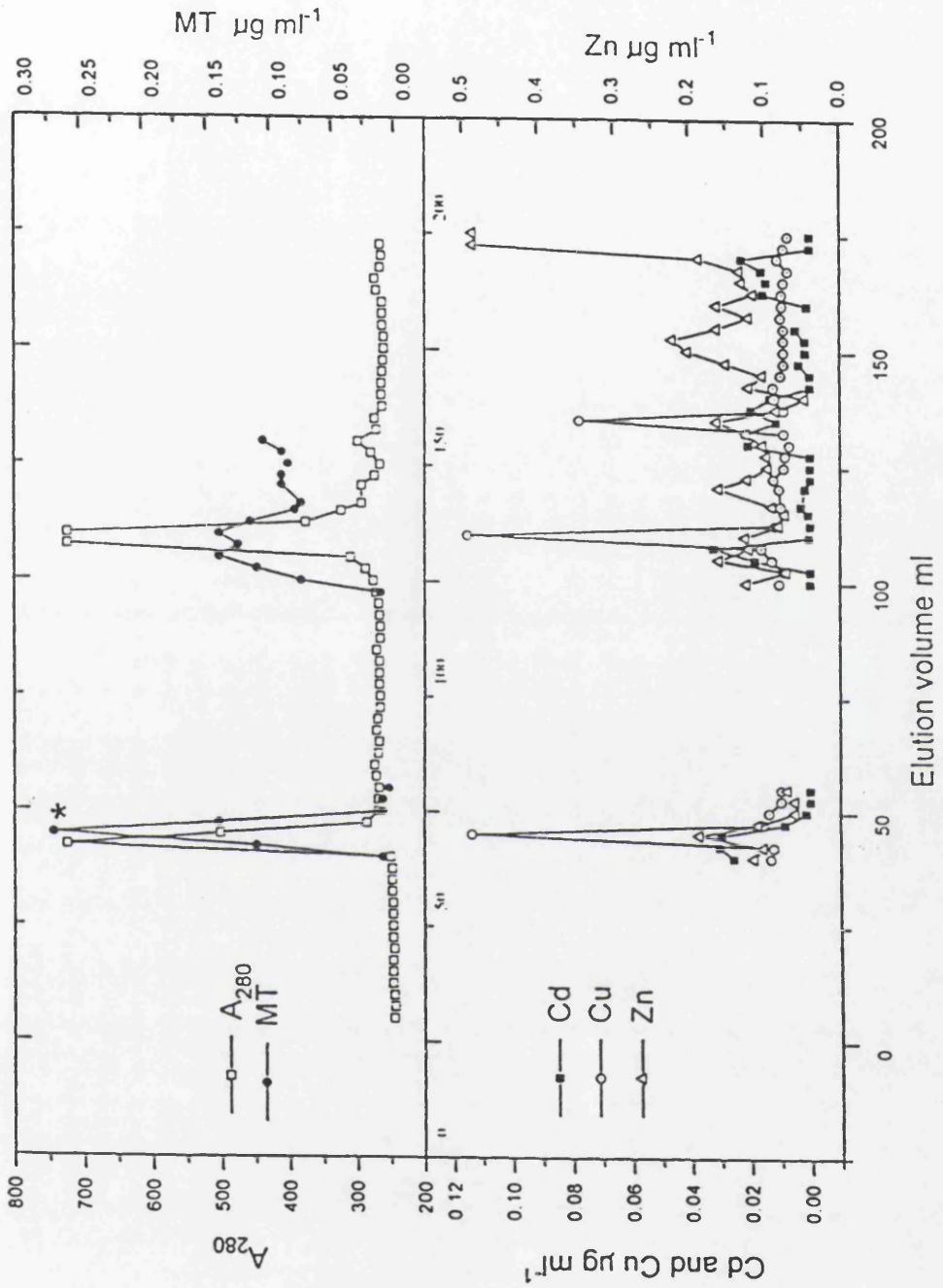
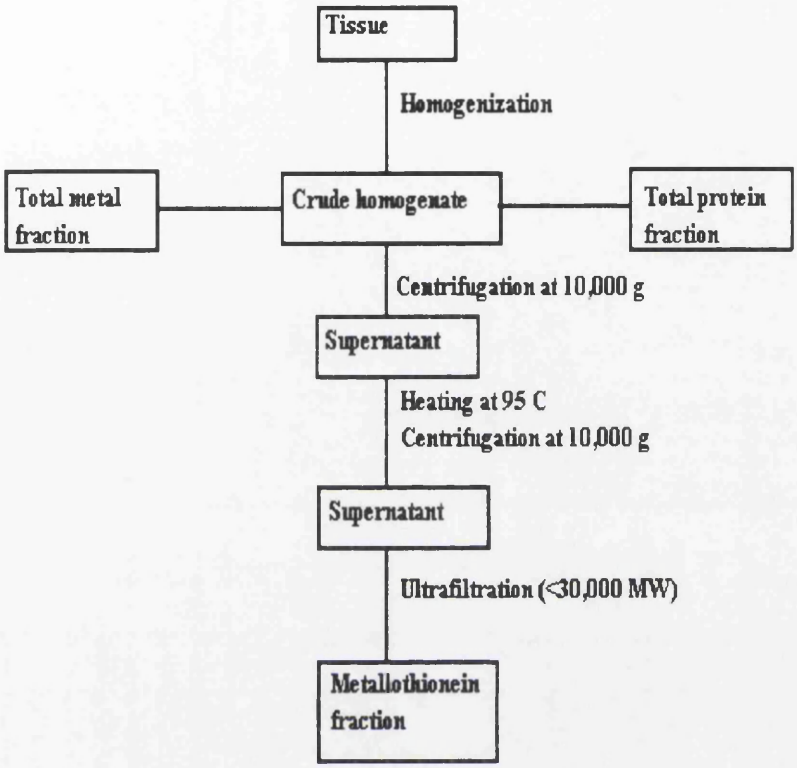


Figure 6.1. Elution profile from gel (Sephadex G-75) filtration on the content of heat-treated cystosol of the gill. * indicates high molecular weight thiol proteins which are not MTs.

Figure 6.2. Schematic representation of tissue preparation for analyses of total metal, total protein and metallothionein.



6.2.2 DETERMINATION OF THE METALS IN CRUDE HOMOGENATE

Concentrations of cadmium, copper and zinc in the crude homogenate of the gill filaments were determined by Flame Atomic Absorption Spectrometry (FAAS) and Graphite Furnace Atomic Absorption Spectrometry (GFAAS) after digestion of samples with nitric acid. Throughout this thesis, concentrations of metals in different Chapters were measured in three different ways which were a) Flame Atomic Absorption Spectrophotometry, b) Graphite Furnace Atomic Absorption Spectrophotometry and c) Atomic Emission Spectrophotometry. Analytical information is given about these methods in the following section.

6.2.2.1 Atomic Absorption Spectrophotometry

Atomic Absorption Spectrometry (AAS) is commonly used to determine the concentrations of metals. This technique involves the fact that free atoms of an element in the ground electronic state absorb radiation in the form of light at a particular wavelength. The amount of radiation absorbed is proportional to the number of atoms present. The concentration of an element can be determined from this relationship. AAS involves producing a cloud of atoms of the metal in a free state, shining a beam of light covering a narrow spectral range through it, and measuring the reduction in the transmitted radiation. The output signal is processed and amplified before being read out as an absorbance value which is proportional to metal concentration.

6.2.2.2 Flame Atomic Absorption Spectrophotometry (FAAS)

In FAAS the sample solution is drawn up through a fine capillary tube and aspirated into a flame. The solution moves up the capillary because of the decreased pressure created by the expansion of the oxidant, in this case air, which is flowing over its upper end (the Venturi effect). A cloud of droplets of approximately 5-10 μm diameter is formed mixed with the fuel/oxidant gas mixture (nebulisation). In the flame the solvent evaporates leaving a dry aerosol consisting of solid or molten particles of the solute. Due to the high temperature of the flame these are volatilized then atomised to form free metal atoms. A light source is required, the most common type being hollow cathode lamp (HCL). The cathode is cylindrical and is made of the metal under investigation. The lamp is filled with a carrier gas (an inert gas) through which an electrical discharge is passed. Positive ions are then generated which collide with the cathode, releasing atoms from it. These atoms radiate light of

specific wavelengths forming characteristic spectral lines. In electrodeless discharge lamps (EDLs), electrons are generated by a discharge in a noble gas which fills the lamp at low pressure. Collisions of the electrons excite the atoms of the analyte. The beams from the light sources pass through the area of atomised metal in the flame and a photodetection system measures the amount of radiation absorbed. This takes the form of a monochromator and photomultiplier tube which isolate the required spectral range and measure light intensity respectively. The signal is processed electronically before being displayed.

6.2.2.3 Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS)

FAAS is not sensitive enough for analysis of metals present at very low concentrations. This is because full atomisation must take place within the few milliseconds during which the volatilized sample is in the flame, and also due to chemical interferences from decomposition products. GFAAS has enhanced sensitivity and has the added advantage that only a small volume of sample is necessary. The basic processes of GFAAS are similar to those of FAAS but instead of the sample being atomised in a flame, a small volume is injected into a graphite tube which is heated in a programmed series of temperature changes. This allows the volatilization steps to take place separately, the solvent and matrix substances being removed by a constant stream of an inert gas (argon). While the furnace is held at the atomised temperature, the flow of gas can be stopped so that atoms of the metal are not diluted by the gas flow and remain in the radiation beam for up to several tenths of a second. The partial pressure of atoms in the light beam is therefore increased, allowing more atoms to absorb light and hence increasing sensitivity.

The instrument used in this investigation uses Zeeman background correction. The application of a strong magnetic field to the atoms causes splitting of the spectral lines absorbed. Measurement of absorbance with and without magnetic field allows detection of the amount of absorbance due to background, which can then be subtracted from the total amount due to the metal atoms plus the background substances present (Zeeman 3030 Operators Manual).

6.2.2.4 Atomic Emission Spectrophotometry

Atomic emission spectra are observed when photons are emitted by atoms in an excited energy state during their return to a less excited state. The frequencies of lines are characteristic of the elements present and can, therefore, be utilized in qualitative and quantitative analyses.

For the excitation in atoms, energy sources of various kinds are used. Low energy sources such as gas/air flames excite relatively few lines, yielding simple spectra which can be resolved with sample apparatus. The sample for analysis is vaporized into the excitation source (for metals this is usually done by using a rod of the metal as one of the electrodes of arc), and the light thus produced is focused on to the entrance slit of the monochromator. The whole spectrum is focused simultaneously on an optical plane where it is recorded on a photographic plate. Alternatively, a series of photosensitive detectors can be placed along the optical plane to receive discrete spectrum lines (Betteridge and Hallam, 1972).

6.2.3 DIGESTION OF CRUDE HOMOGENATE

The digestion of crude homogenate was carried out in a laminar flow hood connected to a fume cupboard (Grundy Equipment) using 10 ml glass beakers (Pyrex). Before use, each beaker was heated at 70 ^{o}C in concentrated nitric acid (Analar) for ten minutes and rinsed in distilled water. They were kept in a covered tray to avoid contamination. 30 sample of crude homogenates were defrosted and measured using micropipettes (Eppendorf). A measured volume of crude homogenate was poured into the beakers. 5 ml nitric acid (Aristar) was added to each sample and beakers were placed on a hotplate (Thermostat, Gallenkamp) in the fume cupboard. Approximately 40 mg of reference material (in duplicate) were also included in 10 ml beakers in each batch of samples (TORT I lobster hepatopancreas, certified reference material, Marine Analytical Chemistry Standards Program, National, Research Council, Canada). Results of these measurements are given in Table 6.1.

Table 6.1. Concentrations of metals (μg g^{-1} d.w.) in the reference material R.C. = Reference concentrations, P.C. = Present concentrations measured in GFAAS for cadmium and copper and in FAAS for zinc.

	Cadmium	Copper	Zinc
R.C.	26.3±2.1	439±22	177±10
P.C.	25.2±2.8	416±30	167±15

Temperature of the hotplate was gradually increased from approximately 50 ^{o}C to approximately 150 ^{o}C. Samples were digested until 0.5 ml of total volume remained in the beakers. They were removed from the hotplate and placed on a cool plate in the fume cupboard to cool the samples. A total of 300 μl hydrogen peroxide (30 % Analytical, FSA) was added to each sample in 100 μl aliquots and the beakers

returned to the hotplate. Samples were kept on the hotplate until all visible reaction had finished. After the third addition the samples were left on the hotplate until dryness but not burning point. 5 ml 10 % nitric acid (Aristar) was added to each sample. Determinations of the metals were carried out on this 5 ml sample after appropriate dilutions of samples to give reading within the range of standards.

6.2.4 ANALYSIS OF CADMIUM AND COPPER

Concentrations of cadmium and copper were determined by Graphite Furnace Atomic Absorption Spectrometry (GFAAS) using a Zeeman 3030 GFAAS (Perkin Elmer). The instrument was allowed adequate warm-up time of the lamps, approximately 25 minutes for Cu HCL (Intensitron hollow cathode lamp, Perkin Elmer) and one hour for the Cd EDL (Electrodeless discharge lamp, Perkin Elmer). The graphite tube and L'vov platform (Perkin Elmer) were placed and conditioned using a conditioning program before each run of samples. Operating conditions of instruments were as follows;

HGA cooling system (Perkin Elmer)

Autosampler (Perkin Elmer AS60)

Printer (Anadex 'Silent Scribe')

Slit Width : 0.7 nm

Signal Processing : peak area

Modifier : 5 % NH4H2PO4 solution

	CD ANALYSES	CU ANALYSES
Wavelength (nm)	228.8	324.8
Lamp current EDL setting	5	15
Atomisation temperature (^{o}C)	1600	2500
Modifier volume	5	10

6.2.4.1 Preparation of Cadmium and Copper Standards

Standards were prepared using commercial stock solutions of the metals (BDH, Chemicals). Stock concentrations of cadmium nitrate, copper nitrate and zinc acetate were diluted appropriately with 10 % nitric acid. 7 concentrations of standards were prepared between 0.0009-0.0074 μg ml^{-1} for cadmium and between 0.009-0.074 μg ml^{-1} for copper. Standards were prepared immediately before each analysis in individual autosampler cups. The instrument was calibrated with a 10 % nitric acid (Primar, FSA) blank and the above standards. Standards were also checked during sample reading and at the end of analysis. Samples were diluted with 10 % nitric acid into the range of the calibration standards if they were higher than the highest standard.

6.2.4.2 Standard Addition

Atomization of metals in the GFAA may be affected by the matrix in the solution. To test this, different tissue digests were analysed using the method of standard additions to check that the samples reacted in the same way as standards. For this, concentrations of metals in digested samples were measured, and at least two different concentrations of standards were added and mixtures reanalysed. Results of this mixture gave "observed values", while the total of individual measurements gave "expected values". These results were statistically analysed to test for any difference. Metallothionein concentrations were also checked in the same way. There was no significant difference ($P>0.05$) in any fraction analysed between "expected" and "observed" values after standard additions. As an example of these measurements, mean values and standard deviation of samples, standard addition, expected values and observed values of metals (copper and cadmium) measured in GFAAS and

metallothionein in the gill of the animals are given in the following table (Table 6.2) indicating results of Mann-Whitney U-tests between observed and expected values.

Table 6.2. Results of Mann-Whitney U-tests between observed and expected concentrations of metals and metallothionein in the gill of *Nephrops norvegicus* after standard additions. Metal concentrations in the crude homogenates are given as μg ml^{-1}, while metallothionein values are given as peak height (mm). ns = not significant (P>0.05).

	Sample	Standard	Expected	Observed	P value
Cadmium					
Mean	0.00153	0.00148	0.00305	0.00292	ns
sd	0.00036	0.00050	0.00066	0.00079	
Copper					
Mean	0.0176	0.0147	0.0322	0.0295	ns
sd	0.0068	0.0050	0.0083	0.0090	
MT					
Mean	19.3	31.4	50.9	49.0	ns
sd	7.7	11.4	11.4	11.0	

6.2.5 ANALYSIS OF ZINC

Concentrations of zinc were determined by Flame Atomic Absorption Spectrometry (FAAS) using a Perkin Elmer 5000 FAAS. Operating conditions of the instrument were ; wavelength (nm) = 213.9, lamp type = HCL, Time (sec) = 2, background correction = on, fuel = acetylene, oxidant = air.

6.2.5.1 Preparation of Zinc Standards

Zinc standard solutions were also prepared in the same way explained for copper and zinc. Zinc standards were prepared as 100 ml stock using acid washed volumetric flask and micropipettes (Eppendorf). 7 concentrations of zinc standards were prepared in the range of 0.1-1.0 μg ml^{-1}. The instrument was zeroed on double

distilled water then 10 % nitric acid, and calibrated with the above standards. Standards were checked during sample reading and at the end of analysis. Samples which showed high zinc concentrations were diluted with 10 % nitric acid to bring absorbance into the calibration range.

6.2.6 CALCULATION OF METAL CONCENTRATIONS

Concentrations of the metals were calculated from the calibration curves obtained by absorbance values and standard concentrations of each metal from each run. Diluted samples were multiplied by appropriate factors to find real concentrations. Finally all samples were multiplied by the amount of acid used (5 ml). This value was multiplied by volume of crude homogenate digested and divided by 1000 μl (1 ml) to give metal concentrations in 1 ml crude homogenate (μg metal ml^{-1}). After this point metal concentrations can be expressed in two ways. First, metal concentration in crude homogenate (μg ml^{-1}) is divided by the protein concentration of crude homogenate (mg ml^{-1}) which will give an expression of μg metal / mg protein. Secondly, concentrations of metals in crude homogenate are multiplied by volume of Tris buffer used and tissue wet weight (1 mg tissue assumed to be 1 μl) and then divided by wet weight of tissues which will give an expression of μg metal g^{-1} wet weight of tissue. Metal concentrations throughout this thesis are expressed as μg metal / mg protein where protein analysis for the tissues was carried out. Wet weights of tissues were not used for the calculation of the result due to possible differences of water level between animals.

6.2.7 ANALYSIS OF METALLOTHIONEIN

Concentrations of metallothionein in the gill filaments and hepatopancreas of *Nephrops* were determined by differential pulse polarography (DPP). The analysis was carried out in ultrafiltered cytosol fraction of the gill and hepatopancreas which were heat-treated and centrifuged to coagulate and remove heat-sensitive proteins, leaving only heat-stable proteins. The DPP is sensitive to any thiol proteins present, but can be assumed to measure metallothionein (MT) or MT-like proteins due to heat sensitive thiol proteins and proteins of molecular weight of higher than 30,000 being removed by heating and ultrafiltering.

6.2.7.1 Polarography

Polarography is an electrochemical technique which can be used in analysis of a wide range simple and more complex molecules, both quantitatively and qualitatively. With a dropping mercury electrode (DME), the working electrode takes the form of drops at the end of a capillary in a controlled, regular manner. This method is advantageous in that each drop has a reproducible surface area and the surface is constantly renewed and remains clean, avoiding build-up of the products of any chemical reactions which might occur. Both these factors reduce the errors found with other types of electrodes. Current flows between the working electrode and the counter electrode, which is made of an inert, conductive material (in this case platinum wire). A third silver/silver chloride reference electrode provides a stable potential with which the changing potential of the working electrode is compared, and also enables compensation to be made for the electrical resistance of the solution.

The electrodes are linked to a polarographic analyzer whose function is to control the applied potential and measure the current. Measured current is plotted against applied potential to form the polarographic wave which is recorded on a chart recorder. The nature of the wave is dependent on the electrochemical reactions (oxidation or reduction) of the sample at the electrode (Princeton Applied Research Application Note P-2, 1980).

When a certain potential is applied, corresponding to the redox potential of the analyte, a sharp rise in the polarographic wave is seen as the current increases. The height of the peak is proportional to concentration. This is because as the electrochemical reaction proceeds it depletes the analyte in the solution immediately adjacent to the electrode surface. It is replaced by diffusion, the rate of which depends on the diffusion gradient which in turn is dependent on the concentration of the analyte in the bulk of the electrolyte.

The potential is applied as pulses of a constant magnitude (corresponding to drops of mercury) although the initial potential is ramped. When the current is measured in the final 17 ms of each 60 ms pulse it consists almost entirely of Faradaic current (the type proportional to concentration) due to the much faster decay rate of the other component, the charging current. This enhances sensitivity by minimising interference from the charging current. The current is actually measured at two points, before each pulse and in the last 17 ms, and it is the difference between these two measurements which is plotted against applied potential on the polarogram. A difference only exists when one of the two current measurements falls on the sharp rise in the polarographic wave due to the reduction/oxidation of the analyte and consequent increase in Faradaic current. This will only happen over a relatively narrow band of applied potential voltage, so the plot of the differences forms a peak,

falling back to zero, i.e. no difference between the two measurements, on both sides. The sample to be analysed is added to a supporting electrolyte which consists of a conductive medium, ensuring minimum current flow. Any oxygen dissolved in this medium will produce interfering current when it is reduced. Purging with nitrogen removes dissolved oxygen and the cell is blanketed in nitrogen to prevent any further dissolution during analysis. The sample and electrolyte are kept at a constant temperature during the procedure as changes in temperature may alter conductivity.

6.2.7.2 Use of DPP for Measurements of Metallothionein

DPP has been widely used in metallothionein quantification (Pelacek and Pechan, 1971; Roch et al., 1982 ; Bebbiano and Langston, 1989; Hogstrand, 1991). Alternative techniques are radioimmunoassay, metal binding assays and enzyme-linked immunosorbent assays (ELISA). Brdicka (1933) investigated the analysis of thiolic proteins by polarography using a buffer containing hexamine cobaltic chloride, ammonium chloride and ammonia. It was later found that proteins form a complex with cobalt from a major component of the polarographic activity observed (Pechan and Palecek, 1971). The protein peak appears as a secondary maximum on the side of the larger peak due to deposition of cobalt, and addition of a surface active agent such as Triton-X100 helps to minimise the cobalt peak and separate it from the protein wave. Thompson and Cosson (1984) confirmed the use of DPP, using a quantitative assay for MT from marine organisms, and found that this adaptation of Brdicka's procedure gave enhanced responses.

6.2.7.3 Metallothionein Analysis

Operating conditions of the instrument were as follows;

Instruments ; Static Mercury Dropping Electrode (SMDE) Model 303A (EG and G PARC).

Polarographic Analyser Model 174A (Princeton Applied Research)

'XY' Plotter Type PL100.

Operating mode : SMDE

Drop size : medium

Purge time: 2 min and 0.5 min

Polarographic Analyser Setting ;

Operating mode ; differential pulse

Clock (drop time) : 1 sec

Initial potential : -1.42 mV

Scan rate : 2 mV/sec

Scan direction : negative

Range : 1.5 V

Current range : 10 μA

Display direction : positive

Low pass filter : on

6.2.7.4 Preparation of Supporting Electrolyte

(Modified Brdicka buffer)

The following reagents were added to a 500 ml volumetric flask and made up to volume with distilled water. The buffer was prepared daily using 'Analar, Primar or BDH' grades throughout.

Compound	Amount added	Final Conc.
Ammonium chloride	26.75 g	1 M
Hexaminecobaltic chloride (cobaltic chloride luteo)	0.1605 g	1.2 mM
Ammonium hydroxide (ammonium solution)	27.6 ml	1 M

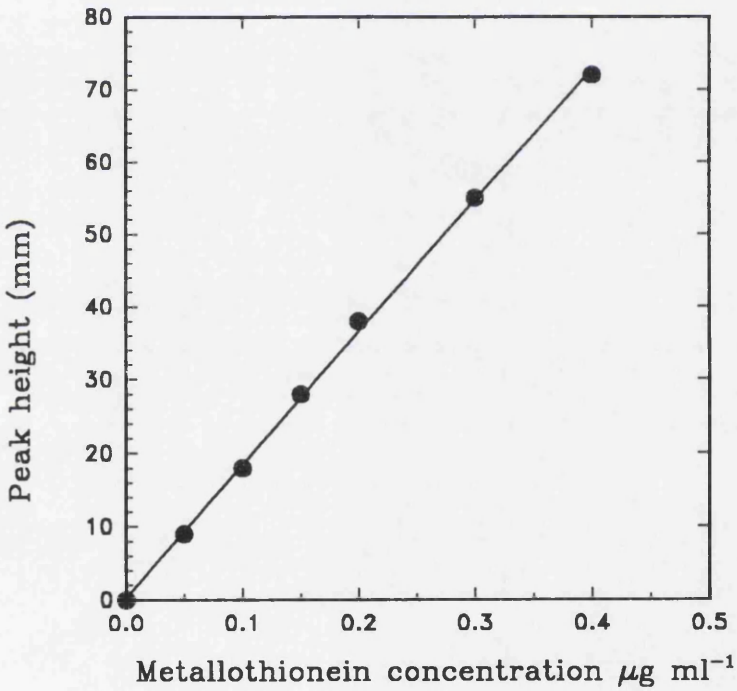
All solutions and samples were kept on ice throughout the procedure to avoid denaturation of the proteins by enzyme activity in the samples. The temperature of the electrolyte was kept constant during readings by the use of a jacketed polarographic cell, through which cold tap water was continually flowing. The oxygen free nitrogen used to blanket the cell and purge its contents was saturated with electrolyte by bubbling it through an electrolyte-filled trap.

For each determination 10 ml electrolyte (modified Brdicka buffer) and 100 μl Triton-X100 (0.0125 % w/v) were added to the cell, these forming the 'blank'. This was purged for 2 min then the applied potential was set to the 'INITIAL' value (-1.42 V) and the recorder allowed time to stabilise before scanning. Samples or standards were injected into the cell via a small side opening and purged for 30 sec to ensure thorough mixing. The applied potential was scanned and the resulting polarographic wave recorded as a peak on the 'XY' recorder. Peak hight was measured as the distance between the highest point of the curve and the baseline in millimetres. After each measurement the mercury was carefully disposed of and the cell, electrodes and housing rinsed with distilled water.

Calibration lines (Figure 6.3) were prepared using at least five nominal concentrations between 0.05 and 0.5 μg ml^{-1} of MT standard solution for each run

using 10 μg ml^{-1} stock metallothionein standards (Metallothionein I from rabbit liver, molecular weight = 6600, Sigma). MT fraction samples were defrosted and injected directly into the cell using micropippetes (Eppendorf). The volume injected was adjusted so that the resultant peak was within the calibration values. One or more standards were run approximately every five samples and at the end of each sample run. Standard additions were also carried out to check that the sample reacted in the same way as the standards during analysis and results are given above (Table 6.2).

Figure 6.3. A typical calibration line between metallothionein concentration and peak height



6.2.8 PROTEIN ANALYSIS

Measurement of protein was carried out as explained in Chapter 5.

6.2.9 STATISTICAL ANALYSIS OF DATA

Data analyses were carried out as explained in Chapter 5.

6.3 RESULTS

Mean values, standard errors of carapace length and numbers of male and female *Nephrops* used in this study are given in Table 6.3 with the results of one way Anova comparisons between groups. There was no significant difference in carapace length of all groups compared for gill and hepatopancreas parameters.

Table 6.3. Mean values and standard errors of carapace length (cl) of male and female *Nephrops norvegicus* used for the gill and hepatopancreas experiments. ns = not significant (P>0.05).

		MALE ANIMALS					FEMALE ANIMALS		
		Control	Treat1	Treat2	Treat3	P	Treat3	Control	P
Gill	No	16	19	16	20		19	7	
	CL	3.50	3.68	3.72	3.64	ns	3.55	3.69	ns
	se	0.06	0.10	0.08	0.07		0.05	0.15	
Hep.	No	15	19	16	20		20	8	
	CL	3.48	3.68	3.71	3.62	ns	3.53	3.68	ns
	se	0.06	0.10	0.08	0.07		0.05	0.13	

6.3.1 Metal and Metallothionein Concentrations in the Gill

Mean concentrations and associated standard errors of cadmium, copper, zinc and metallothionein in the gill of male and female *Nephrops* in controls and in animals exposed to different concentrations of metals are shown in Figures 6.4-6.7 indicating the results of one way Anova. Male animals showed significant increases in concentrations of all the metals and metallothionein in relation to metal exposure, whereas female animals showed only significant increases in the concentrations of cadmium and metallothionein in relation to metal exposure. Male and female animals showed a significant difference in the concentrations of copper in the highest treatment ($P<0.01$), namely male animals had higher concentrations of copper than females (Table 6.5). Mean values and standard errors of comparisons between control and the highest treatment of male and female animals can also be seen in Figure 6.4 to 6.7. Significant results from overall Anova tests for male animals were reanalysed between each treatment (Table 6.4). Results showed that zinc had significant difference only between the control and the highest treatment ($P<0.05$), whereas the copper concentration in the highest treatment was significantly different than in all the other treatments ($P<0.05$). Cadmium concentrations in the highest treatment were much higher than any other treatment ($P<0.0001$), though cadmium concentrations in the medium treatment were only different from the control ($P<0.05$). Concentrations of MT were also significantly different between control and the highest treatment ($P<0.001$). Linear regression analyses were carried out between metal and metallothionein concentrations of male and female animals separately (Figures 6.8-6.13). Results showed that cadmium and metallothionein concentrations in the gill of male and female animals showed positive relationships ($P<0.001$ and $P=0.001$, respectively). Zinc and copper did not show any relationship with metallothionein in the gill of males or females.

Figure 6.4. Zinc concentrations in the gill of Nephrops norvegicus in relation to zinc exposure

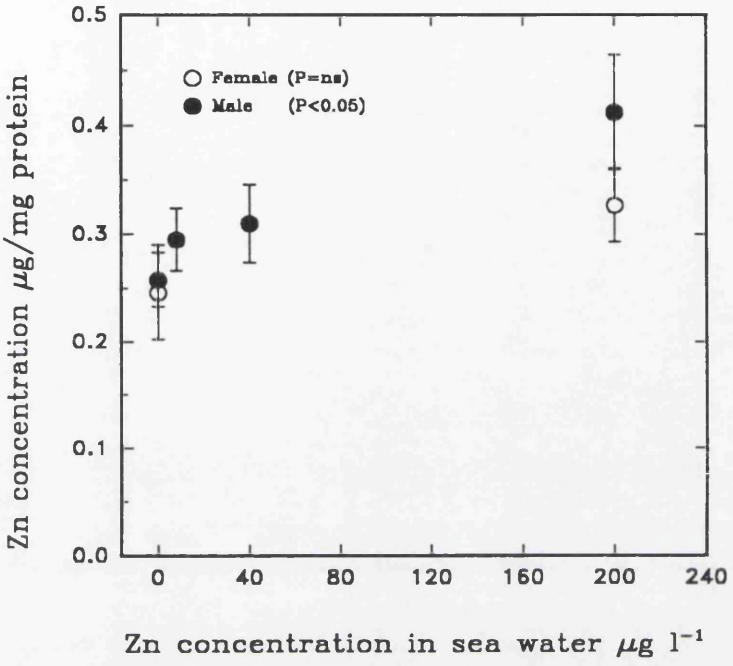


Figure 6.5. Copper concentrations in the gill of Nephrops norvegicus in relation to copper exposure

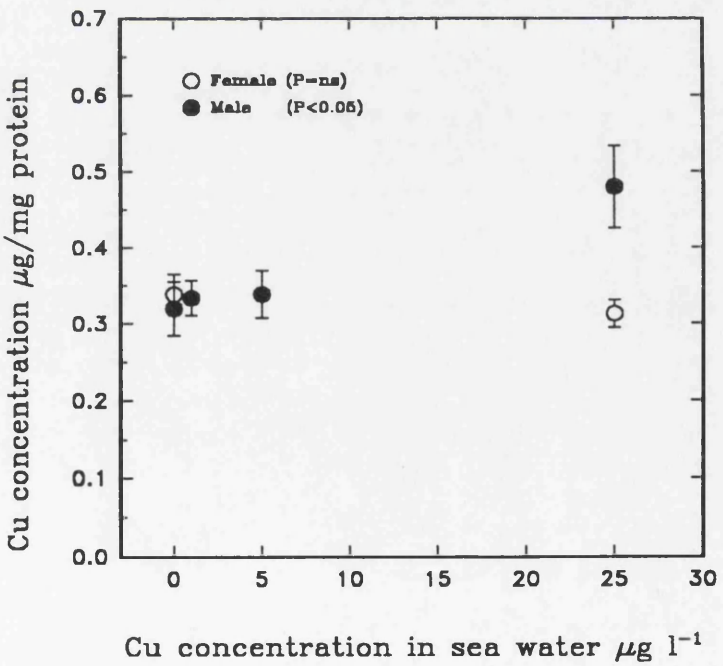


Figure 6.6. Cadmium concentrations in the gill of Nephrops norvegicus in relation to cadmium exposure

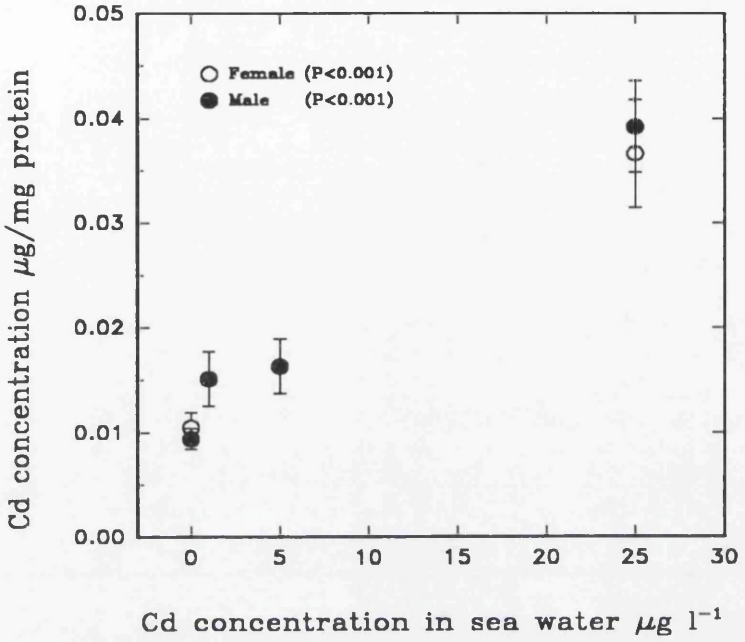


Figure 6.7. MT concentrations in the gill of Nephrops norvegicus in relation to metal exposure

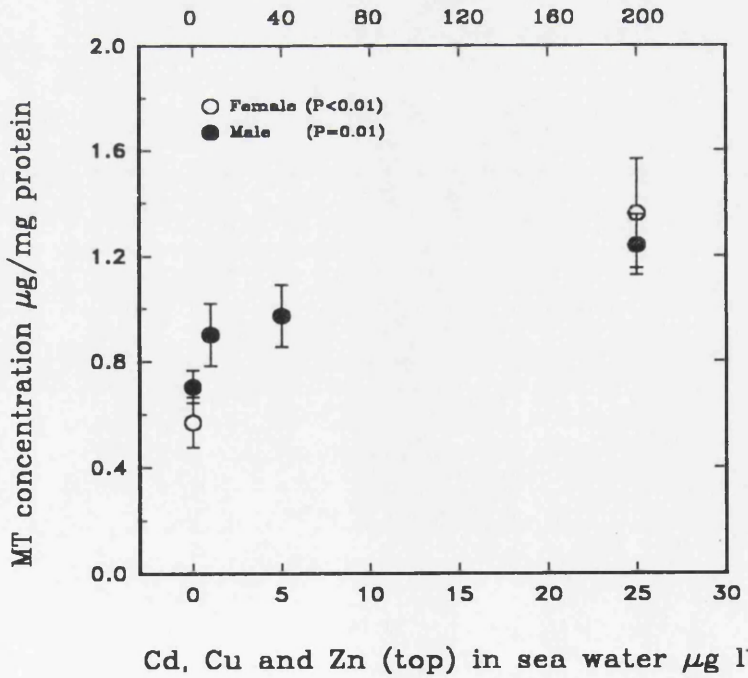


Figure 6.8. Relationship between zinc and MT concentrations in the gill of male Nephrops

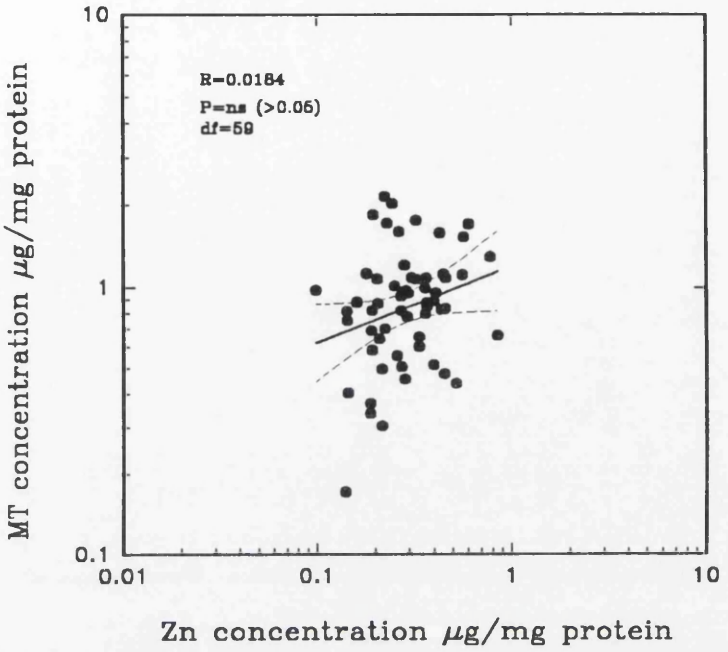


Figure 6.9. Relationship between zinc and MT concentrations in the gill of female Nephrops

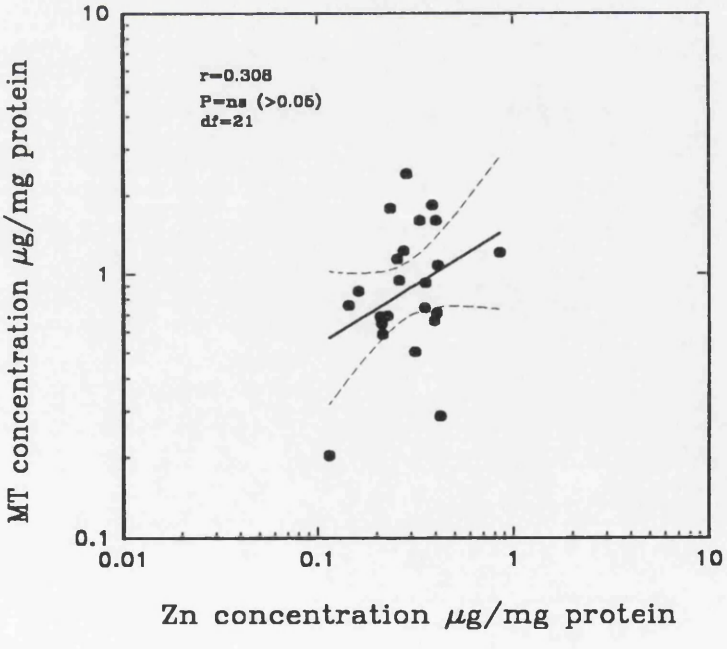


Figure 6.10. Relationship between copper and MT concentrations in the gill of male Nephrops

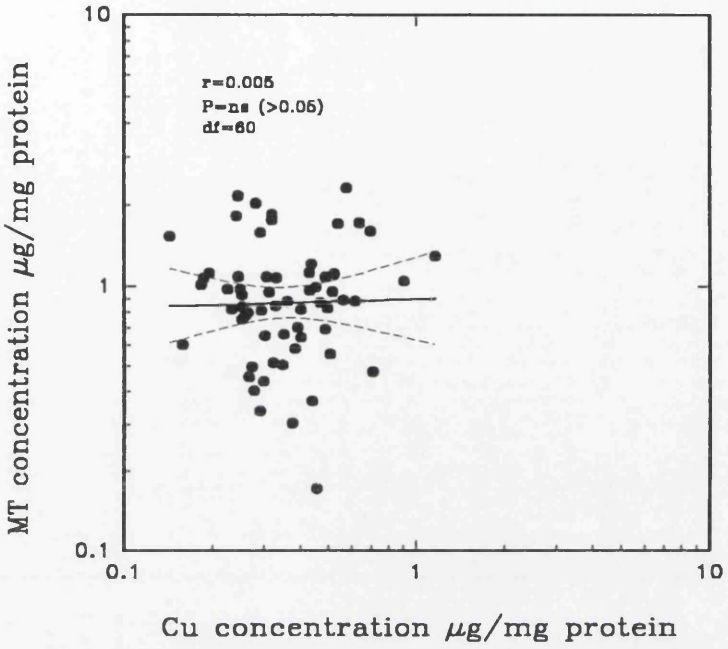


Figure 6.11. Relationship between copper and MT concentrations in the gill of female Nephrops

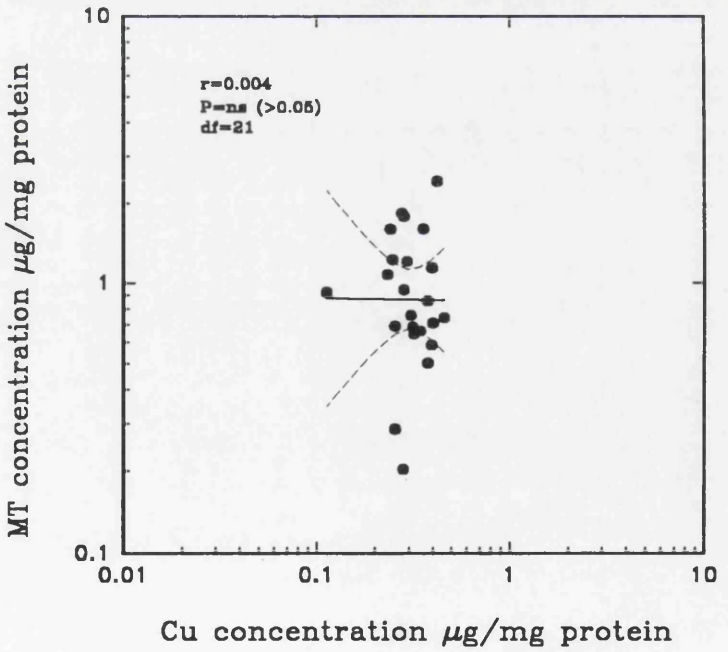


Figure 6.12. Relationship between cadmium and MT concentrations in the gill of male Nephrops

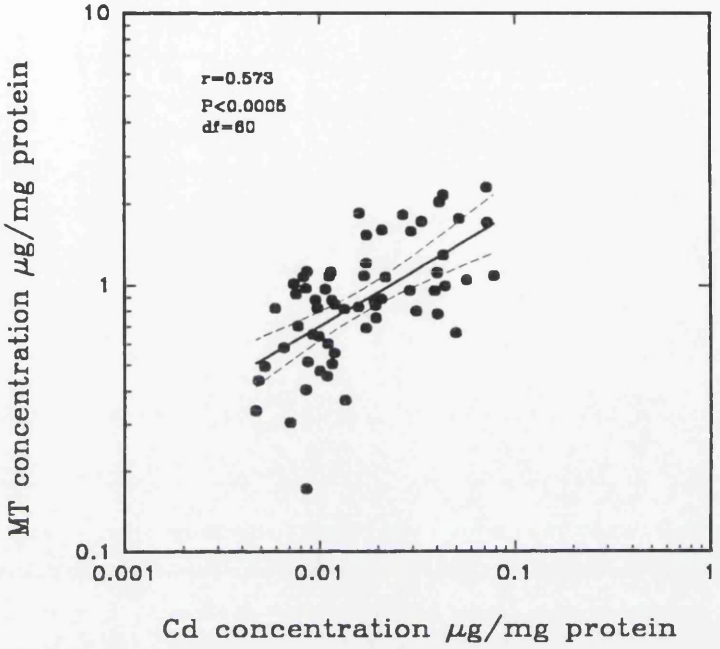
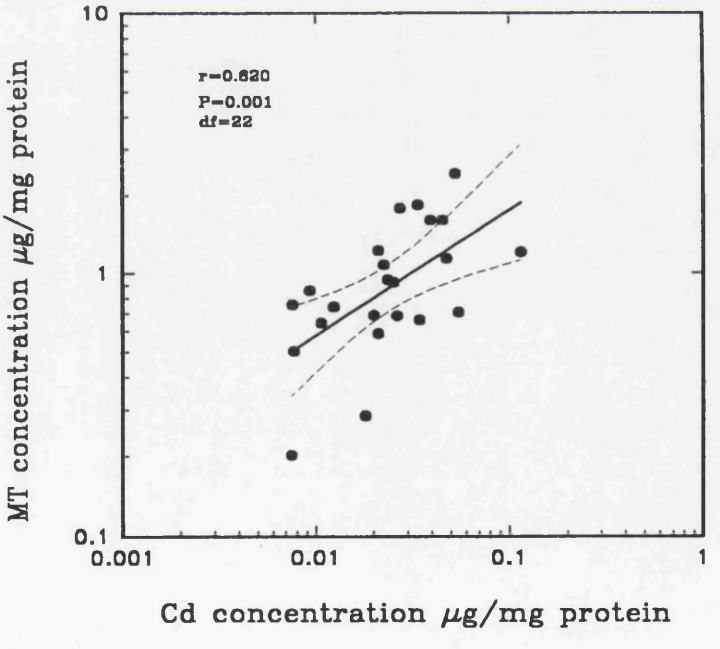


Figure 6.13. Relationship between cadmium and MT concentrations in the gill of female Nephrops



6.3.2 Metal and Metallothionein Concentrations in the Hepatopancreas

Mean concentrations and associated standard errors of cadmium, copper, zinc and metallothionein in the hepatopancreas of male and female *Nephrops* are shown in Figures 6.14 to 6.17 with the results of one way Anova. Male and female *Nephrops* showed significant increases in concentrations of cadmium and metallothionein in relation to metal exposure but copper and zinc did not show the same trend. Male and female animals showed a significant difference in the concentrations of zinc in the highest treatment ($P<0.001$), namely female animals had high concentrations of zinc than males (Table 6.5). Mean values and standard errors of comparisons between control and the highest treatment of male and female animals can be seen in Figure 6.14. Significant results from overall Anova tests for male animals were also reanalysed between each treatment (Table 6.4). Cadmium concentrations in the highest treatment were significantly different than control and the lowest treatment ($P<0.001$), though this difference was less in the medium treatment ($P<0.01$). Cadmium concentrations in the lowest and medium treatments were also different than control ($P<0.05$). Concentrations of metallothionein were significantly different between control and the highest treatment ($P<0.01$). Control and the lowest treatment also showed a significant difference in metallothionein concentrations ($P<0.05$). Regression analyses were also carried out between metal and metallothionein concentrations in the hepatopancreas of male and female animals separately (Figures 6.18-6.23). These analyses showed that cadmium and metallothionein concentrations in the hepatopancreas of male and female animals showed positive relationships ($P<0.001$ and $P<0.01$, respectively). Copper also showed a positive relationship with metallothionein in the hepatopancreas of male animals ($P<0.01$) but not in female animals. Relationship between zinc and MT was not significant ($P>0.05$) in both male and female animals.

Figure 6.14. Zinc concentrations in the hepatopancreas of Nephrops norvegicus in relation to zinc exposure

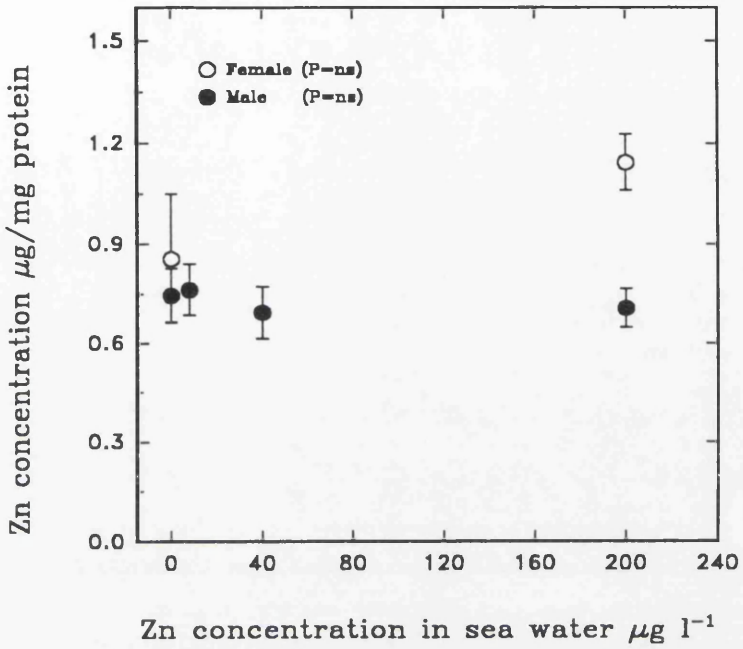


Figure 6.15. Copper concentrations in the hepatopancreas of Nephrops norvegicus in relation to copper exposure

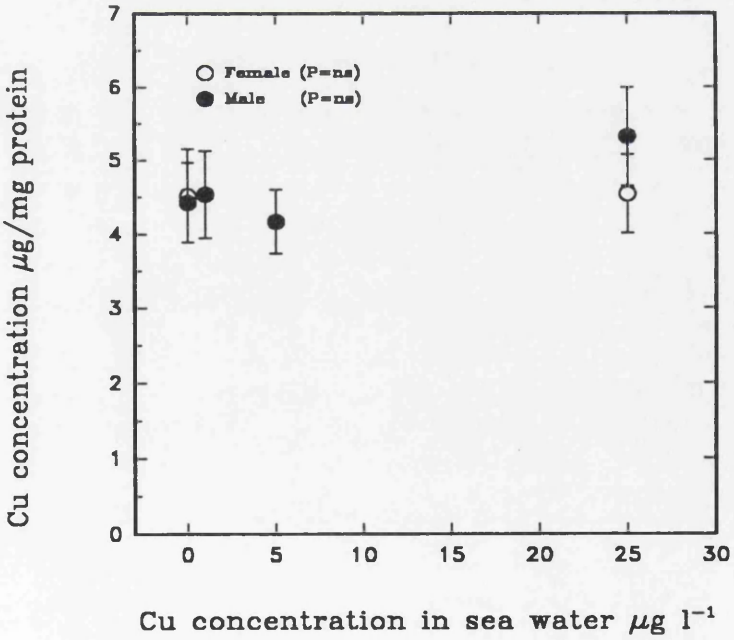


Figure 6.16. Cadmium concentrations in the hepatopancreas of Nephrops norvegicus in relation to cadmium exposure

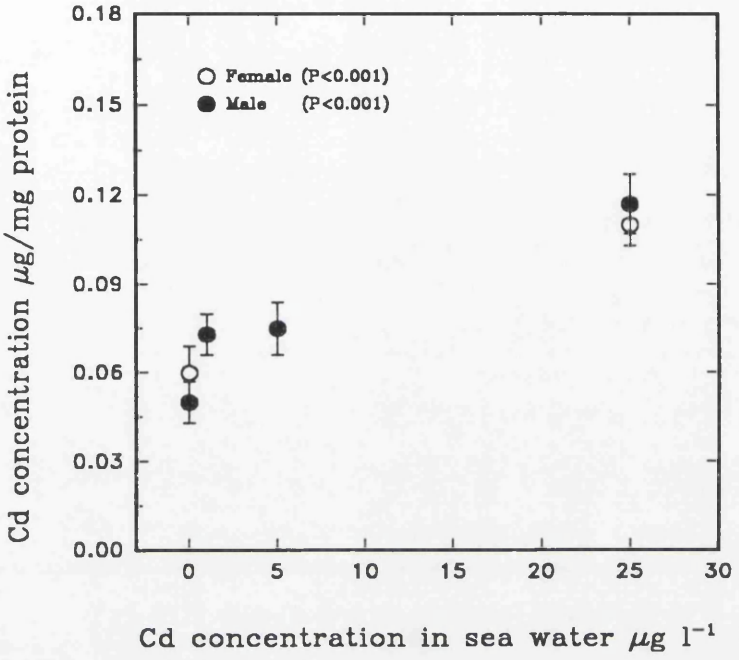


Figure 6.17. MT concentrations in the hepatopancreas of Nephrops norvegicus in relation to metal exposure

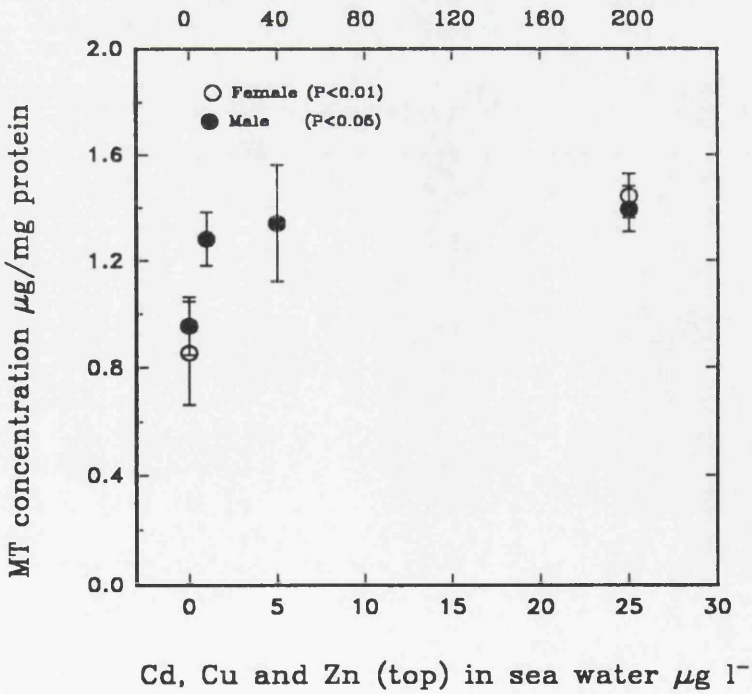


Figure 6.18. Relationship between zinc and MT concentrations in the hepatopancreas of male Nephrops

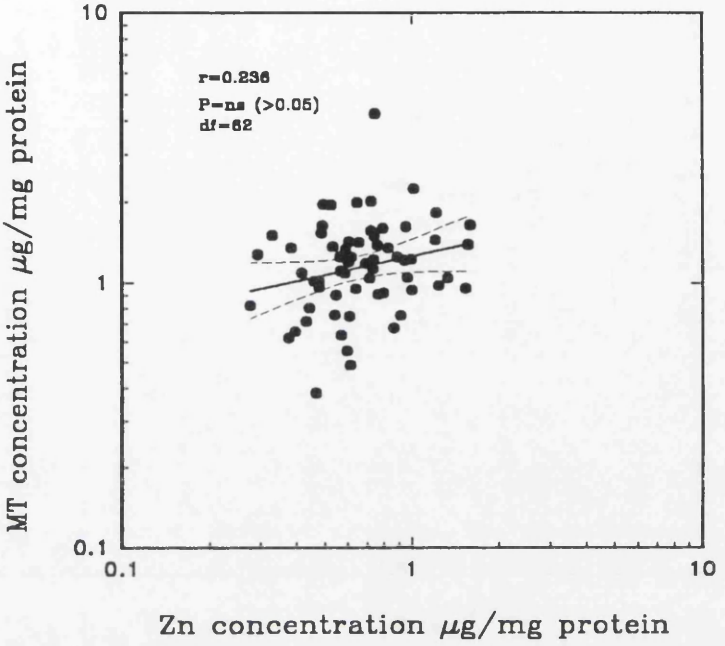


Figure 6.19. Relationship between zinc and MT concentrations in the hepatopancreas of female Nephrops

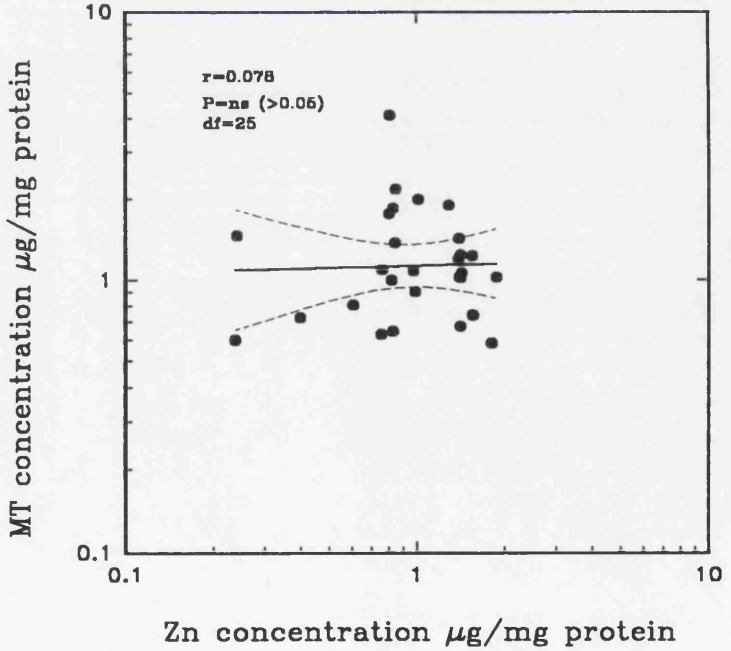


Figure 6.20. Relationship between copper and MT concentrations in the hepatopancreas of male Nephrops

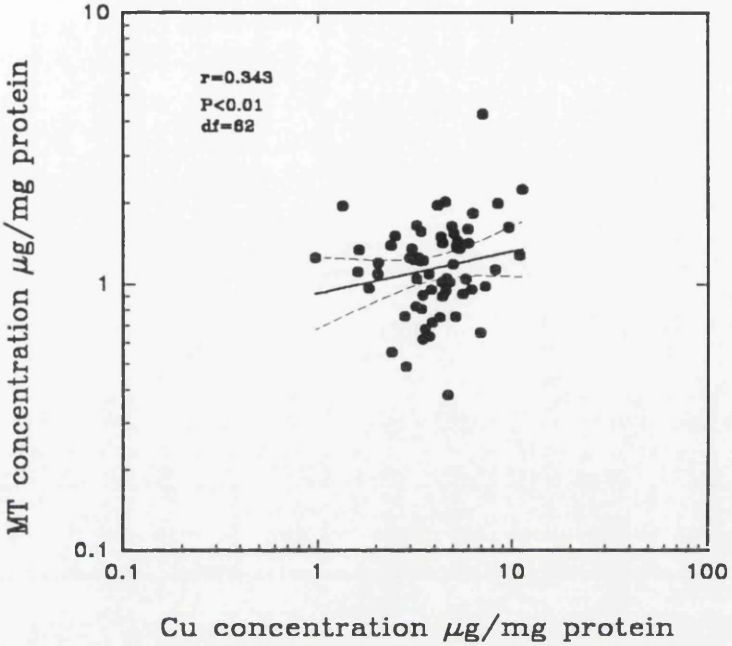


Figure 6.21. Relationship between copper and MT concentrations in the hepatopancreas of female Nephrops

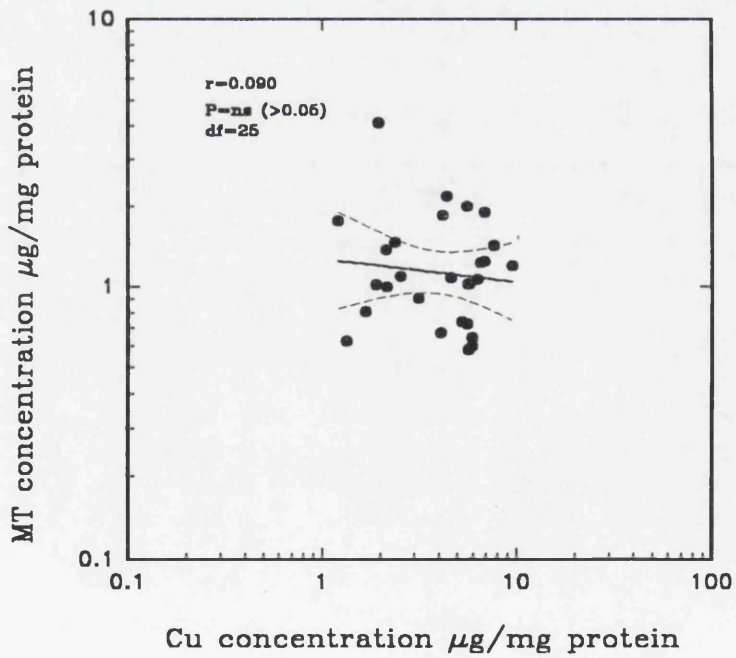


Figure 6.22. Relationship between cadmium and MT concentrations in the hepatopancreas of male Nephrops

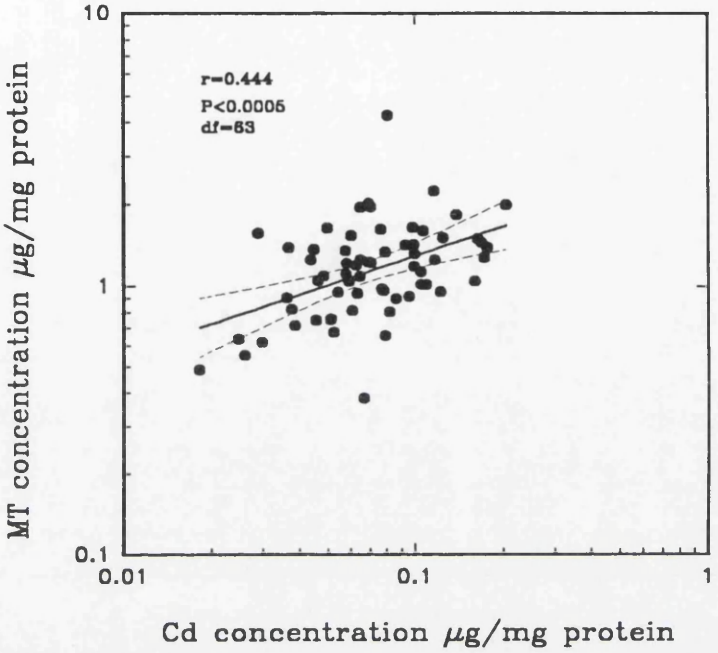
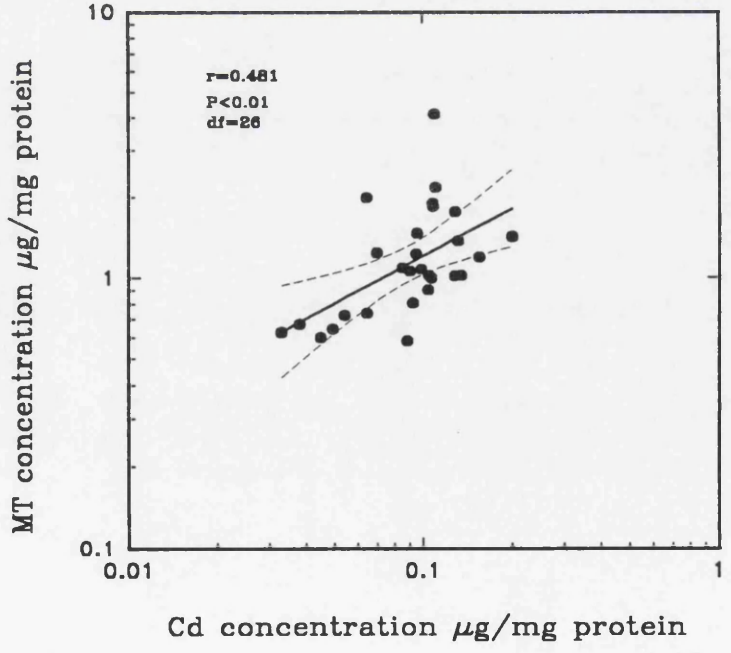


Figure 6.23. Relationship between cadmium and MT concentrations in the hepatopancreas of female Nephrops



6.3.3 Concentrations of Metals in the MT Fraction of the Hepatopancreas

Concentrations of the metals in metallothionein fraction of the hepatopancreas were measured and mean values and associated standard errors are shown in Figures 6.24 to 6.26 with the results of one way Anova. Results showed that cadmium concentrations increased significantly in both male and female animals with increases in exposure concentrations ($P<0.001$), while copper and zinc concentrations did not vary significantly ($P>0.05$). Group comparisons of male animals (Table 6.4) with one way Anova showed that animals from the medium ($P<0.05$) and the highest treatments ($P<0.0001$) had higher concentrations of cadmium than found in controls. Cadmium concentrations in animals from the highest treatment were also significantly higher than in animals from the lowest treatment ($P<0.001$). The highest treatment and the medium treatment, however, did not show any significant differences in cadmium concentrations ($P>0.05$) (Table 6.4). There was no significant difference in the concentrations of metals in the MT fraction of the hepatopancreas between controls and the highest treatment of male and female animals (Table 6.5). Mean values and standard errors of these comparisons can be seen in Figures 6.24-6.26. Ratios between metallothionein and metals in MT fraction of the hepatopancreas were also calculated and statistically analysed with one way Anova. These results are shown in Figures 6.27 to 6.29 indicating the mean ratios of each treatment and their associated standard errors with the results of one way Anova between treatments. Ratios between cadmium and metallothionein were increased significantly in both male ($P<0.001$) and female ($P<0.05$) animals with increases in exposure concentrations, while copper and zinc ratios to metallothionein did not show any increase in either sex ($P>0.05$).

Figure 6.24. Zinc concentrations in the MT fraction of the hepatopancreas in relation to zinc exposure of Nephrops norvegicus

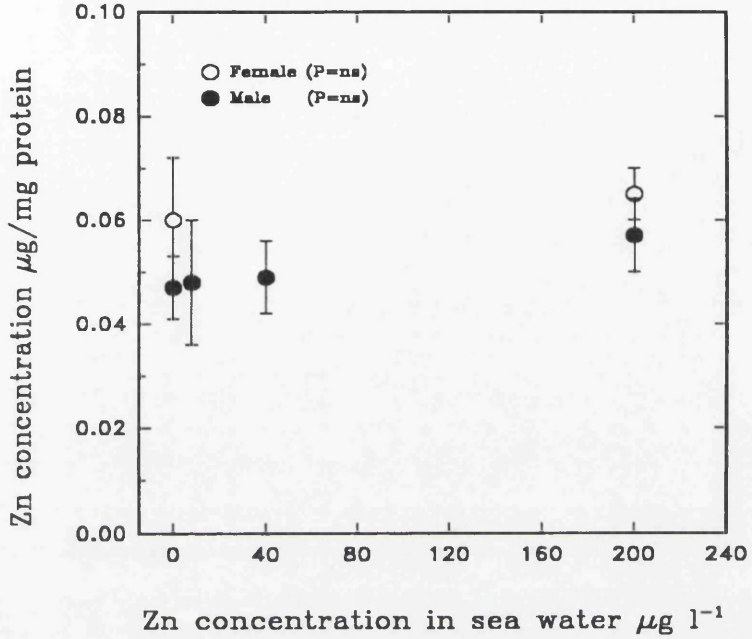


Figure 6.25. Copper concentrations in the MT fraction of the hepatopancreas in relation to copper exposure of Nephrops norvegicus

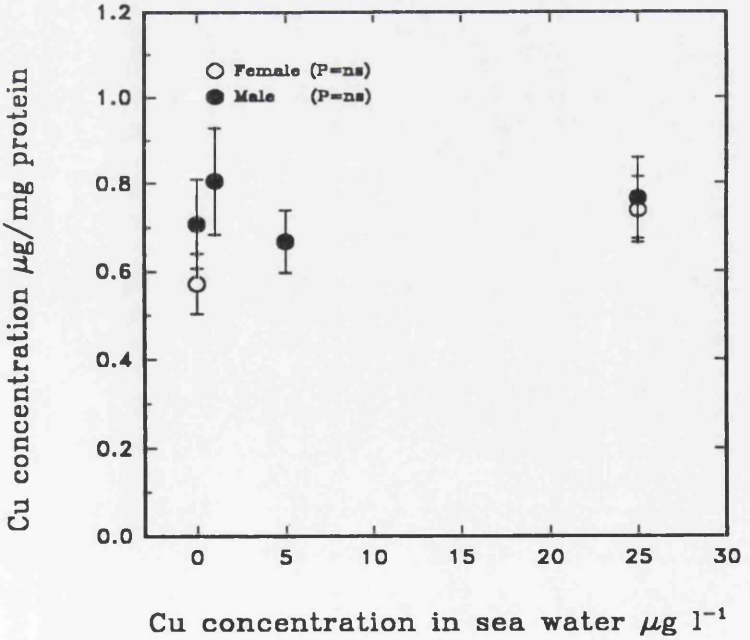


Figure 6.26.Cadmium concentrations in the MT fraction of the hepatopancreas in relation to cadmium exposure of Nephrops norvegicus

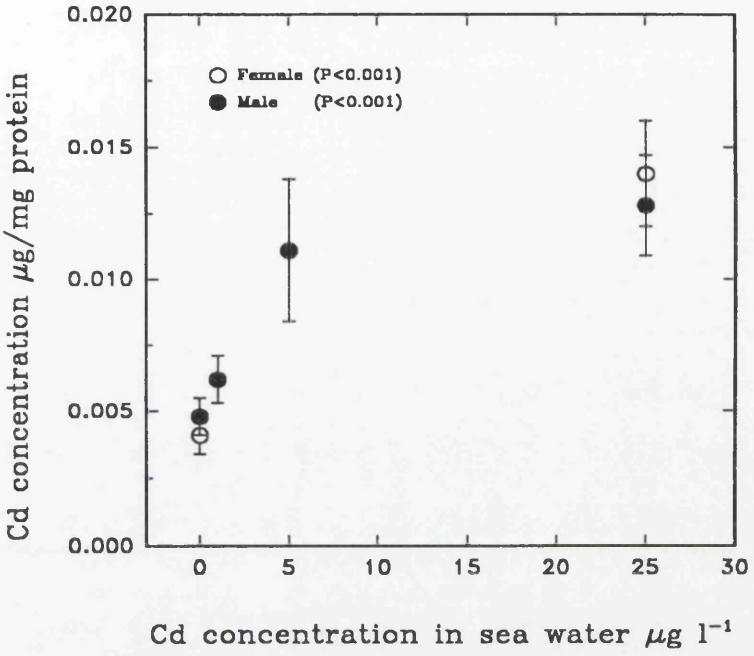


Figure 6.27. Ratios of Zinc to MT in the MT fraction of the hepatopancreas in relation to zinc exposure of Nephrops norvegicus

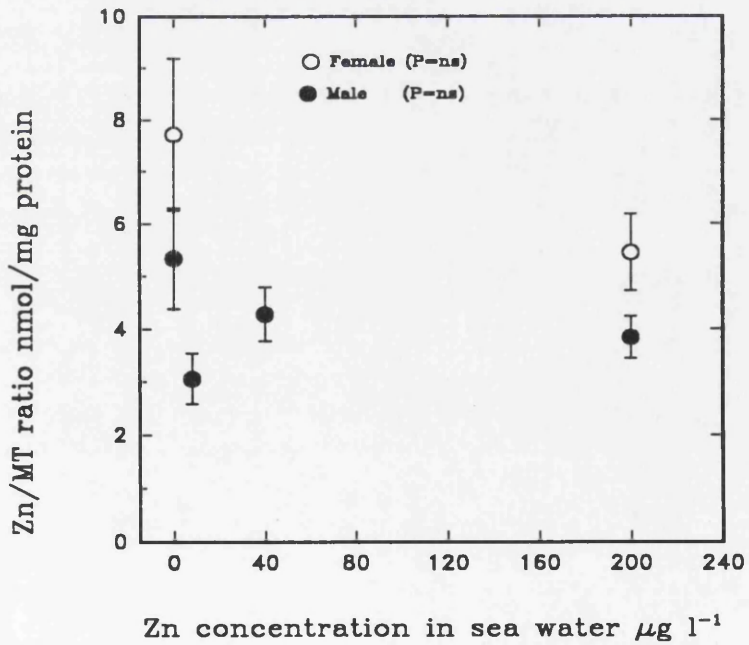


Figure 6.28. Ratios of Copper to MT in the MT fraction of the hepatopancreas in relation to copper exposure of Nephrops norvegicus

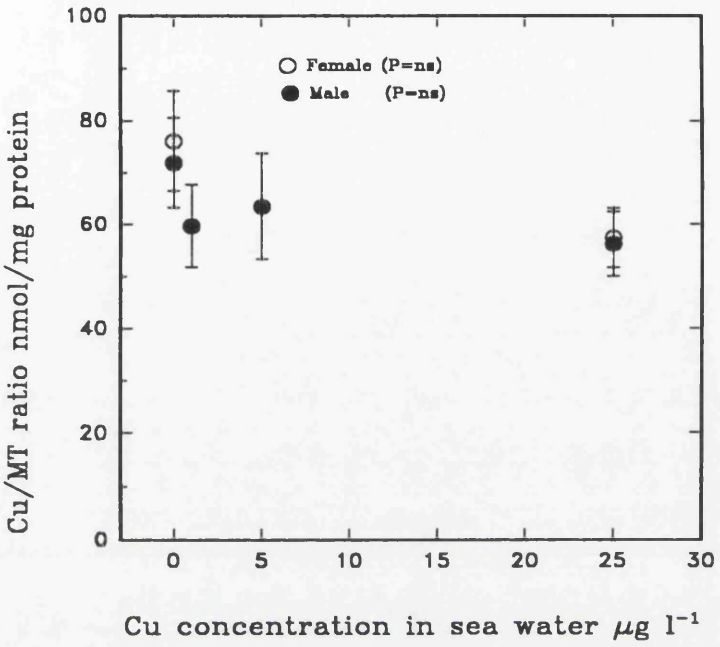


Figure 6.29. Ratios of cadmium to MT in the MT fraction of the hepatopancreas in relation to cadmium exposure of Nephrops norvegicus

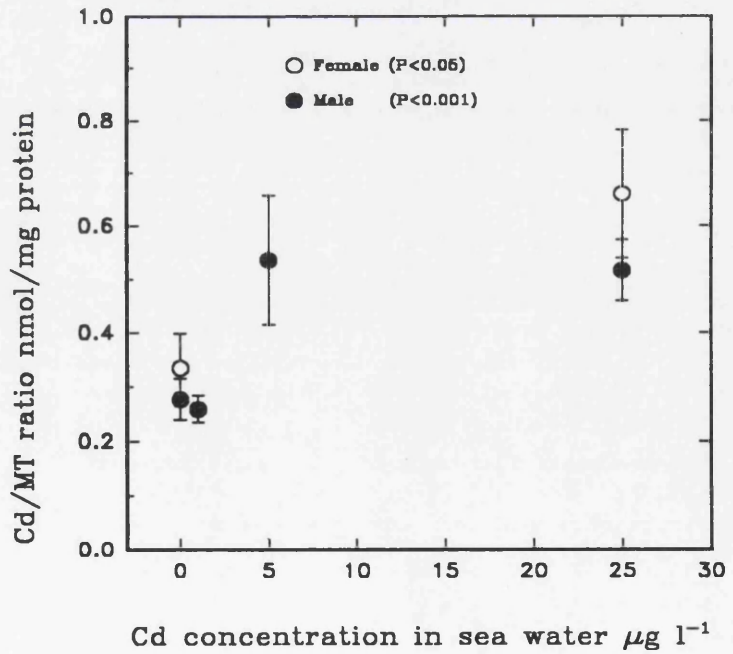


Table 6.4. Comparisons of metal and metallothionein levels between treatments of male animals with one way Anova. Only significant results from overall comparisons (P values are shown in Figures) are tested and their P values are given in the following table. # = total metals.* = MT fraction metals in the hepatopancreas.

		GILL			HEPATOPANCREAS		
		Treat1	Treat2	Treat3	Treat1	Treat2	Treat3
Zn	Control	ns	ns	0.011			
#	Treat1		ns	ns	—	—	—
*	Treat2			ns	—	—	—
					—	—	—
Cu	Control	ns	ns	0.015			
#	Treat1		ns	0.025	—	—	—
*	Treat2			0.045	—	—	—
					—	—	—
Cd	Control	ns	0.012	0.0001	0.026	0.033	0.0001
#	Treat1		ns	0.0001		ns	0.0008
	Treat2			0.0001			0.004
Cd	Control				ns	0.018	0.0001
*	Treat1					ns	0.0008
	Treat2						ns
MT	control	ns	ns	0.0008	0.037	ns	0.003
	Treat1		ns	ns		ns	ns
	Treat2			ns			ns

Table 6.5. Comparisons of metal and metallotionein levels in male and female animals with one way Anova. P values are given in the following table. # = total metals, * = Metals in MT fraction. C M&F = Control male and females. Tr3 M&F = Treated male and females in the highest exposures.

	GILL		HEPATOPANCREAS			
	#	#	#	#	*	*
	C M&F	Tr3 M&F	C M&F	Tr3 M&F	C M&F	Tr3 M&F
CL	ns	ns	ns	ns	ns	ns
Zn	ns	ns	ns	0.0002	ns	ns
Cu	ns	0.008	ns	ns	ns	ns
Cd	ns	ns	ns	ns	ns	ns
MT	ns	ns	ns	ns	ns	ns

6.4 DISCUSSION

Natural concentrations of heavy metals in the tissues of *Nephrops norvegicus* can be affected by sex, size and season. Size and sex of animals could also affect the accumulation of metals from seawater (Chapter 2 and 3). All animals used in this study were caught on the same day and at the same location, and sizes of animals were not significantly different among compared groups. *Nephrops* showed different patterns of metal accumulation in the range of metals used between the tissues and the sexes. Male animals showed significant concentration dependent accumulation of copper and zinc in their gills, while female animals did not show any significant increase in the levels of these metals. Higher accumulation rate of metals (for copper and zinc) in male animals caused a significant difference in concentrations of copper between the sexes namely male animals accumulated higher concentrations of copper than females. This result shows that levels of copper and zinc were regulated in the gill by female animals but not by males. Observations showed that male animals are more active in tanks than female animals. This may cause higher accumulation rates of metals in the gill of male animals. In the hepatopancreas both male and female animals showed the same patterns of accumulation. Levels of copper and zinc did not significantly differ with increases of exposure concentrations, which means that concentrations were regulated in the hepatopancreas of both sexes. Male animals seemed to be better regulators of zinc in the hepatopancreas, as female animals showed some increases in zinc concentration of the hepatopancreas which caused a significant difference between male and female animals in the highest treatment. Regulation of copper and zinc have also been reported in other decapod crustaceans (Bryan, 1964 ; 1967 ; White and Rainbow, 1982 ; Rainbow, 1985 ; Rainbow and White, 1989). They indicated that copper and zinc can be regulated by decapod crustaceans and accumulation of these metals begins only after the regulation

mechanism breaks down. However, it is well known that accumulation of cadmium is dependent on environmental concentrations and there is no evidence for the regulation of this metal by Crustacea (Wright and Brewer 1979 ; White and Rainbow, 1982 ; Rainbow, 1985 ; Amiard et al., 1987 ; Rainbow and White, 1989). In this study, cadmium concentrations increased in the tissues of male and female animals even in the concentration lower than the EQS level.

Metallothionein concentrations of the hepatopancreas and gill from male and female animals showed significant increases with increases of exposure concentrations of metals. However, of the metals studied, these increases showed positive relationships only with cadmium in the gill and hepatopancreas of male and female *Nephrops*. Copper in the hepatopancreas of male animals also showed a positive relationship with MTs. Induction of metallothionein has also been shown in decapod crustaceans after exposure to cadmium either by water and food or by injection (Overnell and Trewhella, 1979 ; Otvos et al., 1982 ; Brouwer, 1984 ; Engel and Brouwer, 1986 ; Howard and Hacker, 1990). As explained earlier, decapod crustaceans can regulate concentrations of copper and zinc up to a threshold level whereas cadmium accumulates in relation to exposure concentrations. Accumulation rate of cadmium, therefore, is much higher especially in the beginning of exposure than copper and zinc which may also increase the affinity to bind MTs. Brouwer (1984) experimented with the blue crab, *Callinectes sapidus* exposed to cadmium for a short time. He indicated that all the cadmium in the cytosolic fraction of the gill was associated with low molecular weight cadmium-binding proteins. However, after 48 h of exposure only 50 % of the cadmium in the cytosol was bound to this protein. The rest was found to be associated with high molecular weight proteins. Copper and zinc, however, are not strong inducers of MTs like cadmium. This might be due to high basal levels of copper and zinc MTs in Crustacea (Engel, 1987 ; Engel and Brouwer,

1987 ; Roesijadi, 1992). Copper and zinc MTs in decapod crustaceans are naturally found in high levels to serve as storage forms for these metals and play regulator roles within the metabolism, especially donor roles for apohaemocyanin and carbonic anhydrase (Engel, 1987 ; Engel and Brouwer, 1987) while cadmium MTs are induced after exposure to this metal for sequestration reason (Roesijadi, 1992). High accumulation rate of copper and zinc may be necessary to be able to increase basal levels of copper and zinc MTs or at least levels should be higher than the levels that can be regulated. Determination of metals in the MT fraction of homogenate of hepatopancreas also showed that copper and zinc did not increase significantly with increases in exposure levels. As a result of this accumulation pattern of the metals, ratios of cadmium to metallothionein increased significantly, while copper and zinc ratios to MTs were not increased. Actually, ratios of copper and zinc in the MT fraction of the hepatopancreas were decreased, though these differences were not significant between treatments. This may be due to replacement of copper and zinc by cadmium for sequestration processes. Otvos et al. (1982) found that metallothionein from the crab *Scylla serrata* contained only cadmium after repeated injection of $^{113}CdCl_2$. This result also shows that copper and zinc bound to metallothioneins were replaced by cadmium. Krezoski et al. (1988) also showed that when cadmium was added *in vitro* to cytosol of fish liver, cadmium displaced zinc but not copper. Sander and Jenkins (1984) indicated that cytosolic copper in copper exposed crabs, *Rhithropanopeus harrisii*, was associated with both MT and high molecular weight ligands, and was independent of external copper. McCarter et al. (1982) showed that the levels of copper in the low molecular fraction of fish exposed to 70 $\mu g\ l^{-1}$ were not significantly increased over control values for 6 weeks. Olsson and Haux (1986) showed that the increased cadmium correlated (R=0.84) with an increased MT level in the liver of perch *Perca fluviatilis* exposed to cadmium, though Zn (r=0.51) and Cu (r=-0.15) showed low correlation with MT in the liver.

MTs can be induced by different factors. Howard and Hacker (1990) studied the combined effects of salinity, temperature and cadmium on the induction of cadmium-binding proteins in the grass shrimp, *Palaemonetes pugio*. They indicated that an increase in Cd-binding protein concentrations corresponded with increases in the cadmium exposure levels and temperature and decreasing salinity. Salinity and temperature may not be prime factors here to increase the levels of MT but are known to be factors which affect the metal accumulation from the marine environment. Higher temperature and low salinity cause higher accumulation of metals which would also indirectly increase MT induction. Stress and the reproductive cycle of marine animals have also been found to affect MT concentrations in marine teleost fish (Bremner and Beattie, 1990 ; Baer and Thomas, 1990). Moulting can be one of the most important periods of the crustacean annual cycle. Engel and Brouwer (1987) indicated that concentrations of metallothionein in decapod crustaceans vary at different times of the year indicating changes of MTs depending on moult stages of the animals.

MTs could be the most promising indicators for heavy metal contamination in the aquatic environment and have been proposed to be used as indicators of heavy metal contamination of aquatic animals (Olafson et al.,(b) 1979 ; Roch et al., 1982 ; Haux and Forlin, 1988 ; Hogstrand and Haux, 1990). However, there would be some conditions for this since not all metals show the same patterns of accumulation in marine animals. MTs could only be used as an indicator tool for heavy metals which accumulate in marine animals in proportion to environmental concentrations and show effective induction of MTs. Additionally, some other factors which could affect MT concentration such as stress, reproductive cycle, moulting and conditions of water should be considered in natural samples. George (1990) also indicated that before measurements of MT levels can be used as an indicator of past metal exposure

in field samples, the characteristics of the response in the test organism must be known. Thus the dose/response, longevity and magnitude of effects (induction and degradation time), as well as information on seasonal variations, effects of reproduction, and other stress factors are required. In respect to this, laboratory experiments indicated that metallothioneins can be used as an indicator for cadmium but not for copper and zinc in the gill and hepatopancreas of *Nephrops norvegicus* since only cadmium increases in the both tissues of the sexes in proportion to exposure concentrations, while copper and zinc are either regulated or slightly increased in this study. Additionally, only cadmium and MT showed strong and constant relationships in both tissues of male and female animals, whereas copper and zinc did not show this trend. Since copper and zinc metallothioneins in Crustacea have been shown to serve as storage forms of copper and zinc for metabolism and, therefore, levels can change during different periods of the year, especially depending on moult stage of crustaceans (Engel, 1987 ; Engel and Brouwer, 1987), copper and zinc metallothionein may not be reliable indicators of copper and zinc contamination in the marine environment. For better understanding of the use of MTs as an indication of metal contamination, it would be essential to study MT concentrations in naturally contaminated and clean areas and look at the relationship with metals.

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CHAPTER 7

GILL ATPASE ACTIVITIES AND SOME PARAMETERS IN THE GILL AND BLOOD OF *NEPHROPS NORVEGICUS* FROM CONTAMINATED AND UNCONTAMINATED AREAS OF THE CLYDE SEA; EFFECTS OF A PARASITE INFECTION

7.1 INTRODUCTION

Heavy metals have been discharged for many years into the seas from industrial effluents, sewage sludge and atmospheric deposition. Concentrations of heavy metals in the marine environment can increase depending on sources of contamination (Langston, 1990). Marine animals accumulate these metals from sea water and through their diet causing elevated concentrations in their tissues. Higher concentrations of heavy metals in the aquatic environment are known to be toxic and can cause physiological alterations in normal metabolism of aquatic animals at sublethal levels (see Chapter 1). Heavy metals are known to inhibit the activity of different enzymes including ATPases *in vitro* (Riedel and Christensen 1979). For example, activities of different ATPases in the gill of fish and crustaceans have been shown to be inhibited by heavy metals *in vitro* (Bouguegneau, 1976 ; Tucker and Matte, 1980 ; Haya et al., 1983 ; Lauren and McDonald, 1987). *In vivo* effects of heavy metals, however, are not so clear, possibly because homeostatic mechanisms cause some compensatory alterations in enzyme activity (Stagg and Shuttleworth 1982). Although the activity of ATPases were altered in the gill of the Norway lobster, *Nephrops norvegicus* by *in vivo* heavy metal exposure, male and female animals showed different patterns (Chapter 5). Heavy metals can also alter concentrations of ions in the blood and tisssues of fish and so can affect the osmolality. Copper, zinc, cadmium and mercury altered normal concentrations of ions in the blood of crustaceans and fish after exposure to the metals dissolved in water (Lewis and Lewis, 1972 ;Thurberg et al., 1973 ; McCarty and Houston, 1976 ; Christensen et al., 1977 ; Inman and Lockwood, 1977 ; Cardeilhac et al., 1979 ; Rombough and Garside, 1984 ; Bjerregaard and Vislie, 1985a :1985b ;1986).

The Clyde Sea on the west coast of Scotland is a major seaway and leisure area and supports valuable fisheries. Sewage sludge consisting of primary settlement material, activated sludge and industrial waste has been dumped (approx. $1*10^6$ tonnes/day) off Garroch Head in the Firth of Clyde. Untreated sewage from inland as well as coastal towns is discharged along much of the coast of the Firth of Clyde. Sewage sludge dumping and other industrial effluents cause elevated levels of heavy metals in sea water, sediment and in fauna which live around in dumping area. (Steele et al., 1972 ; Halcrow et al., 1973 ; Mackay 1986 ; Clark and Davies 1989).

The Norway lobster, *Nephrops norvegicus* is widely distributed in the Clyde Sea and supports an important fishery. *Nephrops* accumulates heavy metals from sea water or food and tissue concentrations can rise many-fold, toxic effects occurring at high metal concentrations (Chapters 3 and 4). A laboratory experiment (Chapter 5) showed that heavy metals in sublethal concentrations inhibit significantly the activity of ATPases in the gill of *Nephrops norvegicus in vivo*. Male and female animals, however, showed different inhibition patterns. After observing inhibition of the activity of ATPases in laboratory conditions, it would be essential to investigate how ATPase activity would be affected in naturally contaminated areas.

Some *Nephrops norvegicus* from the Clyde Sea area are infected by a species of parasitic dinoflagellate related to *Hematodinium perezi* (Field et al., 1992). In advanced stages of this infection, affected lobsters can be easily recognised by the dull orange colour of the body and appendages, and milky-white haemolymph which seems to be due mainly to the presence of increased total cell numbers. A picture of an infected animal is given in Chapter 8 (Figure 8.1). Infected animals show varying degrees of lethality which increases with increasing severity of infection. Severely infected animals are also moribund and has difficulty in walking as well as showing

histopathological changes in some tissues. Copper concentrations of haemolymph and oxygen carrying capacity of haemocyanin were found to be lower in infected individuals than those apparently healthy individuals (Field, 1992 ; Field et al., 1992), presumably related to removal of copper from haemocyanin due to the infection since removal of copper from haemocyanin is known to inactivate this molecule or vice versa (Waterman, 1960). The infection of *Nephrops* by the parasite in the Clyde Sea have showed increases in recent years, infected animals reaching very high percentages of total sampled animals in some areas. Because sampling of the animals in this study was done in different stations in short trawling period, samples contained high percentages of infected animals from stations. Therefore, the infected animals were also used in the present study taking the infection as a factor.

The aim of the present study is to investigate the activities of total ATPase, total Mg-ATPase in its oligomycin sensitive and insensitive components and Na,K-ATPase in the gill of male *Nephrops norvegicus* from contaminated and uncontaminated areas of the Clyde Sea. Infection determination was carried out by looking at the blood colour of the animals. Blue or bluish blooded animals were classified as normal animals, while milky-white blooded animals were classified as infected animals. Thus, blood colour was also involved in statistical analyses. 10 stations in the Clyde Sea were chosen in relation to a pollution gradient containing the dumping ground and relatively clean sites. Concentrations of some ions (Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-) in the blood and gill tissue of male *Nephrops* were measured. Copper and zinc concentrations in the blood of the animals were also measured and possible relationships were investigated between ATPase activity and concentrations of the variables measured in the gill and blood. All investigations were carried out for effects of station and infection on the levels of the parameters and, normal and infected animals were compared for the levels of the parameters.

7.2 MATERIALS AND METHODS

The Norway lobsters, *Nephrops norvegicus* were collected by 30 minute trawl from R.V. Clupea at 10 stations in the Clyde Sea (Figure 7.1) between 13-22 May 1992. Samples from station 11 were only used in Chapter 8. The animals were left in running sea water and then transferred to the University Marine Station, Millport for processing on a daily basis.

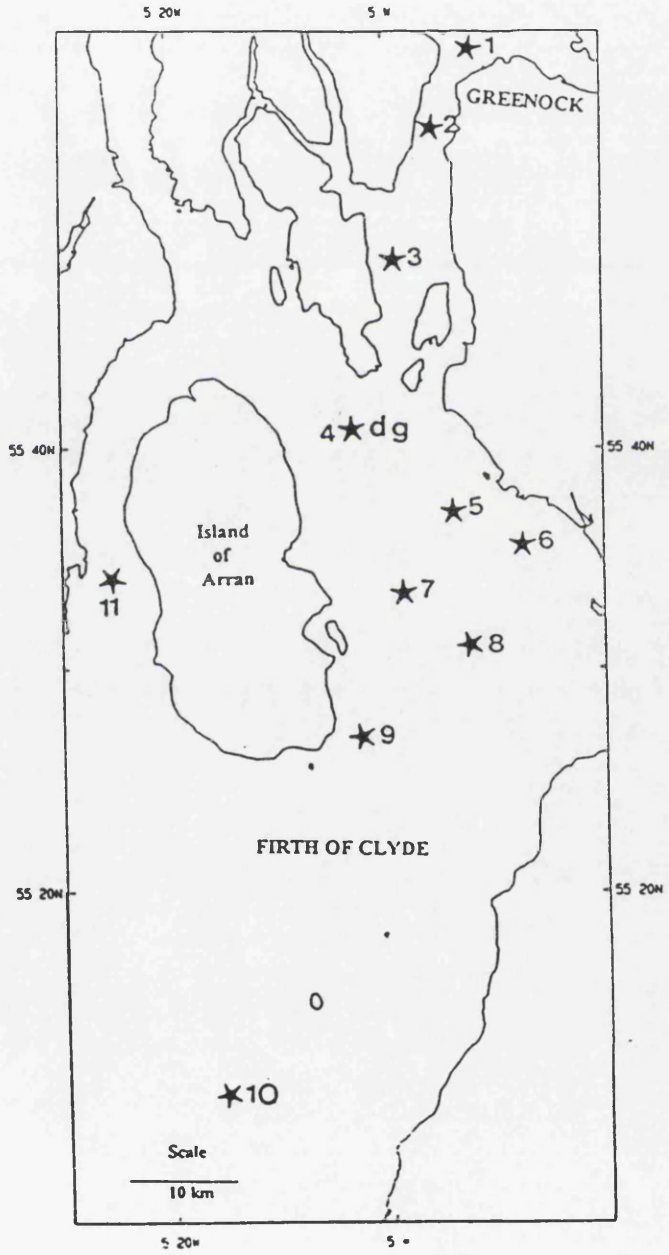


Figure 7.1. Sampling stations of *Nephrops norvegicus* in the Clyde Sea. Stations 1 to 10 were used in this study. dg indicates the dumping ground.

Carapace length was measured and sex of animals was noted. A sample of blood was taken by syringe from the base of the last walking legs or joints of claws and put in eppendorf tubes and frozen at -20 ^{o}C for subsequent measurements of blood ions (Na^+, K^+, Ca^+, Mg^+ and Cl^-) and copper and zinc in the blood. Animals were killed by decapitation and gills were dissected quickly in a cold room (4 ^{o}C). After dissection, some of the gill flaments were snap frozen in liquid nitrogen for measurements of ATPase activity. ATPase activities were measured as described previously in chapter 5 after one or two days storage in liquid nitrogen. Additional samples of gill flaments were also taken to measure the total tissue concentrations of ions Na^+, K^+, Ca^+ and Mg^+. These gill flaments were put into an oven set to 70 ^{o}C for 4 days to dry the samples. Dry samples were used to measure the concentrations of gill ions. The water content (%) of gill flaments was determined from weight of the samples before and after drying.

7.2.1 Measurements of Metals and Ions

Blood samples were diluted 2500 times for sodium and 50 times for the other ions. Analysis of copper and zinc were also carried out in the latter fraction of the blood. Concentrations of the ions in the dry gill were determined in solution obtained after dry gill had been digested in concentrated nitric acid overnight in room temperature. This solution was diluted 300 times for Na^+ and 20 times for the other ions. After appropriate dilutions, all ion and heavy metal levels were brought to the linear range of standards which were prepared using B.D.H. stock standard solutions. Concentrations of standards (as μg ml^{-1} and (μmol ml^{-1})) were in the following ranges; Copper=0.25-4 (0.0039-0.0629), zinc=0.0625-2 (0.00095-0.0305), sodium=0.5-10 (0.0217-0.435), potassium=1-10 (0.0256-0.256), magnesium=0.5-10 (0.0205-0.411), calcium=1-10 (0.0249-0.249).

Copper, zinc and magnesium concentrations in the blood of *Nephrops* were measured with atomic absorption spectrophotometry techniques, while concentrations of sodium, potassium and calcium were measured with atomic emission spectrophotometry techniques (Perkin Elmer 500). Concentrations of metals and ions in the blood were calculated from the linear equations obtained from measurements with a range of concentrations of standards.

7.2.2 Measurement of Osmolality

Osmolality in the blood of animals was measured using a Wescor 5500 vapour pressure osmometer. The micropipettor of the instrument furnished with the vapour pressure osmometer uses air-displacement to dispense a 10 μl volume of liquid for osmolality assay. This volume of liquid is the optimum level for total saturation of sample disk (Wescor manual). Before starting any assay, the instrument was calibrated with standards in concentrations of 290 and 1000 mmol/l using a calibration nomograph for the instrument. A single sample disk was placed on the sample holder for each run using clean forceps and 10 μl of standard or blood sample was aspirated with a pippette (Gilson, France). Then, osmolality was measured directly (mOsmol/kg) after closing the sample chamber. Sample holder was cleaned after each measurement using lint-free tissues.

7.2.3 Measurements of Chloride

Chloride measurements in the blood of *Nephrops* were carried out by using a PCLM3 chloride meter (Jenway ltd.). The instrument works in biological ranges of 10 to 350 mmol/l with a coefficient of variation of $\pm$ 1.5 % at 100 mmol/l level using the titration method. Chloride measurement was started by adding 15 ml of

acid buffer and 10 drops of gelatine (supplied from Jenway) into a cuvette. A metal rod was also put into the cuvette to stir magnetically throughout measurement. The instrument was first conditioned to zero and 20 μl of 100 mmol/l standard (supplied from Jenway) was added to the medium and titration was began. If this volume of chloride standard produced a reading by the instrument of between 98 and 102 units the instrument was set up properly, so measurements of samples were started by adding the same volume of the chemicals. Chloride concentration was expressed as mmol/l. The same buffer can be used for about 15 samples, until the instrument warns that reagents should be changed.

After addition of sample or standard, a constant current passes between two silver electrodes which then liberate silver ions at a constant rate into the solution. These silver ions combine with chloride ions in solution and are precipitated as insoluble silver chloride. When all of the chloride has combined with the generated silver, free silver ions become available in the solution and their presence is detected by two further silver electrodes.

7.2.4 Statistical Analyses of Data

As indicated earlier, some of the Clyde *Nephrops* infected by a fatal parasite related to *Hematodinium*-like species of dinoflagellate. In advanced stages of infection, normally blue coloured blood becomes milky white, though mild infection may not change the colour of the blood (Field, et al., 1992). Investigations were also carried out by separating the normal (blue blood) and advance infected (white blood) animals. Male *Nephrops* were used only throughout this study. Statistical analyses of data were carried out using Minitab 8.2 statistical package program. Any data which would be analysed statistically were first plotted on graphs to see their distribution.

Data not normally distributed were transformed by $\log_{10}$ or square root. General Linear Model was used as specific statistical analysis program which allowed the use of Anova and Linear Regression analysis at the same time. There were significant relationships between ATPase activity and carapace length in Chapter 5, so carapace length was taken into account as a covariate when it differed significantly among stations. Interactions between factors for all variables were also checked before any test. The statistical model was first applied for whole samples to find out effects of station, carapace length and especially to find out if blood colour shows significant effects in the levels of parameters which may be important for description of station and size effects. Since there were differences in levels of many variables in relation to blood colour, the white and normal blooded animals were separated. Normal blooded animals were reanalysed with the same model leaving only one factor (station) and covariate (carapace length). White blooded animals were also compared among stations but with Kruskal-Wallis one way analysis of variance since there was no difference in carapace length of animals from different stations. Mean levels of the variables between normal and infected animals were also statistically compared with the Mann-Whitney U-test. These analyses were carried out only for variables for which stations did not show any effect on levels of the parameters. Carapace lengths of the two groups were not significantly different, so carapace length was ignored for these comparisons. Rank correlation was applied to data between ATPase activities and parameters in the blood and gill. For this test, data were first ranked and correlation was tested for the whole sample and also for normal animals alone.

7.3 RESULTS

Figure 7.1 shows the sampling stations of the Norway lobster, *Nephrops norvegicus* also indicating the dumping ground. Carapace length and number of animals used for

Table 7.1. Mean carapace lengths, standard errors and numbers () of normal (blue blooded) and infected (white blooded) male Norway lobster *Nephrops norvegicus* caught in different stations from the Clyde Sea. Number of samples were different in different parameters for statistical comparisons. nwb=no white blood. nbs=no blood sample.

	C A R A P A C E L E N G T H		
Station	All Animals	Normal Animals	Infected Animals
1	(13) 4.65	(11) 4.60	(2) 4.95
se	0.23	0.23	0.05
2	(10) 4.84	(9) 4.89	nwb
se	0.24	0.26	
3	(9) 4.20	(9) 4.20	nwb
se	0.18	0.18	
4	(10) 4.31	(4) 4.15	(2) 4.20
se	0.19	0.05	0.20
5	(14) 3.89	(4) 3.62	nwb
se	0.16	0.24	
6	(11) 5.19	nbs	nbs
se	0.22		
7	(4) 4.15	(4) 4.15	nwb
se	0.31	0.31	
8	(6) 3.93	(6) 3.93	nwb
se	0.23	0.23	
9	(12) 4.42	(9) 4.37	(2) 4.80
se	0.11	0.13	0.20
10	(21) 3.82	(12) 3.81	(6) 4.06
se	0.06	0.05	0.19
Average	(110) 4.32	(68) 4.25	(12) 4.19
se	0.07	0.08	0.18
P value	P<0.005	P<0.005	ns (P>0.05)

analysis are given in Table 7.1. Carapace length of all animals and normal (blue blood) animals differed significantly among stations ($P<0.005$), while carapace length of the infected (white blood) animals did not show any difference ($P>0.05$).

Table 7.2. Results of linear regression analysis using all samples. Station and blood colour were used as factors while carapace length was used as covariate. ST&BC&CL indicates the interaction if any between blood colour, station and carapace length. + and - signs indicate positive and negative relationship between carapace length and variables. ns = not significant (P>0.05).

Variable	ST&BC&CL	CL	Station	Blood Colour
T-ATPase	ns	ns	0.017	0.0001
T-Mg-ATPase	ns	ns	0.028	0.0001
Ois.Mg-ATPase	ns	ns	ns	0.0001
Os.Mg-ATPase	ns	ns	0.001	0.007
Na,K-ATPase	ns	ns	0.002	0.038
% water	ns	+0.014	0.018	ns
Gill sodium	ns	ns	ns	ns
Gill potassium	ns	ns	0.042	0.003
Gill calcium	ns	ns	ns	ns
Gill magnesium	ns	ns	ns	ns
Osmolality	ns	ns	ns	0.025
Blood copper	ns	ns	ns	0.0001
Blood zinc	ns	-0.024	ns	0.001
Blood sodium	ns	ns	ns	ns
Blood potassium	ns	+0.034	ns	ns
Blood calcium	ns	ns	ns	ns
Blood magnesium	ns	ns	0.034	ns
Blood chloride	ns	ns	ns	ns

Table 7.3. Results of linear regression analysis using only normal blooded animals. Station was used as factor while carapace length was used as covariate. + and - signs indicate positive and negative relationship between carapace length and variables. Comparisons of the parameters among stations in the infected (white blooded) animals were carried out with Kruskal-Wallis one way analysis of variance since CL does not differ among statios.

	Normal Blooded Animals			White Blooded Animals
Variable	ST&CL	CL	Station	Station
T-ATPase	ns	ns	0.015	ns
T-Mg-ATPase	ns	ns	0.009	ns
Ois.Mg-ATPase	ns	ns	ns	ns
Os.Mg-ATPase	ns	ns	0.002	ns
Na,K-ATPase	ns	ns	0.009	ns
% water	ns	ns	0.035	ns
Gill sodium	ns	ns	ns	ns
Gill potassium	ns	ns	ns	ns
Gill calcium	ns	ns	ns	ns
Gill magnesium	ns	ns	ns	ns
Osmolality	ns	ns	ns	ns
Blood copper	ns	ns	ns	ns
Blood zinc	ns	-0.031	ns	ns
Blood sodium	ns	ns	ns	ns
Blood potassium	ns	ns	ns	ns
Blood calcium	ns	ns	ns	ns
Blood magnesium	ns	ns	0.0001	ns
Blood chloride	ns	ns	ns	ns

Table 7.4. Mean values and standard errors of variables in normal (blue blooded) and infected (white blooded) male *Nephrops*. Comparisons of the two groups with Mann-Whitney U-test are also given in the following table. These comparisons could only be carried out for parameters those which station did not show any effect. nsc= no statistical comparison due to station effects. ns= not significant (P>0.05). ATPase activities are expressed as µmol Pi/mg protein/hour. Heavy metal and ion concentrations are expressed as mmol g^{-1} dry weight in the gill and µmol ml^{-1} in the blood. Osmolality (mOsmol/kg).

	NORMAL BLOOD (68)	WHITE BLOOD (12)	P Value
Carapace L.	4.25 (0.08)	4.19 (0.18)	ns
T-ATPase	3.67 (0.13)	5.26 (0.44)	nsc
T-Mg-ATPase	2.76 (0.10)	3.93 (0.33)	nsc
Ois.Mg-ATPase	1.79 (0.07)	2.64 (0.28)	0.003
Os.Mg-ATPase	0.97 (0.04)	1.29 (0.14)	nsc
Na,K-ATPase	0.87 (0.04)	1.21 (0.16)	nsc
% water	89.0 (0.21)	91.0 (0.38)	nsc
Gill sodium	4042.0 (179)	4285.0 (274)	ns
Gill potassium	373.0 (7.9)	440.0 (29.9)	0.004
Gill calcium	153.0 (5.7)	184.0 (12.3)	0.035
Gill magnesium	265.0 (10.0)	306.0 (23.1)	0.017
Osmolality	985.0 (12.2)	1036.0 (13.9)	0.043
Blood copper	1.01 (0.04)	0.595(0.08)	0.0005
Blood zinc	0.072(0.003)	0.047(0.004)	0.001
Blood sodium	515.0 (76.9)	520.0 (26.3)	ns
Blood potassium	9.14 (0.20)	9.31 (0.50)	ns
Blood calcium	12.32 (0.19)	11.92 (0.44)	ns
Blood magnesium	8.28 (0.28)	11.18 (1.39)	nsc
Blood chloride	425.0 (5.67)	447.0 (8.54)	ns

7.3.1 ATPase activity

In all samples, most ATPase activities showed very significant differences ($P<0.001$) in relation to blood colour of *Nephrops*, except for Na,K-ATPase activity ($P<0.05$) (Table 7.2). Stations also showed significant effects on ATPase activity, except for the activity of oligomycin insensitive Mg-ATPase. Activities of Na,K-ATPase ($P<0.01$) and oligomycin sensitive Mg-ATPase ($P<0.005$), total Mg-ATPase and total ATPase differed among stations. There was no interaction among station, blood colour and carapace length ($P>0.05$). There was also no significant relationship between carapace length and any of the ATPase activities ($P>0.05$).

Table 7.3 shows results of statistical analyses when normal and white blooded animals were separated in the analysis. Results for normal animals using the same analyses were similar in the effects of station and carapace length to those with all animals. Except for oligomycin insensitive Mg-ATPase activity ($P > 0.05$), all the other ATPase activities showed significant differences among stations (Table 7.3). There was also no relationship between ATPase activity and carapace length ($P > 0.05$). Mean values and standard errors of ATPase activities in all animals and in the normal blooded animals among stations are shown in Figures 7.2 to 7.6. The activity of ATPases among stations in the white blooded animals was also statistically analysed with Kruskal-Wallis one way Anova. None of the ATPase activities in white blooded animals showed any difference among stations (Table 7.3). ATPase activities between normal and white blooded animals were compared with Mann-Whitney test for those which station did not show any effect (Table 7.4). Carapace length between the two groups were not different significantly, so any relationship between ATPase activity and carapace length was ignored. Mean activities of all the ATPases were higher in the white blooded animals than normal blooded animals

Figure 7.2. Total ATPase activity in the gill of Nephrops norvegicus from Clyde Sea area

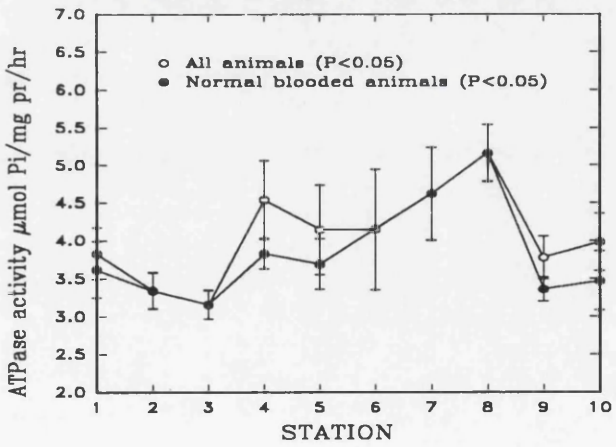


Figure 7.3. Total Mg−ATPase activity in the gill of Nephrops norvegicus from Clyde Sea area

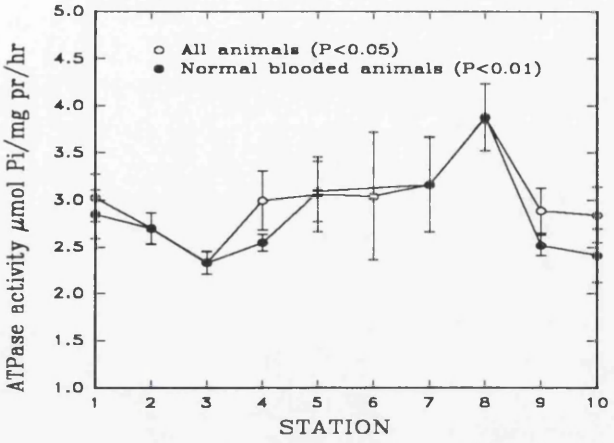


Figure 7.4. OisMg−ATPase activity in the gill of Nephrops norvegicus from Clyde Sea area

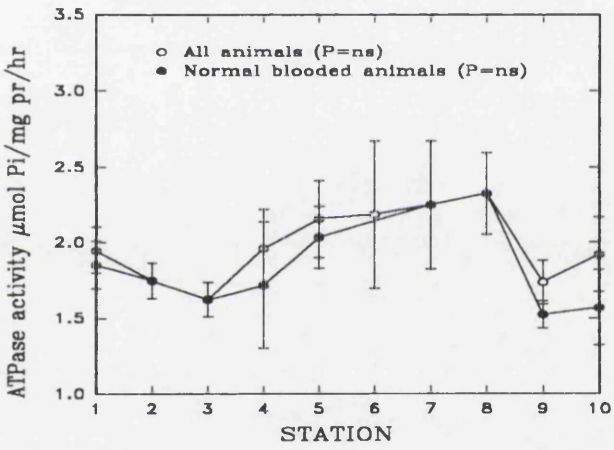


Figure 7.5. OsMg−ATPase activity in the gill Nephrops norvegicus from Clyde Sea area

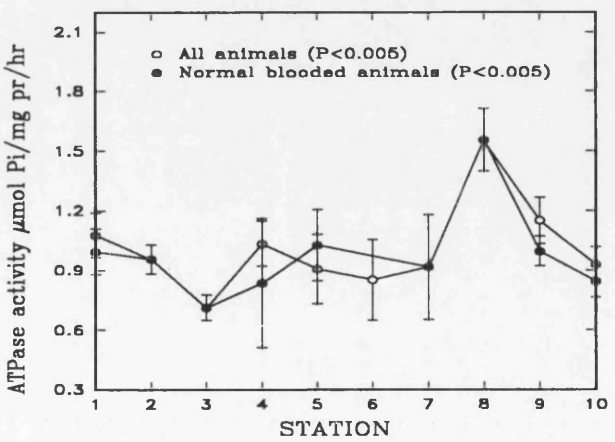
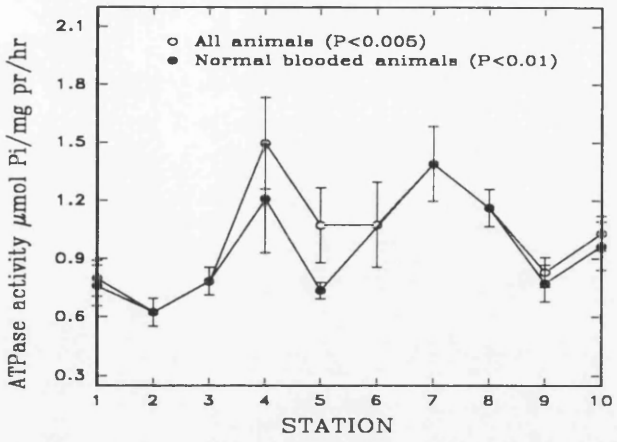


Figure 7.6. Na,K–ATPase activity in the gill of Nephrops norvegicus from Clyde Sea area



(Table 7.4). However, most of the ATPase activities showed significant differences among stations in normal animals which did not allow statistical comparisons between the two groups of animals. The activity of oligomycin sensitive Mg-ATPase was significantly higher ($P < 0.005$) in white blooded animals than in normal animals.

7.3.2 Gill parameters

The same procedure applied for the ATPase activity was also applied for gill parameters such as concentrations of sodium, potassium, calcium, magnesium and % water in the gill of *Nephrops norvegicus*. In whole samples, there was no interaction between station, blood colour and carapace length. Ion concentrations did not show any relationship with carapace length, but % water in the gill had a positive relationship with carapace length. However, station showed significant effect in the levels of gill parameters but only for the concentration of potassium and % gill water ($P < 0.05$). Because % water content of gills differed among stations, all statistical analysis for the gill ions were carried out on the dry tissue values. Blood colour also showed effects but only on % water ($P < 0.05$) and potassium concentrations in the gill ($P < 0.01$) (Table 7.2).

In normal animals alone, results of the analysis were different than the results of whole sample. There were no significant differences in concentrations of gill ions among stations ($P > 0.05$), though % gill water was still significantly different ($P < 0.05$) among stations (Table 7.3). None of the gill parameters showed relationships with carapace length. Gill parameters in the white blooded animals were not significantly different among stations ($P > 0.05$). Mean values and standard errors of these parameters in the gill of all animals and normal blooded animals are shown in Figures 7.7 to 7.11. Comparisons of normal and white blooded animals showed that concentrations of calcium, magnesium ($P < 0.05$) and potassium ($P < 0.005$) were significantly higher in white blooded animals, while concentrations of sodium were not significantly different between the two groups (Table 7.4). Although the mean % water in the gill was higher in white blooded animals, this difference could not be analysed due to station effect in normal animals (Table 7.3).

Figure 7.7. Water percentage in the gill of Nephrops norvegicus from Clyde Sea area

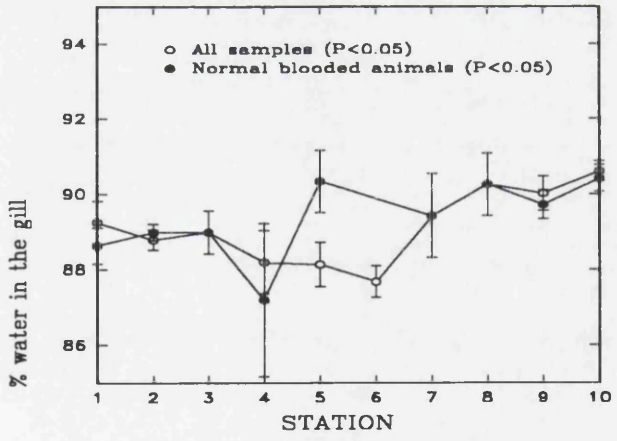


Figure 7.8. Na concentrations in the gill of Nephrops norvegicus from Clyde Sea area

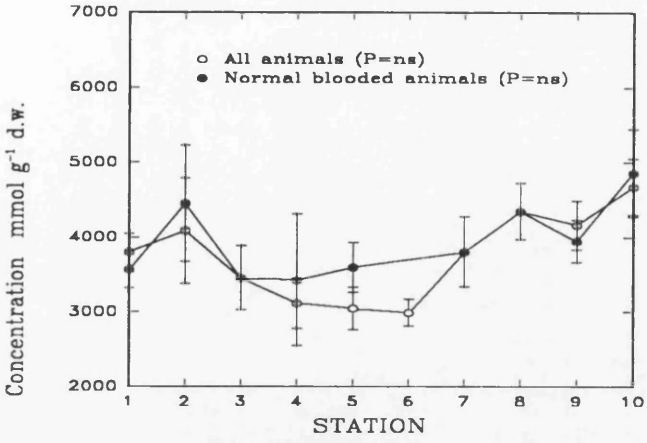


Figure 7.9. K concentrations in the gill of Nephrops norvegicus from Clyde Sea area

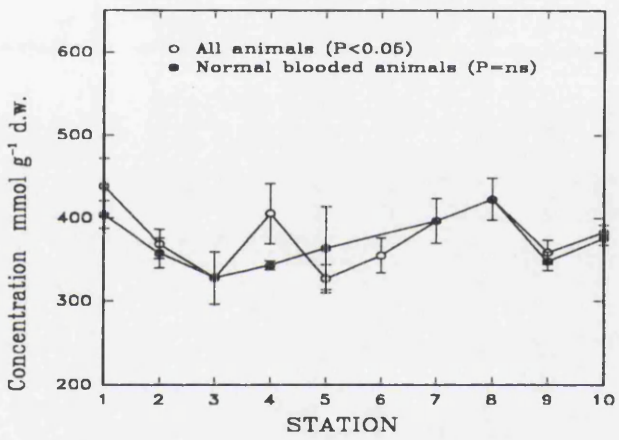
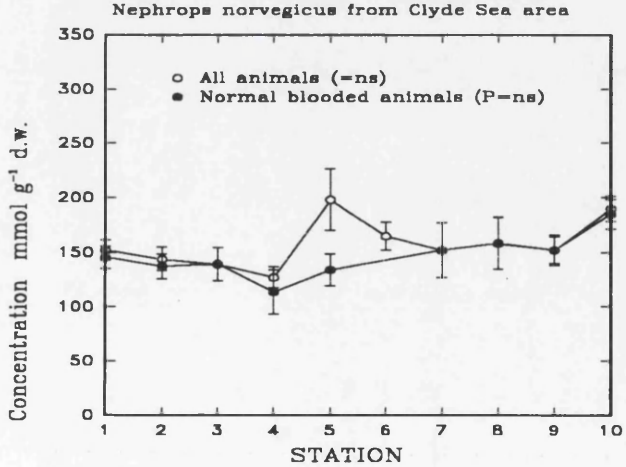
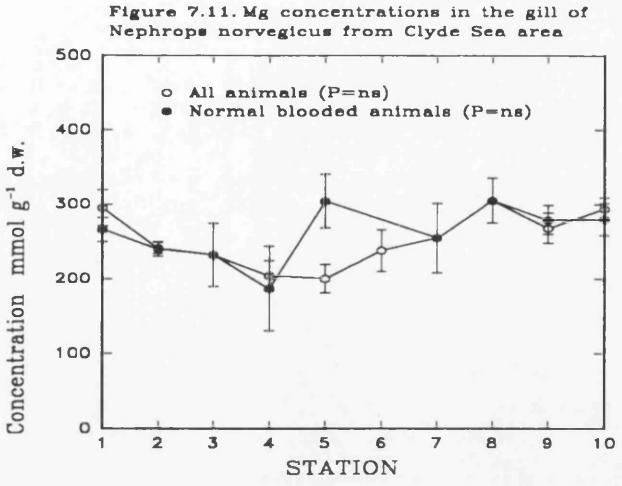


Figure 7.10. Ca concentrations in the gill of Nephrops norvegicus from Clyde Sea area





7.3.3 Blood parameters

Application of the model for blood parameters such as osmolality and concentrations of copper, zinc, sodium, potassium, calcium, magnesium and chloride are also given in the same tables (Table 7.2-7.3). In whole samples, there was also no interaction between station, blood colour and carapace length. The parameters in the blood did not show significant differences among stations except for magnesium concentrations for which there were significant differences ($P < 0.05$) among stations. However, potassium concentration showed a positive relationship ($P < 0.05$), while zinc concentration showed a negative ($P < 0.05$) relationship with carapace length. Blood colour also showed significant effects on levels of osmolality ($P < 0.05$), copper ($P < 0.0005$) and zinc ($P < 0.005$) (Table 7.2).

In normal animals alone, results differed slightly from the results of the whole sample. Carapace length showed only a relationship with zinc ($P < 0.05$). Station also did not affect the blood parameters except for magnesium concentrations ($P < 0.005$). The blood parameters did not differ significantly ($P < 0.05$) among stations in white blooded animals. Mean values and standard errors of the blood parameters in all animals and normal blooded animals are shown in Figures 7.12 to 7.19.

Comparisons of normal and white blooded animals showed some differences in the levels of the blood parameters. For example, white blooded animals had significantly higher levels of osmolality ($P<0.05$) than normal animals, while they had lower concentrations of copper ($P<0.001$) and zinc ($P<0.005$) in their blood. There were no significant differences in the levels of sodium, potassium, calcium and chloride ($P>0.05$) between the two groups. Concentrations of magnesium could not be analysed statistically due to station effect in normal animals.

Figure 7.12. Osmolality in the blood of Nephrops norvegicus from Clyde Sea area

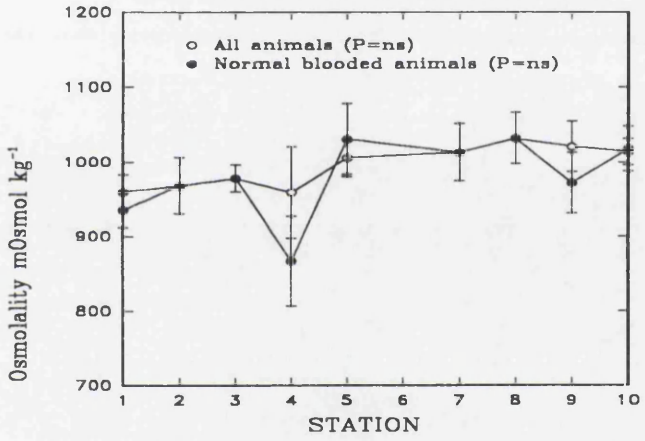


Figure 7.13. Copper concentrations in the blood of Nephrops norvegicus from Clyde Sea area

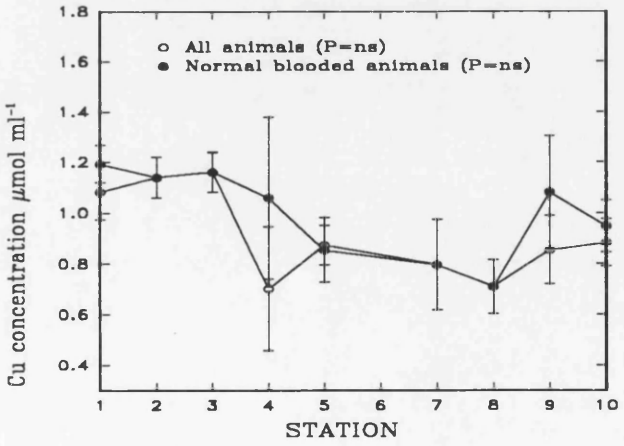


Figure 7.14. Zinc concentrations in the blood of Nephrops norvegicus from Clyde Sea area

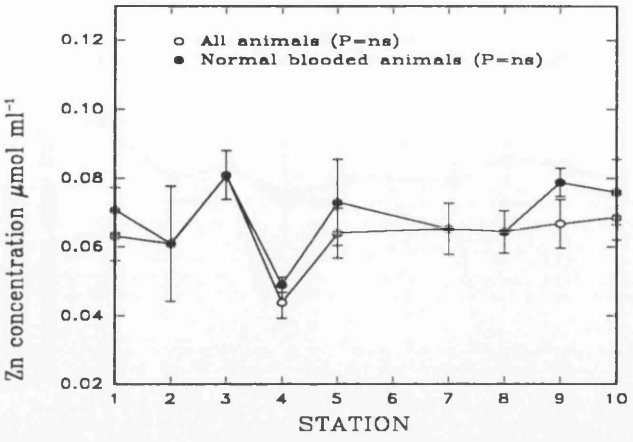


Figure 7.15. Na concentrations in the blood of Nephrops norvegicus from Clyde Sea area

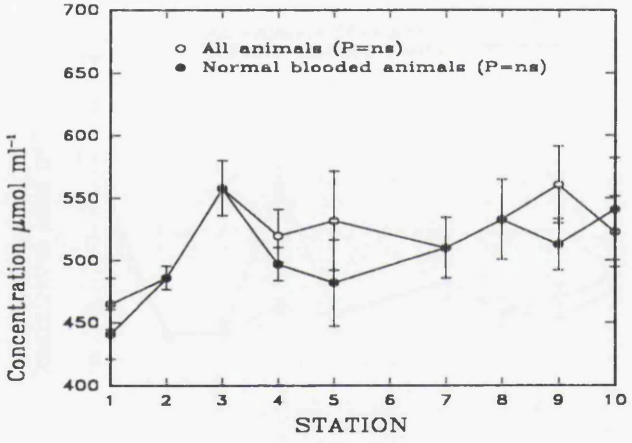


Figure 7.16. K concentrations in the blood of Nephrops norvegicus from Clyde Sea area

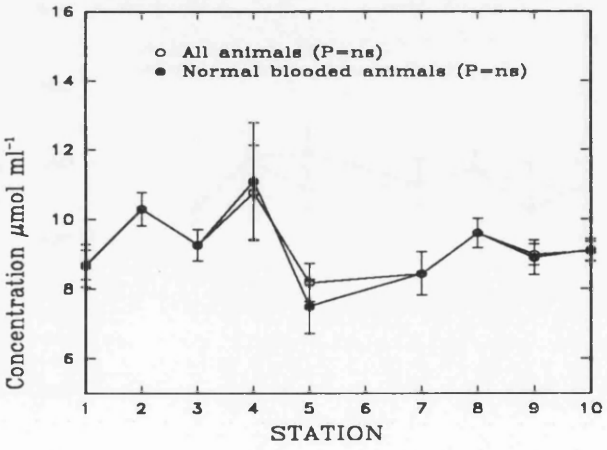


Figure 7.17. Ca concentrations in the blood of Nephrops norvegicus from Clyde Sea area

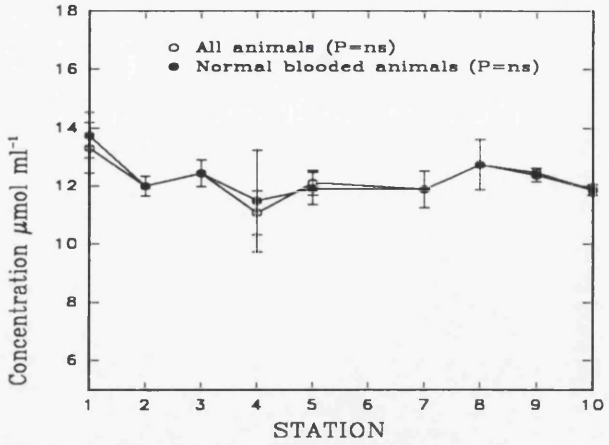


Figure 7.18. Mg concentrations in the blood of Nephrops norvegicus from Clyde Sea area

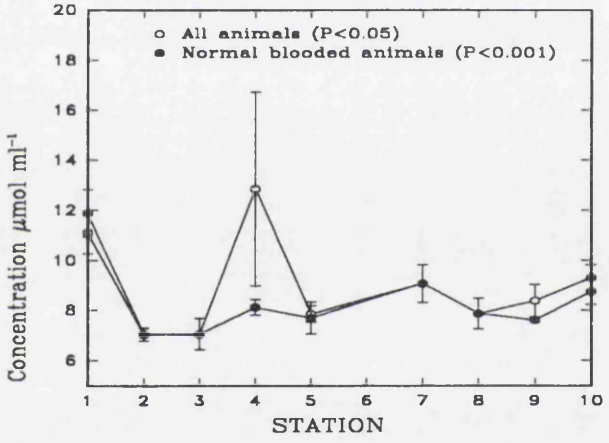
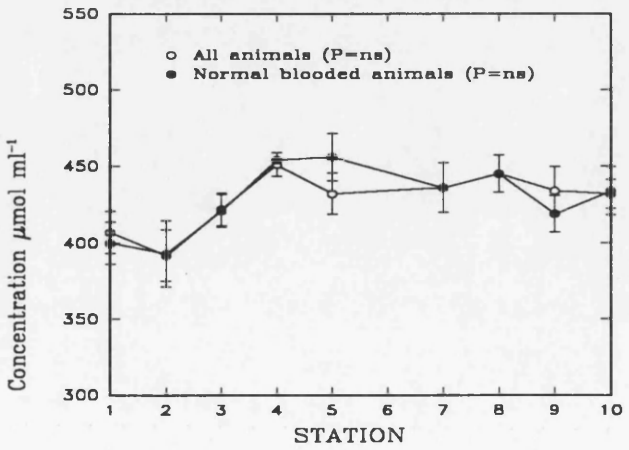


Figure 7.19. Cl concentrations in the blood of Nephrops norvegicus from Clyde Sea area



7.3.4 Correlation between ATPase activity and parameters in the blood and gill

Correlation tests were carried out between the activities of Na,K-ATPase, oligomycin sensitive Mg-ATPase, oligomycin insensitive Mg-ATPase and parameters in the blood and gill of the whole sample (including white blooded animals) and in normal blooded animals alone. Results of these tests are given in Table 7.5. In whole samples, the oligomycin insensitive component of Mg-ATPase showed positive correlations in the blood with osmolality ($P<0.05$), sodium ($P<0.05$) and chloride ($P<0.05$) and also with potassium ($P<0.05$) in the gill, while it had negative correlations with copper ($P<0.001$) and zinc ($P<0.001$) in the blood. Oligomycin sensitive component of Mg-ATPase showed a positive correlation with magnesium ($P<0.01$) in the blood and, also with sodium ($P<0.01$) and potassium ($P<0.01$) in the gill. Copper ($P<0.01$) and zinc ($P<0.05$) in the blood also showed negative correlations with this ATPase. Na,K-ATPase activity showed positive correlations with magnesium in the blood ($P<0.05$) and potassium in the gill ($P<0.01$). It also showed negative correlations with copper and zinc in the blood (both $P<0.001$).

There were some differences in the results of the correlation test when only normal blooded animals were involved in the correlation tests (Table 7.5). The number of correlated cases were much less in normal animals. For example, the significant negative correlations between blood Cu and Zn and ATPase activities were not seen in normal blooded animals. All the ATPase activities tested had correlations with only Ca in the gill or blood. There was no correlation between any parameters in the gill and blood and oligomycin insensitive Mg-ATPase activity, except a negative correlation between gill Ca ($P<0.05$) and oligomycin insensitive Mg-ATPase activity. Oligomycin sensitive Mg-ATPase activity also showed only one correlation which was a positive relationship ($P<0.05$) with blood Ca. Similarly, Na,K-ATPase

Table 7.5. Results of rank correlation between ATPase activities and blood and gill paramaters of all samples and normal samples alone. Gill ion concentrations are presented as dry weight values. Cu, Zn and Cd in the gill from different animals from the same stations. * = P<0.05, ** = P<0.01, *** = P<0.001.

		ois-Mg-ATPase		os-Mg-ATPase		Na,K-ATPase	
		All	Normal	All	Normal	All	Normal
Blood	Osmol	*	ns	ns	ns	ns	ns
Blood	Na	*	ns	ns	ns	ns	ns
Blood	K	ns	ns	ns	ns	ns	ns
Blood	Mg	ns	ns	**	ns	*	ns
Blood	Ca	ns	ns	ns	*	ns	**
Blood	Cl	*	ns	ns	ns	ns	ns
Blood	Cu	-***	ns	-**	ns	-***	ns
Blood	Zn	-***	ns	-*	ns	-**	ns
Gill	water	ns	ns	ns	ns	ns	ns
Gill	Na	ns	ns	**	ns	ns	ns
Gill	K	*	ns	**	ns	**	ns
Gill	Mg	ns	ns	ns	ns	ns	ns
Gill	Ca	ns	-*	ns	ns	ns	ns
Gill	Cu	-**		-*		-*	
Gill	Zn	ns		ns		ns	
Gill	Cd	ns		ns		ns	

activity had only one correlation which was a positive relationship with blood Ca ($P<0.01$).

The correlation tests were also carried out between ATPase activities and concentrations of copper, zinc and cadmium in the gill. However, data for gill heavy metals were from different groups of male *Nephrops* from the same stations. Copper in the gill showed negative correlations with the all ATPases tested (Table 7.5). For example, in all animals copper showed negative correlations with oligomycin insensitive ($P<0.01$) and sensitive ($P<0.05$) and Na,K-ATPase ($P<0.05$) activities. Zinc and cadmium did not show any correlation with any of the ATPases tested ($P>0.05$).

7.4 DISCUSSION

Results of the statistical analyses applied for the whole sample were very interesting when they showed that blood colour was a very important factor to consider in the activities of gill ATPases, and levels of parameters in the gill and blood of male *Nephrops*. Results of statistical analyses in normal blooded animals showed that the activities of all ATPases studied showed significant differences among stations, except for the activity of oligomycin insensitive Mg-ATPase. However, none of the other ATPase activity in white blooded animals differed significantly among stations. This could be due to low numbers of white blooded animals. Normal blooded (blue or bluish blood) animals were sixty eight, whereas white blooded animals were twelve which is not a good number for satisfactory statistical analyses. Percentage of *Nephrops* infected by the parasite in the Clyde Sea area could be very high, though all infected animals may not have white haemolymph which means the infection has several degrees. White colour of haemolymph could be seen in advanced infected

animals (Field et al., 1992). Since the infection has different degrees, classification of the infection by blood colour seemed to be a good method available to me despite disadvantage in number of samples. Mean activities of all the ATPases studied in the gill of infected *Nephrops* were higher than the levels in the normal animals. There may be two ways to explain the situation. First, ATPase activity increased in the infected animals as a result of parasite infection by changing the metabolism of the animals. The statistical analyses for the all animals (Table 7.2) showed that blood colour affected the activities of all the ATPases, though the effect of infection on the activity of Na,K-ATPase was less ($P=0.038$) than all the other ATPase activities investigated which P values varied between 0.0001-0.007. Metabolic activity in infected animals might be increased to demand higher energy consumption due to the parasitic infection which might also increase the activity of Mg-ATPase especially oligomycin sensitive Mg-ATPase activity as this ATPase has been shown to involve oxidative phosphorylation (Boyer et al., 1977). Oxygen consumption of infected animals has been found to be higher than normal animals (Field et al., data in prep.). Also the high ATPase activity in the infected animals could be due to lower levels of ATP in the infected animals than in the normal animals. Field (1992) showed that ATP concentrations of the infected animals were significantly lower than those in the normal animals. As can be seen from the results of this study only the components of Mg-ATPase activity were very much higher in the infected animals than in the normal animals which also affected the total ATPase activity. Although Na,K-ATPase activity was also higher in the infected animals, this difference was slight. Oligomycin sensitive Mg-ATPase is involved in oxidative phosphorylation and therefore is essential for ATP production from ADP. Because low ATP concentrations (Field, 1992) and higher oligomycin sensitive Mg-ATPase activity in the infected animals have been shown in this study, there may be a relationship between these two parameters. Secondly, higher ATPase activity in the infected

animals can be a sum of ATPase activities of *Nephrops* gill and parasite metabolism. Field et al. (1992) showed that the number of parasites in the haemolymph of infected *Nephrops* increases in relation to infection gradient indicating that the white blooded animals (advance infection) can have very high numbers of the parasite. This could mean that infected animals with white haemolymph could have the most concentrated parasite cells in the blood. Increase of cells in the blood could also affect the number of cells in the gill. Therefore, ATPase activity measured here could be a total of *Nephrops* gill activities and metabolic activities of the parasite. Additionally, concentrations of most gill ions such as potassium, calcium and magnesium were significantly higher in infected animals (Table 7.4) which may also affect higher activities of the gill ATPases. However, before trying to find further explanations for the results of this study, one would need to do more studies on the activities of ATPases in the parasite and look at the relationship between ATPase activity in relation to infection gradients. In fact, each measurement used in this study should also be measured in the parasite to be able to find out if the metabolism of the parasite affected the ATPase activity and the other parameters in the gill and blood of the infected animals in this study. To study relationship between ATP concentrations and ATPase activity in the gill of the both infected and normal animals would also be essential for further explanations.

The activities of ATPases have been shown to be affected by various factors in the gill of *Nephrops* including size and sex of animals and heavy metal exposure (Chapter 5). However, in this study there was no relationship between ATPase activity and carapace length of the animals. This could be due to less variation of size groups from the stations in this study. In Chapter 5, the negative relationships between carapace length and activities of oligomycin sensitive Mg-ATPase and Na,K-ATPase were found in a group of male animals for which carapace length

showed wider variation. The effects of stations on the activities of the ATPases were also significant factor especially for the normal animals which nearly all ATPases showed significant variations. Samples from the dumping ground (station 4) and from station 7 showed higher Na,K-ATPase activity, while oligomycin sensitive Mg-ATPase activity was higher in station 8. The activities of the other ATPases were generally lower in stations away from the dumping ground such as stations 1, 2, 9 and 10. Interestingly, before seeing the results of this study, I expected to see lower ATPase activities in the dumping ground since ATPase activities have been reported to be inhibited in aquatic animals by a range of chemicals including heavy metals, pesticides and detergent (Davis et al., 1972 ;Riedel and Christensen, 1977 ; Jurkowski, 1977 ; Jowet et al., 1981 ; Christensen et al., 1982 ; Heath, 1987 and Chapter 5) which might be at higher levels in sewage sludge dumping areas. Therefore, it is very diffucult to speculate that activities of ATPases were affected by heavy metals present in the stations. Additionally, except for cadmium, concentrations of copper and zinc did not vary significantly among stations in the Clyde Sea (Chapter 8). This is important because especially copper and ATPase activities had negative relationships in this Chapter and in Chapter 5. Therefore, changes in copper concentrations in the gill of the animals from different stations could also show effects on the activities of ATPase.

Correlation tests were carried out for the parameters in the gill and blood with ATPase activities in all animals and normal animals alone. Most striking result were negative correlations between blood copper and zinc concentrations and activities of Na,K-ATPase, oligomycin sensitive and insensitive Mg-ATPase in all animals. These relationships, however, became non-significant when only normal animals were involved in correlations which clearly indicates that negative correlations were due to white blooded animals. As indicated earlier blood copper and zinc concentrations

were very much lower in white blooded animals, while all the ATPase activities were higher. This may also show that they could show negative correlations. In general, there were more correlations between variables in the gill and blood with ATPase activities in all animals than only normal animals were involved (Table 7.5). Correlation tests were also carried out between the gill heavy metal concentrations (Cd, Cu and Zn) and ATPase activities but using different groups of male *Nephrops* from the same stations. In these correlation tests, copper also had negative correlations with the all ATPase activities. Negative correlation between copper and Na,K-ATPase activity in male *Nephrops* was also found in chapter 5. It seems that higher copper concentrations cause lower ATPase activity or vice versa. However, low concentrations of copper and high ATPase activity in this study are also characteristics of infected animals. From these results, it may be concluded that high ATPase activities in the present study are due to low copper concentrations which is indirectly related to infection of *Nephrops*. Additionally, high ATPase activity in the infected animals might be due to the effects of the infection for the two reasons explained on previous pages.

Concentrations of blood and gill ions were also measured in this study because these parameters have been reported to be affected by heavy metal exposure of aquatic animals. For example, concentrations of blood ions in decapod crustaceans have been shown to be sensitive to heavy metal exposure. Bjerregaard and Vislie (1985a and 1986) exposed *Carcinus maenas* to copper and mercury and found that levels of osmolalility, Na^+, K^+ and Cl^- were reduced up to 90 %. Thurberg et al (1973) indicated that the addition of copper to the medium resulted in a loss of the normal osmoregulatory patterns of the crab *Carcinus maenas* until normally hyperosmotic blood-serum became isosmotic with the surrounding medium. However, cadmium elevated blood-serum osmolality above its normal hyperosmatic state. In the same

crab, Bjerregaard and Vislie (1985b) showed that cadmium altered haemolymph ion concentrations in higher exposure regime though results were not consistant. Inman and Lockwood (1977) also showed that sublethal concentrations of methylmercury reduced concentrations of Na^+ in haemolymph of *Gammarus duebeni*. At this point, there are questions to ask. Are low concentrations of bood copper and zinc concentrations affecting the higher levels of gill ions in infected animals or, are these due to infection ? In this study, there was no evidence for the reduction of ions in the gill and blood due to heavy metal exposure. In fact, there were increases in the levels of these parameters, though these increases might be related to the parasite infection. However, to be able to answer these queries, one would need further studies such as, especilly on the determinations of ATPase activity and ion concentrations of the parasite and find out relationships between these variables and infection gradients in the gill and blood of infected and healthy *Nephrops norvegicus*.

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CHAPTER 8

HEAVY METAL AND METALLOTHIONEIN CONCENTRATIONS IN THE GILL OF *NEPHROPS NORVEGICUS* FROM CONTAMINATED AND UNCONTAMINATED AREAS OF THE CLYDE SEA; EFFECTS OF A PARASITE INFECTION

8.1 INTRODUCTION

The induction of metallothionein (MT) synthesis by various physical and chemical factors is known in animals, though real role(s) of MTs have not been established (Bremner and Beattie, 1990). One of the most important inducers of MTs is heavy metals such as cadmium, copper and zinc. Heavy metal exposure of marine animals can cause elevated levels of metallothionein in their tissues. The induction of MTs are generally dependent on metal concentrations and exposure time as well as being dependent on other physical and chemical factors (see Chapter 6).

It seems likely that metallothionein levels can be used to demonstrate of heavy metal contamination in the aquatic environment, as has been suggested by several authors (Olafson et al., 1979 ; Roch et al., 1982 ; Hogstrand and Haux, 1990). This suggestion was supported in an experiment in the gill and hepatopancreas of *Nephrops norvegicus* (Chapter 6). However, some restrictions were suggested for the use of metallothionein as a monitoring tool of cadmium contamination in the marine environment since metallothionein can also be induced by factors such as stress, steroid hormones and bacterial infection, very hot or cold environment (Sobocinski, 1978 ; Karin, 1985 ; Klaassen and Lehman-McKeeman, 1989 ; Bremner and Beattie, 1990). Therefore, physical conditions of animals studied for metallothionein determination may be crucial to determine due to possible stresses caused factors such as a disease or infection.

As indicated in Chapter 7, *Nephrops norvegicus* from the Clyde Sea area were infected by a parasite related to *Hematodinium perezi*. This parasite invades the haemal spaces, and is fatal to *Nephrops* (Field et al., 1992). The body colour of infected animals becomes a dull orange colour, while haemolymph becomes milky-

white. However, this colour of haemolymph is generally seen in severely infected animals, this may not be seen in mildly infected animals (Field et al., 1992).

The aim of this study was to measure metallothionein, copper, zinc and cadmium in the gill of *Nephrops norvegicus* from different stations (including sewage sludge dumping ground) in the Clyde Sea and to investigate levels of these parameters in relation to contamination gradients. Relationships between metallothionein and metal concentrations were also investigated to see if metallothionein can also be induced in the gill of *Nephrops* in relation to metal concentrations in natural conditions. Since the Clyde *Nephrops* were infected by the parasite and this infection has been shown to effects the survival of the animals as well as some other parameters (Field et al., 1992 and Chapter 7), it may be a very strong stress factor for the infected animals. Because stress can increase the levels of metallothionein, the infection of the animals was taken into account in statistical analysis of data.

8.2 MATERIALS AND METHODS

Male *Nephrops norvegicus* were caught in the same stations from the Clyde Sea area as shown in Chapter 7 using all 11 stations. Therefore, animal capture, study period and maintenance of the animals were the same as explained in Chapter 7. However, infection determination was different in this study. Since no blood was taken from *Nephrops* in the present study, determination of the infection was done by looking at the body colour of the animals and therefore the infected animals contain both mild and severe infections. Body colour of infected animals becomes dull orange, while the haemolymph becomes milky-white. Body colour differences between normal and infected animals can be seen in Figure 8.1. Male gonads were carefully separated and measured to the nearest 1 mg using a Mettler AE240 balance.

Figure 8.1. Pictures of heathy (H) and infected (A) *Nephrops norvegicus* (After Field, 1992).

The methods for metallothionein, protein, copper, zinc and cadmium analyses in the tissues of *Nephrops* were the same as described in Chapter 6.

The same statistical procedure used in Chapter 7 was also applied for this study unless otherwise indicated.

8.3 RESULTS

Table 8.1. Mean carapace lengths (mm), standard errors and numbers () of normal and infected male Norway lobster *Nephrops norvegicus* caught in different stations from the Clyde Sea. Results of one way Anova among stations are also given in the following table. ns = not significant, nid = no infection determination.

	Total Samples	Normal Animals	Infected Animals
1	(9) 48.1	(2) 45.2	(7) 48.9
se	1.63	4.35	1.73
2	(10) 46.0	(5) 46.8	(5) 45.3
se	1.23	2.30	1.13
3	(10) 35.7	nid	nid
se	1.24		
4	(10) 47.2	nid	nid
se	1.47		
5	(10) 39.5	(8) 38.9	(2) 42.3
se	1.90	1.84	7.60
6	(11) 51.9	(7) 53.7	(4) 48.9
se	2.18	2.82	3.33
7	(10) 49.9	nid	nid
se	2.51		
8	(10) 40.5	nid	nid
se	1.39		
9	(9) 41.7	(6) 43.0	(3) 39.2
se	1.46	1.75	2.25
10	(8) 44.6	(5) 46.9	(3) 40.8
se	1.17	2.09	0.95
11	(12) 44.7	(5) 46.1	(4) 45.9
se	0.99	1.00	1.98
Average	(109) 44.6	(38) 45.6	(28) 45.4
se	0.65	1.11	1.04
P value	P=0.0001	P=0.001	ns

Mean carapace length and numbers of infected and normal male *Nephrops norvegicus* caught from different stations are given in Table 8.1. Results of one way Anova among stations are also given in this table. Results showed that carapace lengths of whole samples ($P<0.001$) and normal samples ($P<0.005$) were significantly different among stations (Table 8.1).

Table 8.2. Results of linear regression analysis using all samples. Station and infection were used as factors while carapace length was used as covariate. ST&INF&CL indicates the interaction between infection, station and carapace length. + and - signs indicate positive and negative relationship between carapace length and variables. ns = not significant (P>0.05)

Variable	ST&INF&CL	CL	Station	Infection
Gonad	ns	+00001	ns	ns
MT	ns	ns	ns	0.002
Zn	ns	ns	ns	0.05
Cd	ns	+0.012	0.001	ns
Cu	ns	ns	ns	0.001

The statistical model applied to whole data using station and infection as factor and carapace length as covariate showed that there was no interaction for the variables among station, infection and carapace length (Table 8.2). Metallothionein concentrations did not show any relationship with carapace length of *Nephrops* and did not vary among stations. However, the infection played a significant ($P<0.005$) role in metallothionein concentrations of the animals. Copper concentrations did not show any relationship with size of *Nephrops* and did not vary among stations. Like metallothionein concentrations, copper concentrations were also affected significantly ($P<0.001$) by the infection. Similarly, zinc concentrations also did not vary among stations and there was no relationship with carapace length. However, the infection showed a slight effect ($P=0.05$) on zinc concentrations of the gill. In contrast, cadmium concentrations were not affected ($P>0.05$) by the infection but showed a positive relationship ($P<0.05$) with carapace length and levels differed significantly

($P < 0.005$) among stations (Table 8.2). Mean concentrations and standard errors of copper, zinc, cadmium and metallothionein in the gill of whole samples, infected animals and normal animals from 11 stations are shown in figures 8.2-8.5 indicating P values among stations. Male gonads from the animals were also dissected and relationships were investigated. Station and infection did not show any effect in gonad weight while carapace length showed a significant positive ($P < 0.0001$) relationship with gonad weight.

Table 8.3. Results of linear regression analysis after separation of normal and infected animals. Station was used as factor while carapace length was used as covariate.

	Normal Blooded Animals			Infected Animals		
Variable	ST&CL	CL	Station	ST&CL	CL	Station
Gonad	ns	+0.0001	ns	ns	+0.0001	ns
MT	ns	ns	ns	ns	ns	ns
Zn	ns	ns	ns	ns	ns	ns
Cd	ns	ns	ns	ns	ns	0.005
Cu	ns	ns	ns	ns	ns	ns

Since the infection significantly influenced the levels of variables, animals were separated into two groups (infected and normal groups) and the same statistical model was applied to these new groups. Results of these analyses are given in Table 8.3. There was no interaction between station and carapace length in both normal and infected animals. There was also no significant relationship between carapace length and concentrations of metals and metallothionein of either the infected or the normal *Nephrops*. Concentrations of the variables among stations did not show any significant variation, except for cadmium concentrations in infected animals ($P = 0.005$). Cadmium concentrations in the gill of infected and normal *Nephrops* were similar, though cadmium variations in infected animals were wider than for the normal animals among stations (Figure 8.2). Zinc concentrations of both groups were similar and showed similar patterns of variations in most stations. However, in

station 8 and 9 zinc concentrations in infected animals were higher than at remaining stations (Figure 8.3). Copper concentrations in the gill of normal and infected animals were generally different in most stations and followed different patterns of variations among stations. In general, infected animals had lower concentrations of copper in stations (Figure 8.4). Metallothionein concentrations of infected animals were higher in most stations than normal animals, though they showed similar patterns of variations among stations. In station 9 and 10 metallothionein levels were sharply increased in infected animals but came to the same levels in station 11 (Figure 8.5). Gonad mass did not differ among stations and was not affected by the infection whereas it showed a positive relationship ($P < 0.0001$) with carapace length in both normal and infected animals.

Figure 8.2. Cadmium concentrations in the gill of Nephrops norvegicus from the Clyde Sea

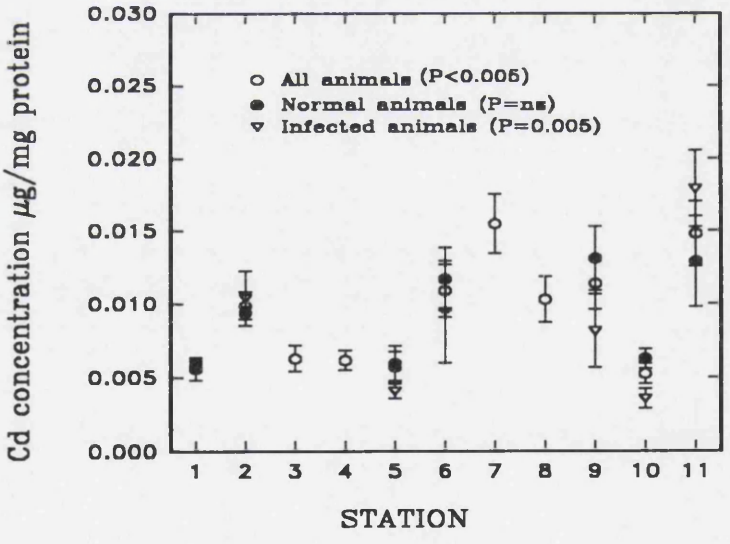


Figure 8.3. Zinc concentrations in the gill of Nephrops norvegicus from the Clyde Sea

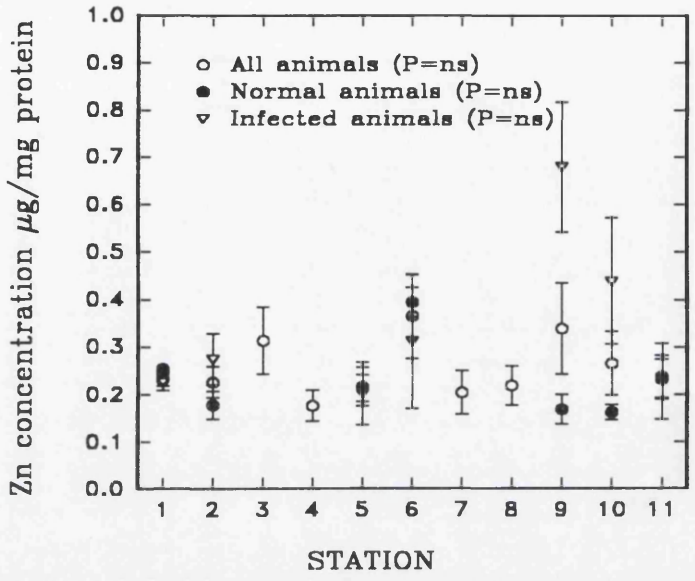


Figure 8.4. Copper concentrations in the gill of Nephrops norvegicus from the Clyde Sea

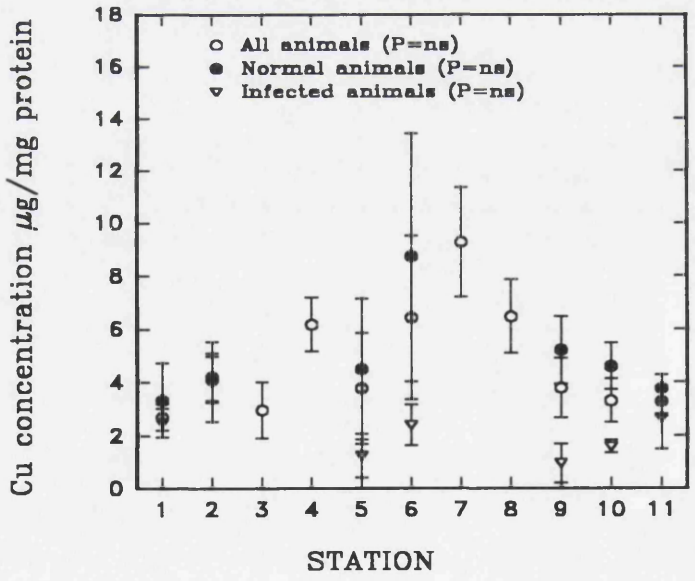
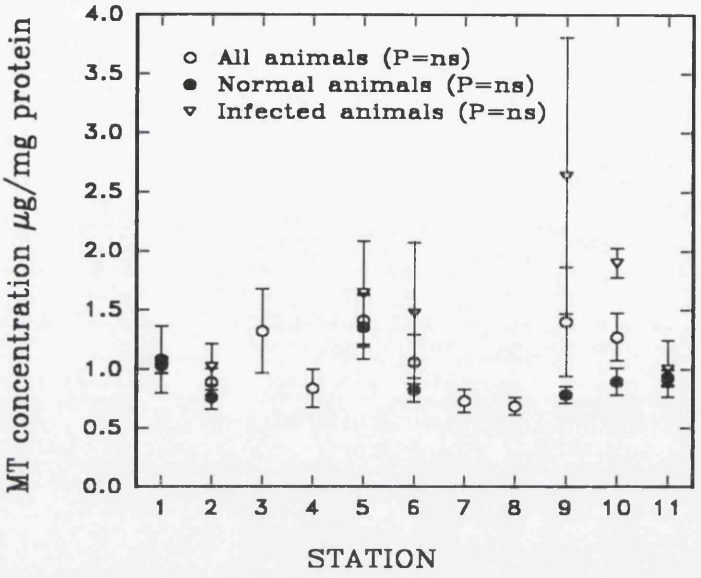


Figure 8.5. MT concentrations in the gill of Nephrops norvegicus from the Clyde Sea



Concentrations of the metals and metallothionein in the gill of infected and normal *Nephrops* were also compared with Mann-Whitney U-tests and results of this test are given in Table 8.4. Carapace length was not involved in these comparisons since both groups had similar size distributions ($P > 0.05$). This table shows that cadmium concentrations of normal and infected animals were similar, though these could not be analysed statistically due to the station effect in the infected animals. However, there were significant differences in the levels of metallothionein ($P = 0.01$), zinc ($P < 0.05$) and copper ($P = 0.001$) between the normal and infected *Nephrops*. The infected animals had higher concentrations of metallothionein and zinc, whereas they had low concentrations of copper in the gill. Mean gonad masses did not show any difference between normal and infected animals (Table 8.4).

Table 8.4. Mean values and standard errors of variables in normal and infected male *Nephrops norvegicus*. Comparisons of the two groups with Mann-Whitney U-test are also given in the following table. CL = carapace length (mm). nsc=no statistical comparison due to station effect. Gonad weight is given as gram.

	NORMAL ANIMALS		INFECTED ANIMALS		P Value
CL	45.6	(1.11)	45.4	(1.04)	ns
Gonad	0.410	(0.038)	0.339	(0.030)	ns
MT	0.961	(0.073)	1.411	(0.177)	0.011
Zn	0.235	(0.028)	0.302	(0.041)	0.044
Cd	0.0099	(0.00084)	0.0086	(0.0011)	nsc
Cu	5.30	(1.09)	2.36	(0.39)	0.001

Regression analyses were also carried out between metallothionein and metal concentrations of the gill from the whole samples and for the infected and normal animals alone. Results of regression analyses for whole samples are shown in Figures 8.6-8.7. Metallothionein concentrations showed a positive relationship ($r=0.349$, $df=99$, $P=0.001$) with zinc levels (Figure 8.6). The relationship between metallothionein and cadmium concentrations was not analysed statistically due to the effect of station on the levels of cadmium and therefore is not shown on a graph. Copper concentrations showed a strong negative relationship with MT concentrations ($r=-835$, $df=100$, $p<0.0001$) as seen in Figure 8.7. Regression analyses in infected and normal animals alone were also shown separately in figures (Figures 8.8-8.12). Zinc and MT concentrations did not show any relationship in normal animals, while in the infected animals there was a positive relationship between zinc and MT concentrations ($r=0.474$, $df=24$, $p=0.01$) (Figures 8.8 and 8.9). Cadmium concentrations also did not show any significant relationship with MT concentrations in normal animals (Figure 8.10). Relationships between cadmium and MT concentrations in infected animals were not analysed due to the station effect on the levels of cadmium in the infected animals. However, copper concentrations in both normal and infected animals showed negative relationships with metallothionein concentrations ($r=-729$, $df=34$, $P<0.0001$ and $r=-904$, $df=24$, $P<0.0001$ respectively). These relationships are shown in Figures 8.11 and 8.12.

Figure 8.6. The relationship between zinc and metallothionein in whole samples of Nephrops norvegicus from the Clyde Sea

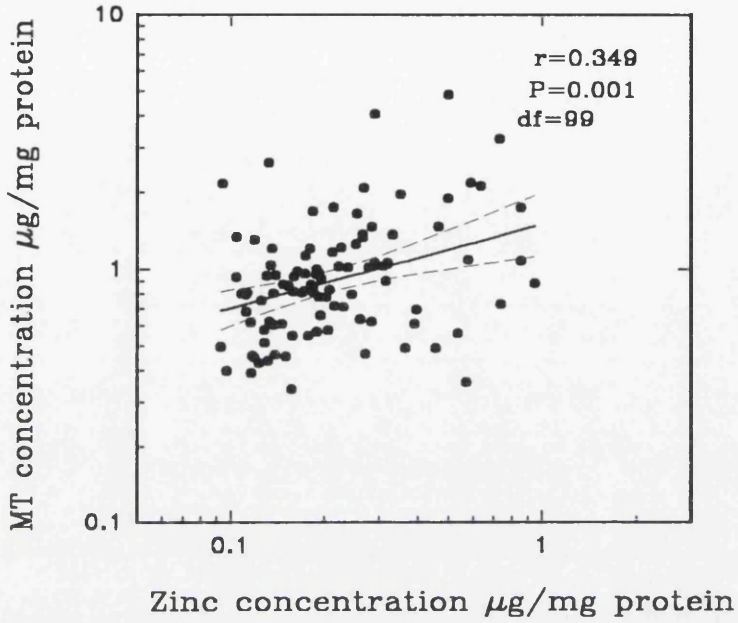


Figure 8.7. The relationship between copper and metallothionein in whole samples of Nephrops norvegicus from the Clyde Sea

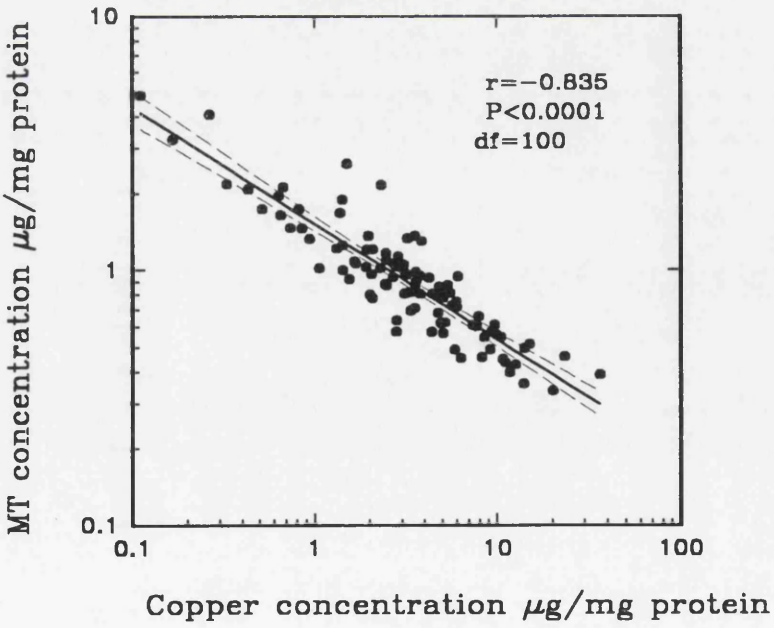


Figure 8.8. The relationship between zinc and metallothionein in the gill of normal Nephrops norvegicus from the Clyde Sea

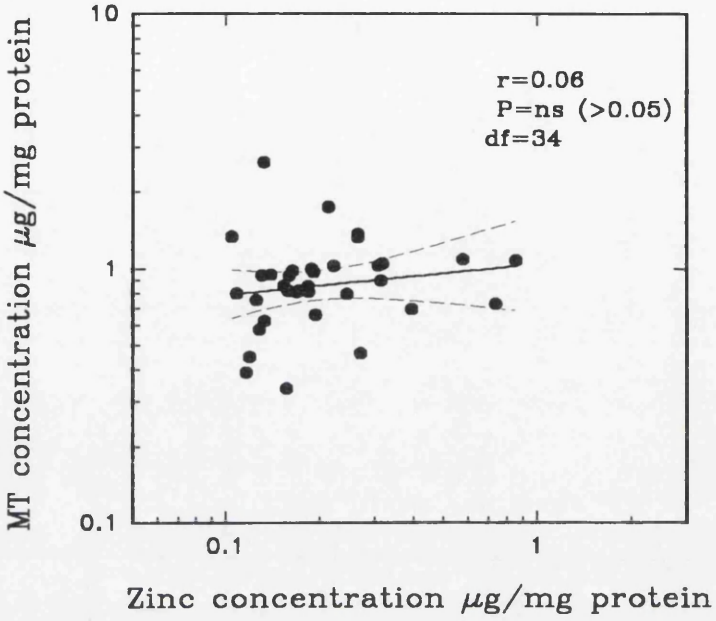


Figure 8.9. The relationship between zinc and metallothionein in the gill of infected Nephrops norvegicus from the Clyde Sea

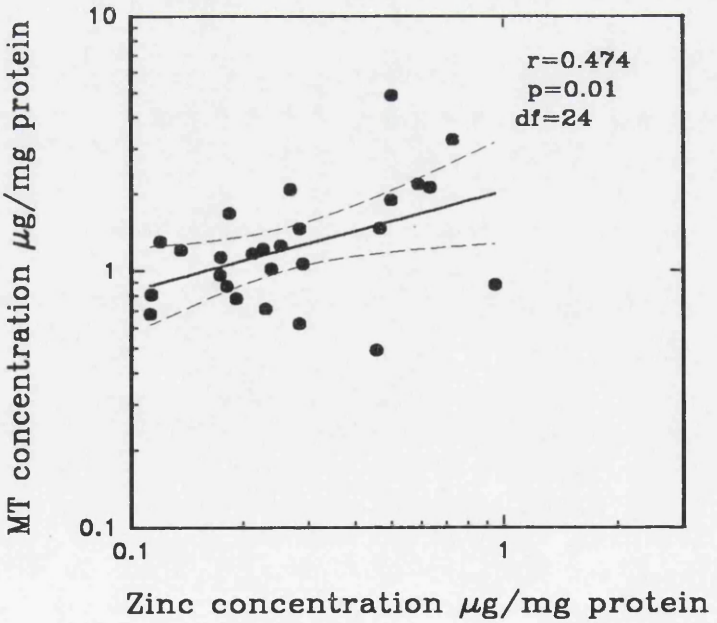


Figure 8.10. The relationship between cadmium and metallothionein in the gill of normal Nephrops norvegicus from the Clyde Sea

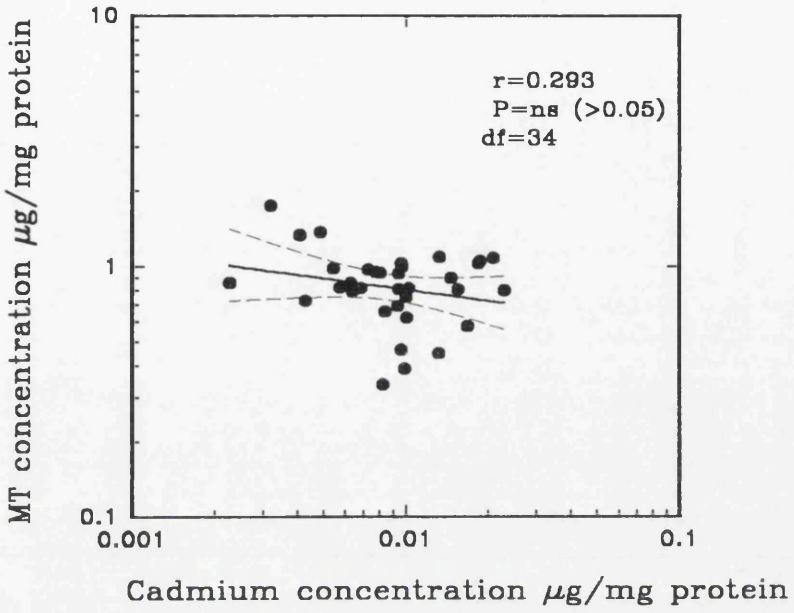


Figure 8.11. The relationship between copper and metallothionein in the gill of normal Nephrops norvegicus from the Clyde Sea

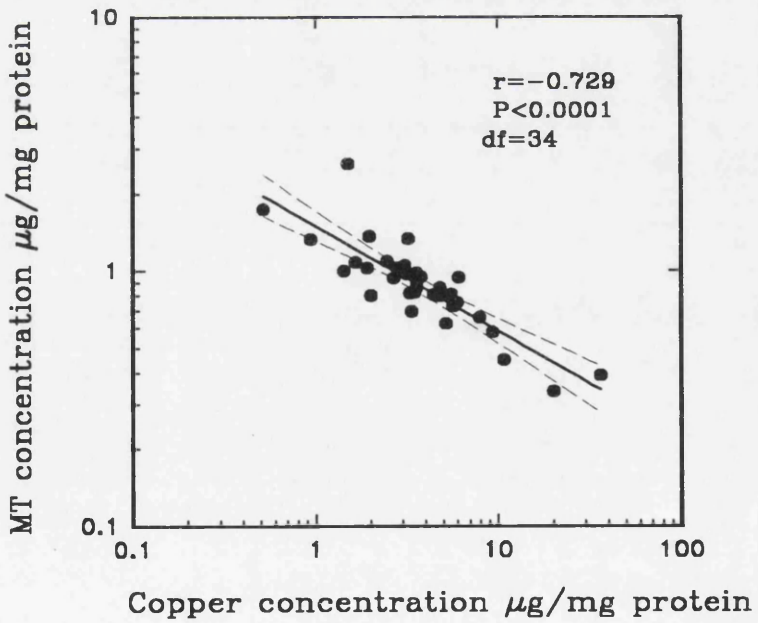
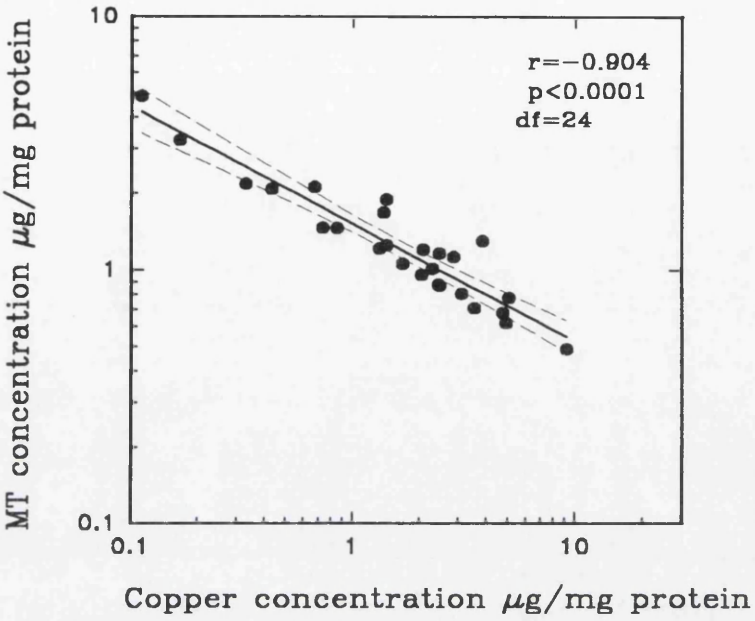


Figure 8.12. The relationship between copper and metallothionein in the gill of infected Nephrops norvegicus from the Clyde Sea



Relationships between gonad mass and metallothionein concentrations were also investigated and results given in a table (Table 8.6). There was no significant relationship between gonad mass and metallothionein in whole animals and in normal animals but there was a negative ($P<0.05$) relationship between gonad mass and metallothionein concentrations in infected animals. There were positive relationships between gonad mass and cadmium concentrations in whole samples ($P<0.0005$) and in infected animals ($P<0.05$), while there was no relationship in normal animals. Copper concentrations also showed positive relationships with gonad mass in whole samples ($P<0.01$) and in infected animals ($P<0.05$) but not in normal animals. Zinc concentration and gonad mass showed a negative relationship in infected animals while there was no relationship in normal animals and in whole sample (Table 8.6).

Ratios of metals to metallothionein (nmol/mg protein) were also calculated and given in Table 8.5. Mean ratio of copper to MT was 951 in the normal animals while this was 301 in the infected animals which gave significant difference ($P<0.005$) between the two groups. Mean ratio of zinc was 28.3 and 27.4 in normal and the infected animals respectively which was not significantly different between the two groups. Mean ratio of cadmium was 0.77 and 0.53 in normal and in the infected animals respectively. However, statistical comparison was not carried out due to the station effect on the cadmium levels in the infected animals.

Table 8.5. Mean ratios and associated standard errors of metals to metallothionein (nmol metal/MT/mg protein) in the gill of of normal and infected male *Nephrops norvegicus*. Comparisons of the two groups with Mann-Whitney U-test are also given in the following table.

	NORMAL ANIMALS	INFECTED ANIMALS	P Value
Cu	951 (313)	301 (79.2)	0.002
Zn	28.3 (3.34)	27.4 (4.58)	ns
Cd	0.77 (0.078)	0.53 (0.102)	nsc

Table 8.6. Relationships between gonad weight and concentrations of metals and metallothionein in the gill of male *Nephrops norvegicus*. + and - indicates the positive and negative relationships respectively.

	WHOLE SAMPLE			NORMAL ANIMALS			INFECTED ANIMALS		
	r	df	P	r	df	P	r	df	P
Gon&MT	0.179	102	ns	0.109	35	ns	-0.448	25	0.019
Gon&Cd	+0.342	99	0.0001	0.114	33	ns	+0.406	24	0.040
Gon&Cu	+0.261	99	0.0008	0.031	33	ns	+0.483	24	0.012
Gon&Zn	0.192	100	ns	0.044	34	ns	+0.481	25	0.011

8.4 DISCUSSION

Metallothioneins are cysteine-rich low molecular weight proteins that are characterized by high capacity to bind heavy metal ions such as cadmium, copper and zinc. This definition is the most important characteristic of metallothionein. Ever since its discovery, metallothionein has been related to many physiological processes. Among those, there is evidence that metallothioneins are involved in copper and zinc metabolism in animals (Karin, 1985). In decapod crustaceans, metallothioneins have been shown to be involved in the synthesis of haemocyanin and carbonic anhydrase as donors of copper and zinc (Brouwer et al., 1986 ; Brouwer et al., 1989). However, much attention have been paid to the role of metallothionein as a detoxification tool of heavy metals. This is not surprising because many experimental studies including a study with *Nephrops* (Chapter 6) have showed that elevated levels of heavy metals can increase the levels of metallothionein in relation to exposure period and concentration (see Chapter 6). From the definition so far, one would expect to see high metallothionein levels when high concentrations of heavy metals such as cadmium, copper and zinc are present in tissues of marine animals. In the present study, metallothionein concentrations showed a negative relationship with copper ($r=-0.835$ $P<0.0001$) and a positive relationship with zinc ($r=0.349$ $P=0.001$) concentrations in all samples, though the relationship between cadmium and MT levels could not be analysed statistically due to the effect of stations on the levels of cadmium. These relationships were also tested in the normal and infected *Nephrops* separately and, some differences were found in the relationships. For example, the relationship between MT and zinc was not significant in normal animals, while this relationship was significant in infected animals ($r=0.474$ $P=0.01$). There was also no significant relationship between cadmium and MT in the normal animals, though this relationship in the infected animals could not be

analysed due to station effect on cadmium levels. Most interesting results were the relationships of copper and metallothionein concentrations in the normal and infected animals which were very significant negative relationships ($r=-0.729$ $P<0.0001$ and $r=-0.904$ $P<0.0001$ respectively). Before attempting to explain these results, it would be essential to look at the factors which affect directly or indirectly the induction of metallothionein. MTs are known to be induced by a variety of factors including heavy metals, stress, bacterial infection, high temperature and low salinity and some other extreme conditions (see Chapter 6). In fact, all these factors are stress factors. In this point, there may be some questions to ask such as; does MT induction indicate a factor of stress caused by different sources or how much does stress play roles in metallothionein induction ?

Stress could give some explanation for the results obtained from this study. As indicated earlier *Nephrops norvegicus* from the Clyde Sea area were infected by a parasite related to *Hematodinium perezi*, a dinoflagellate which can be fatal to its host. This parasite occupies haemal species and numbers of parasites increase with the severity of infection (Field et al., 1992). It is clear that the infection is a severe stress factor for the infected animals. Since stress has been shown to cause an increase in metallothionein levels, one would also expect to see high levels of MTs in the infected *Nephrops*. Copper concentrations in the blood and gill of infected animals were found to be lower than in normal animals (Chapter 7 and the present study). Lower concentrations of copper in the blood were also found by Field et al. (1992). So it is not surprising to see a negative relationship between copper and metallothionein in all animals and in the infected animals. However, it is surprising to see the negative relationship with normal animals. To be able to explain this, one would need to see the relationship between parasite invasion and metallothionein concentrations by means of counting the parasite in the blood using a microscope. In

this way, a more reliable determination of the infection could be obtained. This may be essential especially when an animal has recently been infected and body colour is still normal though stress has already begun. Infection determination in this study was carried out by looking at the body colour of the animals, so it is an arbitrary determination and therefore early stages of infected animals might be taken as normal animals. This might cause the negative relationships between metallothionein and copper in the "normal" animals. However, there was no relationship between cadmium and metallothionein in the normal animals. Calculation of ratios of the metals to MT showed that mean copper ratio in the normal animals was more than three times higher than those from the infected animals, while zinc ratios were not significantly different. Although mean cadmium ratio in the normal animals was slightly higher than in the infected animals, these could not be analysed statistically due to the station effect in the infected animals. It is possible that copper metallothioneins in the infected animals might be degraded to give its copper to haemocyanin which has shortages of copper in the blood. The donor role of copper metallothionein for the synthesis of haemocyanin has been shown in decapod Crustacea (Brouwer et al., 1986 ; Brouwer et al., 1989). In this study total metallothioneins were measured regardless of bound metals. If copper metallothioneins were degraded to give their copper for haemocyanin synthesis or copper bound to metallothionein was replaced by other metals, these would also affect the relationship between copper and metallothionein as well as affecting the relationships with the other metals in both normal and infected animals.

There is no information in this study on metallothionein concentrations of the parasite. This could be crucial to find a better explanation of what is going on with the relationships between metals and metallothionein. If there is metallothionein in the parasite this could also affect the concentrations of metallothionein in the gill of

infected animals depending on infection gradients. Because there are many things which are not clear, discussion of this study, therefore, is wide open.

Relationships between gonad mass and metallothionein concentrations were not significant in whole sample or normal animals alone but there was a negative relationship ($P<0.05$) in infected animals suggesting that infection was higher in smaller animals. Generally, heavy metals especially cadmium concentrations also showed positive relationship with gonad mass which also supported the positive relationships between size and cadmium in tissues of *Nephrops* in Chapter 2.

This study initially was intended to investigate the relationship between heavy metals and metallothionein in contaminated and uncontaminated areas of the Clyde Sea. After laboratory experiments on healthy animals (Chapter 6) in which there were constant positive relationships between cadmium and metallothionein in the gill and hepatopancreas of both male and female *Nephrops norvegicus*, it would be essential to investigate if metallothioneins show positive relationship with heavy metal which might lead one to conclude that metallothioneins could be used as determinators of heavy metal pollution particularly for *Nephrops norvegicus*. There was evidence for fish from the literature that MT levels increased in relation to heavy metal contamination in the aquatic environment and this increase showed positive relationships with heavy metals. Therefore, it was suggested that MTs could be used as a useful quantification of the degree of heavy metal exposure of fish (Roch et al., 1982 ; Roch and McCarter, 1984 ; Olsson and Haux, 1986). For the Clyde *Nephrops* this investigation could not be done precisely due to the infection. However, findings in this study are very interesting and suggests the need to carry out some further studies such as, relationships between metals and metallothionein in relation to infection gradient using parasite count in the blood and comparisons with healthy

animals without parasite. Determination of metal and metallothionein concentrations in the parasite could be very useful to obtain a clearer explanation for the relationship between metals and metallothionein in the gill of *Nephrops norvegicus*.

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