ECOTOXICOLOGY AND ECOPHYSIOLOGY OF MYSIDS, WITH SPECIAL REFERENCE TO COPPER TOXICITY IN *Praunus flexuosus*

by

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A thesis submitted to the University of Southampton
for the degree of Doctor of Philosophy

School of Ocean and Earth Sciences
Faculty of Science

January 2000

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE
SCHOOL OF OCEAN AND EARTH SCIENCE

Doctor of Philosophy

ECOTOXICOLOGY AND ECOPHYSIOLOGY OF MYSIDS, WITH SPECIAL REFERENCE TO COPPER TOXICITY AND *Praunus flexuosus*

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Toxicity of dissolved copper was examined in a common coastal mysid population (*Praunus flexuosus*). The life cycle and ecophysiology were studied under natural conditions throughout the year, and responses to dissolved copper were determined in the laboratory. Pronounced and ontogenic seasonal differences in copper toxicity for the mysid *Praunus flexuosus* were observed. Sublethal and lethal parameters (mortality, behaviour, metabolism, reproduction, and bioaccumulation) showed seasonal variation in response to copper toxicity, being highest toxicity in summer.

Changes in the form of dissolved copper were measured during toxicity testing, using the chelating resin method to provide a measurement of total and labile (Chelex-available) dissolved copper in the natural seawater used in the toxicity tests. Labile dissolved copper did not show significant variations in the test seawater, confirming that organisms were exposed to constant labile copper concentrations during the toxicity test. The total dissolved copper concentration was significantly higher than the labile form, as organic complexation occurred in natural seawater and during toxicity tests. The labile fraction could be less than 50% of the total fraction. The total dissolved copper concentration decreased significantly when the organism was under stress.

While the mortality of the population was insignificant after 10 days of copper exposure (0, 5, 25, 75 and 200$\mu g\ l^{-1}$) in winter, lethal effects occurred at every copper exposure level after 24 hours (96h LC_{50} =30.8 $\mu g\ l^{-1}$) in summer. The effects of copper on metabolism (respiration and excretion) were very sensitive indicators of sublethal toxicity, which resulted in lethal effects with a prolonged time of exposure. Metabolism shifted to a greater reliance on protein catabolism under copper exposure in both seasons, demonstrating a stronger effect in summer. Total copper content accumulated in the organism increased with increasing copper concentration in solution. Copper accumulation rate was higher in summer than in winter, increasing to rates of 7.9$\mu g\ g^{-1}$ dry weight day^{-1}. Reproductive processes were severely disrupted at any copper treatment. Production of juveniles was reduced to zero, because of the high abortion rate, reduction on brood survival and damage to fertilisation processes.

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ACKNOWLEDGMENTS

My warmest and sincere thanks to Professor Lloyd Peck and Professor Paul Tyler (British Antarctic Survey, Cambridge and School of Ocean and Earth Science, Southampton, respectively) for their valuable guidance and help, and to Dr. Peter Statham (S.O.E.S.) for guiding me through chemistry.

I extend my thanks to Professor Patrick Holligan and Professor Mike Collins for their support to access to supervision, and to technical equipment needed for this study.

I am very grateful to John Ward, Min Gordon and Elisabeth Porthero-Thomas (B.A.S.) for their help throughout the course of an intense experimental work and to Dr. Philip Pugh and Nadine Johnston for the kind welcome when I always returned to the office, and for the interesting discussions. Thanks to Ken Robinson for the assistance in the SEM microscopy analysis.

I would like to acknowledge the help I received in the laboratory of chemistry at the S.O.E.S. by Dr. L.M. Nair (University of Cochin, India) and Dr. A Tappin (University of Plymouth), and to Professor Philip Rainbow and to Brian Smith (Natural History Museum, London) for the help in the bioaccumulation analysis.

I greatly appreciate the understanding, help and support of many friends throughout this thesis, especially to Harald Gnewuch, Karen Meidlinger, Simon Keogh, Mark Lee, Antonio Soares, Cristina Vina-Hebron, Emilio Maranon, Jose Da Silva, Jasminder Kaur, Grace Bailey, Jande Anderson, Susan Peace, Virginia Beramendi, Nerea Foncea, Radhakrishnan Periasami.

I am grateful to many students and staff from Southampton University and British Antarctic Survey. Thanks for the smiling to Eva Ramirez, Cathy Allen-Copley, Dawn Powell, Maria Baker, Ben Wigham, Beth Greenaway, Magali Lecouturier. Thanks to the members of staff Bob Stringer, Mike Crowfoot, Gary Fisher, Paul Goody, Kate Davies, Amanda Taylor and Graham Leggatt.

My sincere thanks to Dr. Jean-Claude Sorbe (University of Bordeaux) and Dr. Javier Urrutia (University of Basque Country) for the preliminary discussions about this project.

I acknowledge the scholarship provided by the Basque Government.

Finally, my thanks ❁ to Itxas, Tara, Harry and my family.

CHAPTER 1

INTRODUCTION

CHAPTER 1. INTRODUCTION

Copper is an essential trace metal required by organisms for metabolic functions. It is a component of crustacean respiratory pigments such as hemocyanin and different enzymes (White & Rainbow 1985). Copper concentrations in the coastal marine environment may reach toxic levels to both animals and plants. Increases in copper concentration in estuarine and coastal areas have resulted from, *inter alia*, industrial and domestic waste discharge, disposal of metal mining and refineries (Langston 1990); atmospheric flux from anthropogenic emissions (Chester & Murphy 1990); and use of copper as a base compound for antifouling paints (Anderson 1993, Claisse & Alziew 1993, Isensee *et al.* 1994, Powell 1994). Southampton Water and the Solent, on the south coast of England, are subjected to significant localised anthropogenic influences from industrial and domestic discharges, shipping activity and local marinas. Trace metals research in Southampton Water and the Solent (Armannsson *et al.* 1985, Fang 1995) has shown an increase of copper concentration in sediment and water around the industrial areas, and the recreational marina of the Beaulieu estuary.

Mysids, selected for the present study, are common in coastal and estuarine areas (Mauchline 1980), and are important food chain components (Sorbe 1981, McLachlan & Bate 1984, Lasiak 1986), forming a link between benthic and pelagic systems.

Mysids are sensitive test organisms to pollutants and their sensitivity is high compared with other species commonly used in pollution studies such as Cladocera (e.g. *Daphnia*), copepods, bivalves, decapods and fish (Nimmo *et al.* 1981, von Oertzen *et al.* 1988. Daniels *et al.* 1993, Nipper *et al.* 1993, Cripe 1994).

The majority of previous toxicity studies have been episodic pollution events, using non-indigenous species. A local population of mysids, not exposed to contamination, could provide a representative and realistic monitor to estimate levels of toxicity, and the associated ecological consequences.

1.1 MYSIDS (general aspects)

Mysids were originally classified as euphausiids because of their similar appearance. Subsequently, based on anatomical grounds, the Euphausicea were placed with the Decapoda and the mysids in the peracarids, such as amphipods or isopods. They were introduced as "Opossum Shrimps" by Tattersall & Tattersall (1951), but the first description of a species

was *Praunus flexuosus* (Muller 1776). They are Sub-phylum Crustacea, Class Malacostraca, Subclass Peracarida, Order Mysidacea.

Mysids live in estuaries, coastal waters, mid-oceanic water and a few species in the deep-sea. They are omnivorous, feeding on phytoplankton, zooplankton, detritus and organic matter from sediment and the water column.

Mysid shrimps are an important component of the biota along shores. Rich concentrations occur in inshore waters and they are important sources of food for many fish species (Smale & Kok 1983, Lasiak 1986). They may constitute the main source of food for demersal fish in the nearshore zone (McLachlan 1983, Wooldridge 1983, Brown & McLachlan 1990), and also in deeper areas of the continental shelf (Sorbe 1981).

1.1.1 Taxonomy

The Order Mysidacea has 2 suborders, the Lophogastrida and the Mysida. Lophogastrida have gills on the thoracic limbs. They comprise only 2 families restricted to offshore waters below 100m., but usually much deeper. The Mysida are more advanced, do not have gills, and include the remaining subfamilies of mysids. The first fossils described (von Münster 1839) were from the upper Jurassic lithographic limestone of Solenhofen (Bavaria) and the morphology of the fossils shows a greater similarity with the Lophogastridae than with the other subfamilies of mysids.

1.1.2 Distribution

Mysids are dominant in littoral and shallow shelf environments. Euphasiids are dominant in the epipelagic and upper mesopelagic regions, while mysids dominate again in the lower mesopelagic and bathypelagic zones (Mauchline 1980). Mysids are good swimmers, swimming near the bottom (suprabenthic) over rocks, weeds, sand, and in the surf zone of beaches, lagoons, marinas, docks and estuaries. In the water column (plankton), horizontal and vertical migrations occur, as well as tidal migrations (Tattersall & Tattersall 1951, Mauchline 1980). Mysids often swim against the current and are found in swarms. Light intensity appears to be the dominant factor controlling the vertical distribution of mysids, which generally avoid bright light, with a few exception such as *Praunus flexuosus*. Mysids live in a wide range of habitats (benthic boudary layer, epibenthical, pelagic) and exhibit a wide range of behaviour. Many aspects of their behaviour influence the distribution and migrations of each species. Because they inhabit a wide variety of habitats with a high

diversity of food resources, the form of the feeding appendages have been modified for the successful exploitation of the range of food resources available. However, the morphology of mysids is very conservative, compared to other peracarids, such as amphipods and isopods.

1.1.3 Internal Anatomy

The cerebral ganglia lie anteriorly and dorsal to the mouth and the large paired antennular and antennal nerves. The muscular system, which supports an active escape response, is well developed and complex in the abdomen. The alimentary canal of mysids has the same general structure as that of decapods. The stomach is divided into an anterior cardiac region and a posterior pyloric region (Mauchline 1980). Both regions are usually armed internally with spines and setae. Digestive and reproductive glands are located in the cephalothorax.

1.1.4 Respiration

The Mysida lack gills, therefore respiration is accomplished by active movement of the exopods of the thoracic limbs. This results in two powerful currents, ventilating the respiratory chamber formed between the line of attachment of the caparace in the region of the maxillules and the caparace fold. The caparace is richly supplied with an ample circulation of blood spaces and sinuses. The inner wall of the caparace is also thin and not chitinous, and gaseous exchanges take place through this wall (Mauchline 1980).

1.1.5 Excretion

Excretion is principally effected by means of the antennal gland. A tube leads from the gland into a small bladder just behind the external aperture, which is situated on the second segment of the antennal peduncle. Excretion is also carried out by groups of mesodermal cells at the bases of the thoracic limbs (Mauchline 1980).

1.1.6 Blood vascular system

The heart is formed from a single tube-like contractile chamber, and is situated in the posterior dorsal region of the thorax. Valves are present at each end and mark the extent of the heart. A blood vessel continues anteriorly as the *aorta cephalica* and posteriorly as the *arteria abdominalis*. There are 3 systems of nerve elements in the heart of *Praunus flexuosus* (Alexandrowicz 1955). The first is a local system situated on the outside of the dorsal wall of

the heart, the second consists of the nerves connecting this local system to the central nervous system, and the third comprises the nerves of the arterial valves. The *aorta cephalica* supplies the antennules and antennae, and the hepatic arteries. The *aorta* descends as the *arteria abdominalis,* to the telson and uropods. The various arteries sub-divide into arterioles within the tissues that they supply and the blood is voided from their ends into a system of sinuses. Delage (1883), concluded that oxygenation of the blood takes place in the sinuses under the caparace. Tattersall & Tattersall (1951) studied the spaces and sinuses in the inner wall of the caparace where the gaseous exchanges take place and suggested the possibility that gaseous exchange could also take place through the thin walls of the body in the thoracic region. There is a lack of more recent anatomical and morphological studies on the Order Mysida. When gills exist (Order Lophogastrida, *Gnathophausia ingens*), blood circulates through the branchial sinuses (Belman & Childress 1976).

1.1.7 Sensory system

Sensory capacity is highly developed in the Mysidacea, especially to light. Littoral mysids in their natural surroundings exhibit a general tone of colour, which blends with that of their environment. If the colour of their environment is changed the animal adopts a similar colour. The changes in colour are brought about by a system of chromatophores. Each chromatophore consists of a group of cells, usually between five and nine, which contain pigment and reflecting substances and they are closely related to the nerve ganglia. The chromatophore system consists of three main groups, the neural, visceral, caudal and accessory group. They are connected to the brain and neural system, glands, thoracic and abdominal somites and optic centre. A study of the chromatophores shows that light also influences the movement of the pigment (Keeble & Gamble 1904). The chromatophores responded to light in two ways, directly and through the eye. Experimental evidence suggests that the pigment hormones in some Crustacea are secreted from the eye-stalk (Mauchline 1980).

The eyes are stalked and movable, consisting of a number of omatidia, each of which is covered distally by a transparent piece of cuticle forming a facet. The facets together make up the cornea. Sensory hairs are present on the bases of the other flagella of the antennules. It is possible that these may serve an olfatory function, for they are more developed in males. In many species there is evidence that the male finds the female by means of chemotaxy. The sensory pore X organ and sinus gland which exert hormonal control over the chromatophores

occur within the eyestalk. A gland, termed a Y-organ, in the ventral region of the head of *Praunus flexuosus* appears to be analogous to the cnidamental gland of insects that secretes a hormone controlling the moulting cycle (Gabe 1952). There is some evidence suggesting that mysids are able to locate sources of food using chemoreceptors and glands on the integument (Clutter 1969, Fuzessery & Childress 1975). Associated with chemoreceptors, there are sub-cuticular and intra-cuticular structures of sensilla and glands. Sub-integumental tissue is connected to the outside environment by pores.

Statocysts or gravity receptors are present in the endopods of the uropods. The statocysts comprise a vesicle inside which there is a lith suspended on sensory hairs. The lith consists of a protein matrix surrounded by a calcareous shell, which is rich in calcium fluoride and has an undetermined organic base. The sensory hairs are under tension when the animal is orientated horizontally. The statocysts operate in conjunction with the eyes to control orientation of the mysid in space. In addition, orientation responses include the inputs from integumental receptors such as tactile receptors on the thoracic legs. Behaviour is complex. Mysids form aggregations, shoals, swarms and school. They perform active vertical and horizontal migrations.

1.1.8 Reproduction

Mysids, like other peracarid crustaceans, carry their embryos in a marsupium in which the entire embryonic development takes place. The ovary of the Mysidacea is located dorsally in the cephalothorax between the alimentary canal and the pericard. The testes of the male are located in the same general region of the thorax as the ovary in the female (Mauchline 1980, Wittmann 1981). The eggs are continuously invested with yolk. The ovarian tubes expand, the female moults and eggs are extruded from the oviducts into the marsupium. Sperm in the marsupium may be introduced by the male or may be draw in from the surrounding water by the female. Fertilisation of the eggs occur and the female starts carrying the brood in the marsupium.

1.1.9 Role in the food chain

The importance of mysids in the food webs of the seas is indicated by their predators. Littoral mysids mobilise organic particles between the benthic and planktonic environments. The mysids are involved in a short food chain: organic debris - mysids - fish. The impact of mysids on the quantity of organic debris in the area may be significant. Very common littoral

and sublittoral predators of mysids are amphipods, shrimps and other decapods, jellyfish, squid and fish. Mysids in the Arctic Ocean form the main diet of salmon and whales (Ross 1835, Tattersall & Tattersall 1951) and other cetaceans (Saemundsson 1937). Mysids are regularly found in the stomach contents of large animals and birds such as Antarctic penguins, because these animals prey on fish that have fed on mysids. It is as the food of fishes that mysids play their most important role. Records from the stomachs of marine fishes are very numerous and from all parts of the world (Tattersall & Tattersall 1951, Sorbe 1981, McLachlan 1983, Lasiak *et al.* 1987, Cockcrofort *et al.* 1988, Brown & McLachlan 1990). The common shrimps, like *Crangon*, or *Palaemonetes* also feed frequently on mysids (Tattersall & Tattersall 1951, Mauchline 1980).

The importance of inshore waters as nursery areas for marine teleost fish is that they provide optimal conditions in terms of food (suprabenthic community- mysids) and shelter (the surf zone may be advantageous in affording protection from predators) (Lasiak 1986).

At the base of the food chain are the primary producers, followed by the macroscopic food chain (large crustacean zooplankton and suprabenthic community which includes mysids) and the interstitial fauna, in which fish are top predators (McLachlan & Bate 1984). Mysids are a major food source for commercially important fish such a striped bass (*Dicentrachus labrax)*, herring (*Clupea harengus)*, mackerel (*Scomber scombus)*, plaice (*Pleuronectes platessa)* and several species of flounder (*Platichthys* sp.) (Markle & Grant 1970, Sitckney *et al.* 1974, Mauchline 1980). Mysids have been recorded in the diets of many other marine fish and decapod species in every ocean. Mysids also aid in the resuspension of sediments and resultant mobilisation of organic particles (Mauchline 1980).

In some cases mysids have been used by humans, such as in Jersey, Channel Isles, where *Praunus flexuosus* was collected and made into a paste called cherve used as bait for catching mullet. This industry has been carried out since the Norman Conquest. In Calcutta, mysids are sold in the markets as food and in some other areas cooked with rice and turmeric as a dish. In Japan there are fisheries for mysids, similar to those taking prawns (Omori 1978). Mysids have been used also in prawn, shrimp and fish farming projects as a live food source and they have been introduced in some ecosystems benefiting fish production and growth rates.

1.1.10 Biochemical composition of mysids

The biochemical composition of mysids is similar to euphasiids, which occupy a similar niche in oceanic waters. Analysis of *Praunus flexuosus*, showed the following composition in percents of dry weight: Protein 72.4%, Lipid 12.6%, Carbohydrate 3.1%, Ash 6.9%, Chitin 6.1% and the water content is 74.3-77.8% (Seguin 1968). Low concentrations of carbohydrates occur in mysids (Moore 1976) because glycogen stores appear to be utilised rapidly under reduced feeding conditions. Phospholipids account for 24-27.5 of total lipids on *Neomysis integer* and wax esters are virtually absent (Raymont *et al.* 1964).

1.2 TRACE METALS IN THE MARINE ENVIRONMENT

Metal contamination of aquatic ecosystems is a global problem. Virtually all pollutants eventually reach the marine environment via rivers or other freshwater run off or via the atmosphere. In the case of trace metals, man is increasing the rate of natural input to the aquatic environment, with mining and production of domestic and industrial wastes. Metals such as Cu and Zn are being mined and mobilised at rates over ten times those expected from natural geological weathering processes (Phillips 1980a). Industrial and cultural activities have led to the widespread dispersal of trace metals and substantial quantities of these materials are ultimately deposited in the sediments of lakes, rivers and in coastal areas. Trace metals released into the atmosphere are a primary source of the dissolved Cu, Fe and Zn in the ocean (Brown & Depledge 1990, Chester & Murphy 1990). Metal concentrations in the surface microlayer of the sea were found higher than at the subsurface of the oceans (Hardy *et al.* 1987), exceeding by orders of magnitude at near urban sites. Chester & Murphy (1990) showed that atmospheric flux of some metals to surface coastal water can equal or exceed the riverine flux.

Biogeochemical processes are dominant factors on the composition of the natural waters (Bricker & Jones 1995). The interactions of the water with the gases, liquids and solids contacted during the hydrologic cycle will determine its composition. Atmospheric, geological and biological factors affect the chemical environment in which trace metals are found. Trace metals physico-chemical forms (speciation) change as metals participate in biogeochemical processes. The particular form in which the metal originally entered the environment may no longer exist, but the total amount of the metal will be present as other

forms. Despite their low general abundance in natural waters, trace metals represent a group of biologically active elements.

Trace metals have been the subject of increasing research activity to determine, and ultimately to control, their concentration in estuarine and coastal marine habitats. Interest has focused on trace metals because of their persistence in the environment, their toxicity at high concentrations, and their tendency to accumulate in the biota with potential hazards to man (Kennish 1992, Sobral & Widdows 1997).

The design of studies to measure trace metal toxicity and solution chemistry has generally been overlooked. Studies have typically used oversaturated solutions. Metal complexation, adsorption and contamination have been uncontrolled and pH variations occurred during experiments (Tessier & Turner 1995). It is important to control the chemical speciation in the exposure medium external to the organism. The study of the interactions of trace metals with aquatic organisms requires interdisciplinary approaches. It is obvious that the effects of trace metals on aquatic organisms (accumulation, nutrition, toxicity) depend on metal speciation in the external environment. Animals can potentially obtain metals from ingestion of food as well as from water.

The interaction of a metal with an aquatic organism (Campbell 1995) involves: (1) advection or diffusion of the metal from the bulk solution to the biological surface, (2) diffusion of the metal through the outer protective layer, (3) sorption/surface complexation of the metal at passive binding sites within the protective layer, or at sites on the outer surface of the plasma membrane, (4) uptake or internalisation of the metal (transport across the plasma membrane). For example, the metal complex in solution and/or the free metal ion approach the biological surface, encountering polysaccharide or glycoprotein layer. The diffusion of the metal through the external biological layer is facilitated by the variety of simple functional groups containing oxygen atom donor groups which can be ionised, providing a matrix of sites through which the metal can migrate. The metal will meet the plasma membrane barrier of hydrophobic, phospholipidic character and some proteins which may traverse the lipid bilayer. The transport of material across cell membranes is associated with their competitive binding to the various ligands in the membrane. Potential binding sites are phospholipids, amino acids, carrier or transport proteins and/or ion pumps or channels that facilitate the movement of the ions across the cell membrane (Campbell 1995). Once the metal is within the cell it may interact with a wide variety of intracellular sites.

Chemical speciation is defined as the distribution of an individual chemical element between chemical species or groups of species (Turner 1995). Chemical speciation in surface waters reflects the chemical complexity of these media. Copper can be used as an example, dissolved Cu^{2+} can be reduced e.g. CuCl or complexed into dissolved inorganic complexes, dissolved organic complexes e.g. amino acids, or Cu-fulvic acid, and Cu-humic acid complexes. Knowledge of speciation is very important because different oxidation stages and chemical forms undergo very different biological and geochemical interactions. Copper exhibits redox changes in seawater Cu (I) and Cu (II). The higher oxidation state is the thermodynamically stable form of the element in oxidising seawater (Moffett & Zika 1988a). Thermodynamic equilibrium considerations predict that nearly all of the dissolved Cu in seawater should exist as Cu (II). However Cu (II) may be reduced to Cu (I) in seawater by a number of reactions, many of which are photochemically induced. Moffett and Zika (1988b) provided evidence of photoreduction of Cu (II) organic complexes in Cu (I) and or reduction of Cu (II) by photochemically produced reductants in Cu(I).

The speciation of trace metals in natural waters is controlled by the interaction of the metals with a complex and varying mixture of inorganic anions, organic ligands, reducible or oxidizable dissolved chemical species, surfaces and organisms. Biogeochemical processes of trace elements in seawater include photomediated processes, such as photochemical control of Mn, Fe and Cu redox cycling, and the formation of, and interactions of dissolved trace elements with colloids.

Inorganic speciation, as the inorganic form of metal includes a) different oxidant states of the metal (redox stage), b) hydrated metal ions (OH), c) complexes with inorganic ligands Cl^-, SO_4^{2-}, CO_3^{2-}. Organic speciation, as organic form of the trace metal or organometallic compounds, includes a) metal covalently bound to carbon such as methylforms of As, Ge, Hg, ethyl, butyl forms and b) complexes with organic ligands such as proteins and humic substances (Donat & Bruland 1995).

The organically complexed fraction for Cu dominates the dissolved speciation of copper in seawater (Sunda & Ferguson 1983, Buckley & Van den Berg 1990, Moffett *et al.* 1990, Donat & Bruland 1992, Donat & Van den Berg 1992, Donat *et al.* 1994).

Vertical profiles in central NE Pacific for the speciation of copper and zinc show that the chemical speciation of these two elements is dominated by organic complexation, although the chemical speciation of the complexing ligand remains unknown (Donat & Bruland 1995)

The chemical context for trace metal element speciation modelling involves the chemical composition of natural seawater, and the status of the master variables, pH, redox potential and ionic strength. Trace metal speciation modelling considers, in turn, complexation by small inorganic and organic ligands, complexation by natural organic matter, adsorption onto particle surfaces and redox reactions. The metal speciation and biological availability or toxicity are in function of the tendency of the metal to react, and it has been quantified by the free metal ion activity.

Copper is known to be taken up by biological systems. There are two main models proposed for metal uptake from solution. 1) taken up passively, as transport across the membrane, with diffusion facilitated by a membrane protein within the lipid bilayer (Simkiss & Taylor 1989, Depledge & Rainbow 1990), and 2) active uptake via energy-requiring ionic/enzymatic pumps (Simkiss & Taylor 1989, Rainbow & Dallinger 1993). Copper ions have a strong affinity for organic ligands. Copper binds with intracellular organic ligands and there is relatively low release of free metal ions from the equilibrium to allow back transport out of the cell (Rainbow 1997). The metal will continue to enter the cell in the absence of a transmembrane energy pump even though the total metal concentration within the cell is much higher than the external metal concentration. Copper becomes trapped in most tissues so that it cannot be mobilised from, for example, mucosal cells. It can cause a reduction in activity of many proteins, thus producing symptoms of disease (Sanders *et al.* 1983). Trace metals are usually transported internally to a particular organ, for instance the hepatopancreas or digestive gland or an excretory organ (Rainbow 1990, Rainbow & Dallinger 1993). Part of the trace metal accumulation is the detoxification of the trace metal ion in the tissues. This detoxification could be transient as copper passes from metallothionein to haemocyanin or more long term as an insoluble deposits in granular form (Rainbow 1997).

1.3 MYSIDS AND THEIR USE IN TOXICOLOGY

Mysids are excellent experimental organisms for Toxicology. They are sensitive to much lower concentrations of contaminants than those reported for other organisms, including copepods, *Daphnia*, shrimps, other decapods and fish (Nimmo *et al.* 1981, von Oertzen *et al.* 1988, Daniels *et al.* 1993, Nipper *et al.* 1993, Cripe 1994).

Many investigations have found species of mysids to be sensitive to a variety of substances at acute and chronic exposures (Lussier *et al.* 1985). Some species may be

extremely useful experimental organisms in studies of the potential impact of various pollutants on the environment. The sensitivity of mysids has already been used to demonstrate the toxicity of a wide range of materials, sediment and water quality (Table 1.1). Mysid test data have been used in hazard assessments of new chemicals as required by "The Toxic Substances Control Act" of 1976. Such test data help to determine the issue of permits for drilling fluids, in establishing Water Quality Criteria and for the issuance of natural pollution Discharge Elimination Systems in USA as governed by their Environmental Protection Agency.

Table 1.1. Toxicity tests on mysids

Acute Toxicity: 24h-96h. exposure
Chronic Toxicity: Life-cycle exposure

Test Material	Species of Mysid	Toxicity Test	Author
Cd	*Mysidopsis bahia*	Acute and Chronic Toxicity	Nimmo *et al.*1978
DEF, Diazon, Dimilin, EPN, Kepone, Leptophos, Methyl Parathion, Phorate, Sevin, Toxaphene, Trifluran	*Mysidopsis bahia*	Acute and Chronic Toxicity	Nimmo *et al.*1981
Hg, Cd, Cu, Cn, Ag, Zn, Ni, As, Cr, Pb	*Mysipodsis bahia*	Acute Toxicity	Lussier *et al.*1985
Cr, Ni, Zn	*Praunus flexuosus*	Acute Toxicity	McLusky & Hagerman 1987
Cd	*Mysidopsis bahia*	Acute toxicity	De Lisle *et al.*1988
Falisan	*Neomysis integer*	Acute Toxicity	von Oertzen *et al.* 1988
Industrial, Municipal Effluents	*Mysidopsis bahia*	Chronic Toxicity	Fisher *et al.*1989
Zn	*Mysidopsis juniae*	Acute Toxicty	Nipper *et al.*1993
Pyrethroids, Organophosphate, Cd, Cu, Zn	*Mysidopsis bahia*	Acute Toxicity	Cripe 1994
Oil	*Mysidopsis bahia*	Acute Toxicity	Daniels *et al.*1993

1.4 AREA OF STUDY

The area of study corresponded to Southampton Water and the Solent (50°40.00' N to 50°55.00' N and 1°35.00' W to 1°00.15' W). Southampton Water is the most important estuary linked to the Solent with its rivers Test, Itchen and Hamble (Fig.1.1). Other local estuaries include the Beaulieu, and the Medina estuary in the Isle of Wight. The width of the Solent is 4 km in the Western portion and 5.5 km to the East whilst Southampton Water has a width at high water of 2 km. Water depths are from 20m to 60m in open waters. The physical conditions along the shoreline range from coastal to estuarine, with a corresponding variation in degree of exposure to waves and tides. The tidal characteristics of the English Channel are the controlling factor with respect to the Solent. During a tidal exchange experiment at the entrance to Southampton Water, Westwood & Webber (1977) found that the proportion of "new water" entering the estuary on a neap tide was 32% of the flood tidal prism, which indicates a reasonable degree of flushing. The tidal features of the Solent estuary are very complex (Webber 1980). There is a sheltering effect of the Isle of Wight and the prevailing winds coming from the Southwest. The differences between minimum and maximum natural temperature in summer and winter are 14°C and 21°C respectively (Carr *et al.* 1980).

In the Solent, marine dredging for sand and gravel is a major activity with several dredging areas between the Needles and Foreland. The dredging activity which supports a considerable amount of shipping is, however, likely to have effects on the distribution and abundance of many species of fish and invertebrates.

The sediments of Southampton Water and the East Solent consist mainly of sandy-muds or muds with extensive patches of sand at the mouths of the estuary and some of the banks (Savari 1988).

Millbrook and Slowhill Sewage disposal works are located on the Test estuary. The upper Itchen estuary receives sewage effluent from the Portswood sewage disposal station. The Woolston sewage disposal works discharges at the mouth of the Itchen estuary (Fig.1.2). Southampton Water also receives waste and cooling waters from the industrial complex in the Fawley area, Hythe, the Esso Refinery as well as the Fawley power station and waste is also received from the outfalls at Netley, Hamble, Ashlett and Hook. The Beaulieu estuary receives only minor inputs of sewage waste.

Investigations of trace metals distributions (Tankere 1992) suggested that sewage effluents and oil refinery outfalls are important additional sources for Cu and Ni in the Test

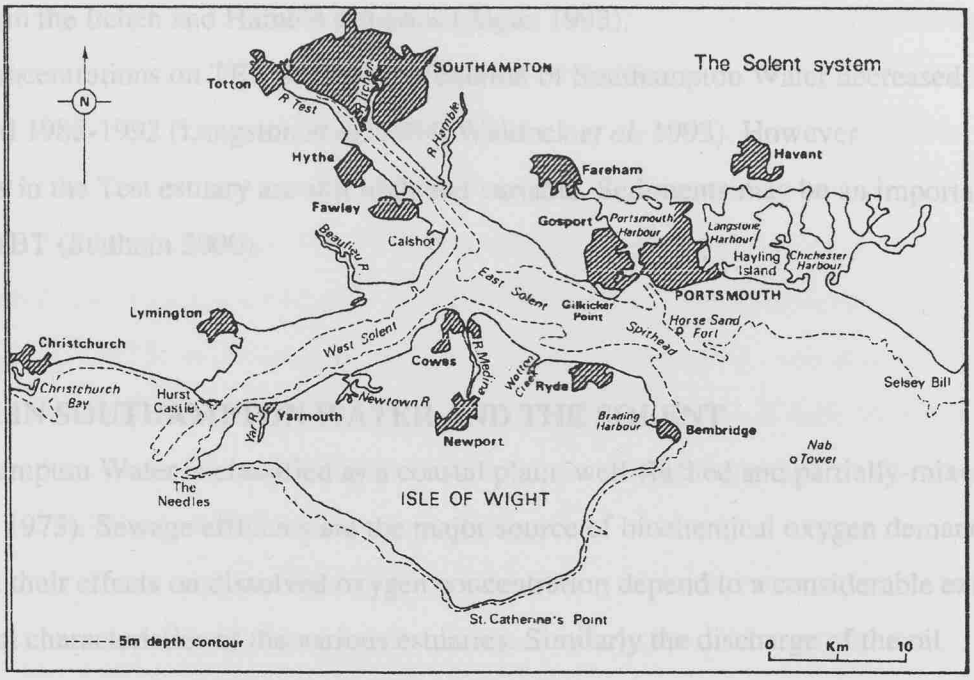


Fig. 1.1 Area of study. Southampton Water and the Solent.
(50°40.00'N to 50°55.00'N and 1°35.00'W to 1°00.15'W)

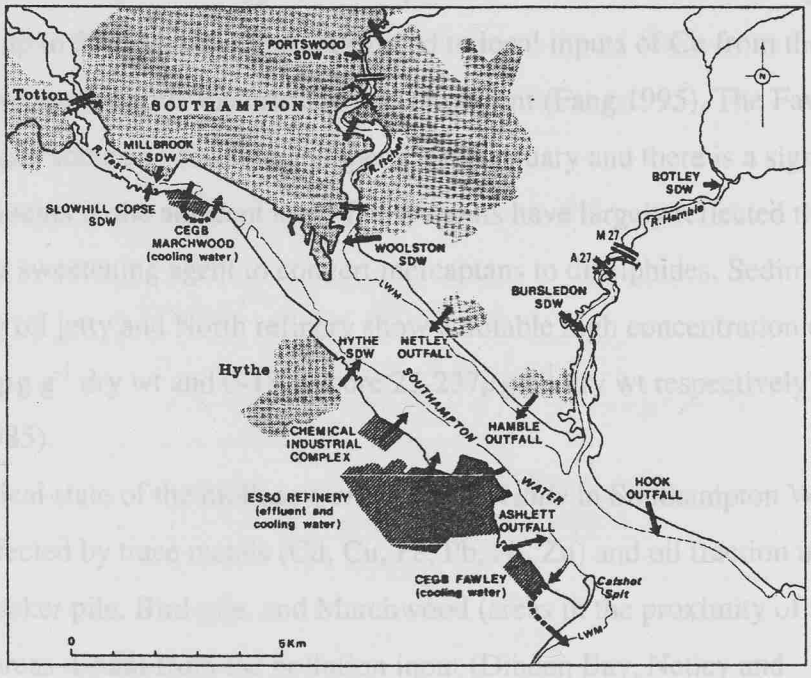


Fig. 1.2 Effluent discharges into Southampton Water and its estuaries.
(Figure adapted from Webber 1980)

and Itchen estuaries. High concentrations of Cu, Pb and Zn in the surficial sediments were also observed in the Itchen and Hamble estuaries (Algan 1993).

The concentrations on TBT in the water column of Southampton Water decreased over the period 1988-1992 (Langston *et al.* 1994, Waldock *et al.* 1993). However concentrations in the Test estuary are still high and variable. Sediments may be an important reservoir for TBT (Statham 2000).

1.5 COPPER IN SOUTHAMPTON WATER AND THE SOLENT

Southampton Water is classified as a coastal plain, well-flushed and partially-mixed estuary (Dyer 1973). Sewage effluents are the major source of biochemical oxygen demand in the region but their effects on dissolved oxygen concentration depend to a considerable extent on the physical characteristics of the various estuaries. Similarly the discharge of the oil refinery at Fawley is an important source of nitrogen, mainly as ammonia. A similar amount comes from sewage effluent. However the majority of the nitrogen supply comes from the rivers in the form of nitrate. Although pollution by trace metals is not considered a major problem in the Solent, the oil refinery appeared to be a major source of dissolved copper (Matharu 1975, Phillips 1980b).

With reference to the inputs of copper, it is important to mention that some high concentrations of Cu, up to 375nM, have been attributed to local inputs of Cu from the Esso oil refinery, which were much higher historically than at present (Fang 1995). The Fawley refinery has been a major source of dissolved copper for the estuary and there is a significant contamination of sediments in the adjacent area. These inputs have largely reflected the use of copper chloride as a sweetening agent to convert mercaptans to disulphides. Sediment taken from the Fawley oil jetty and North refinery show a notable high concentration of Cu (0-72cm core 88-362 $\mu g\ g^{-1}$ dry wt and 0-18cm core 23-237$\mu g\ g^{-1}$ dry wt respectively), (Armannsson *et al.* 1985).

The physiological state of the mollusc *Cerastoderma edule* in Southampton Water (Savari 1988), was affected by trace metals (Cd, Cu, Fe, Pb, Ni, Zn) and oil fraction in solution at Fawley, Tucker pile, Bird pile, and Marchwood (areas in the proximity of a pollution source). In areas distant from the pollution input (Dibden Bay, Netley and Woolston) the energetic budget (scope for growth) of *Cerastoderma edule* was ten times greater than of the ones located near Fawley.

Fang (1995), studied the behaviour of trace metals, during mixing in some estuaries of the Solent region particularly the Southampton estuary and part of the Solent and Beaulieu estuary. Dissolved copper concentrations in the River Test and Itchen were about 15-20 and 25 nM, respectively (Fang 1995). Direct inputs to the estuary from the chemical industrial sources probably account for some of the pattern observed. The high concentrations of dissolved Cu in the Beaulieu estuary (29-69 nM) were attributed to inputs from antifouling material, which is also a source for organic compounds, that may form complexes with the metal. Young *et al.* (1979), examining the effects of antifouling material, indicated that about 3 litres of copper-based antifouling paint is used to treat wetted surfaces of each recreational vessel per year. Assuming 400 vessels in the Beaulieu estuary and the typical loss of coatings, estimated a localised input of particulate copper of 3kg y^{-1}.

A recent review of trace metals in water and sediment of the Solent system (Statham 2000) found that concentrations of dissolved metals in Southampton Water appear to be below environmental quality standards (EQS). Sediments in Southampton Water, particularly on the western shore, show high concentrations of copper (99-67 $\mu g\ g^{-1}$) (Savari 1988, Algan 1993). In the case of TBT concentrations in the water of the Test are still high and not below EQS. The dissolved copper concentration in seawater in the study area is typical of coastal areas (Table 1.1.) and the copper concentrations in sediments are not high in comparison with other locations (Table 1.2.) subjected to anthropogenic inputs. Environmental Quality Standard (EQS) for copper in seawater is 5 $\mu g\ l^{-1}$ (National Rivers Authority 1994). EQS for copper in sediments has not been defined yet.

Table 1.2: Copper dissolved concentration ($\mu g\ l^{-1}$) in seawater at different locations

Location	**Cu ($\mu g\ l^{-1}$)**	**Reference**
Oceanic waters	0.03-0.64	Burton & Statham 1982
North Sea	0.01-6.8	Topping *et al.* 1980, Brugman 1981
Baltic Sea	0.31-0.95	Brugman 1981
Mediterranean	0.04-5.8	Huynh-Ngoc & Fukai 1979
Bristol Channel (U.K.)	0.6-5.4	Abdullah & Royle 1974
Poole Harbour (U.K.)	0.2-28	Langston 1990
Derwent estuary (Tasmania)	10-27	Bloom & Ayling 1977
Restronguet Creek (U.K.)	3-176	Bryan *et al.* 1985
Taiwan	>370	Hung & Tsai 1991
Southampton Water and the Beaulieu estuary	0.86-24.8	Tankere 1992, Tappin *et al* 1993, Fang 1995

Table 1.3: Copper concentrations in marine sediments ($\mu g\ g^{-1}$) at different locations.

Location	**Cu ($\mu g\ g^{-1}$)**	**Reference**
Bristol Channel (U.K.)	>54	Bryan *et al.* 1985
Port Pirie (Australia)	>151	Ward *et al.* 1984
Mersey estuary (U.K.)	>144	Langston 1986
Baltic Sea	>283	Brugman 1981
Derwent estuary (Tasmania)	>400	Bloom & Ayling 1977
Los Angeles (USA)	>940	Hershelman *et al.* 1981
Restronguet Creek (U.K.)	>2540	Bryan *et al.* 1985
Southampton Water	33-99	Savari 1988, Algan 1993

1.6. OBJECTIVES OF THE STUDY

The aim of this thesis was to study the toxicity of a common pollutant present in the study area (copper) on a common coastal population of hyperbenthos and zooplankton species (mysids).

The specific objectives of this study are:

- To study the life cycle and ecophysiology (metabolism and reproduction) of *Praunus flexuosus* under natural and experimental conditions, and evaluate the copper effects (Chapters 3, 7 and 8).
- To study the copper speciation and bioavailability in relation to toxicity testing (Chapter 4).
- Identify lethal and sublethal toxicity levels of copper for different seasons (Chapter 5).
- Quantify copper bioaccumulation at different seasons (Chapter 6).
- Evaluate copper effects on the physiology (metabolism and reproduction) of the mysid at different seasons (Chapters 7 and 8).

CHAPTER 2

GENERAL MATERIALS AND METHODS

CHAPTER 2. GENERAL MATERIALS AND METHODS

This chapter describes the general materials and equipment used in the experiments of this thesis, and the collection and maintenance of mysids. More detailed information on specific experiments are given in the relevant chapters.

2.1 SAMPLING METHODOLOGY FOR MYSIDS

Mysids, as part of the hyperbenthos (or suprabenthos), are caught by benthic and planktonic sampling gears but specific devices have to be used to sample the hyperbenthos effectively. Depending on the topographical features of the sites, hand and plankton nets were used. Samples were collected at different depths, in estuaries, coastal waters, beaches, and marine lagoons. Collections from the boats used plankton nets, because the nature of the bottom did not allow the use of epibenthic sledges.

Once collected, samples were transported live, in seawater from the collection site in a cool box to the laboratory to avoid temperature fluctuations and minimise the decrease in oxygen levels. Reduced light levels were used to keep the animals calm during transport. Separate seawater samples were collected from the field and transported using acid washed polypropylene containers to the laboratory.

Experimental work concentrated on one mysid population. The study of the different aspects of this thesis required the regular collection of specimens of the same population and the establishment of an aquarium system for mysid maintenance at field conditions and controlled water quality.

Collection of the specimens for experimentation was performed once or twice per month using a 0.5 mm mesh size hand net, from a marine lagoon at Keyhaven, West Solent, Southampton, (50°40.00'N to 50°55.00'N and 1°35.00'W to 1°00.15'W). To minimise physiological stress, individuals were transported to the aquarium within 1h and maintained at ambient conditions similar to field conditions, including water quality.

In order to work with experimental animals that were representative of the field population, regular comparisons were made with field (Keyhaven) population and the population maintained in the laboratory (originally sampled from Keyhaven). Growth (length), animal condition and behavioural patterns of both populations were monitored and field temperature changes were parallel in the laboratory.

2.2 AQUARIUM MAINTENANCE

Techniques for the laboratory culture of *Mysidopsis* species (Lussier *et al.* 1988), note the importance of water quality for the maintenance of mysids in healthy condition. Salinity, temperature, dissolved oxygen and nitrogen levels must be maintained at optimum laboratory culture conditions.

The culture of mysids used in this study, was performed in glass tanks with an undergravel filter and gentle aeration. 50 litre tanks were used, with increased surface area to depth ratios because the epibenthic behaviour of mysids requires a benthic surface area. Natural seawater collected from the sample location (field) was filtered through a 1.2µm filter (GF/C) and UV irradiated before use as seawater aquarium. The substratum used was coral gravel. To maintain the water quality, a biofilter, undergravel filter and UV light filter were used. Careful monitoring of the nitrogen compounds was carried out twice per week as were other variables such as pH, temperature, salinity, oxygen and light period and intensity. It was intended to reproduce the field conditions as closely as possible, to reduce stress and avoid any effects on the physiology of the mysid. Water movement helped maintain normal behaviour in the mysids including tactile responses and normal orientation to currents. It also aided the resuspension of food and particulate matter in the water column.

Water quality parameters were maintained at pH ~ 8, dissolved oxygen > 80 % saturation , ammonia ~ 0.1mg l^{-1}, nitrite < 0.05 mg l^{-1}, nitrate ~10 mg l^{-1}. Light intensity - 350 lux and salinity of 33.

Water movement and levels of dissolved and particulate organic matter were also controlled. Copper and other metal contamination were controlled at the sampling, working environment, laboratory materials, reagents, experiments and analysis following procedures in Howard and Statham (1993).

Feeding procedures and the culture management are important factors in maintaining the mysids. Mysids are filter feeders, on suspended particles or preying on copepods and other zooplankton. *Artemia* gave the best results as a diet. Fresh and live 24h-old or 48h-old *Artemia*, have a high nutritional value and the live food maintained the feeding stimulus and normal behaviour. The maximum population density of mysids in the tanks was 50 adults l^{-1}. Mysids will cannibalize their own juveniles, or injured adults, if not enough food is provided or if the population density is too high. Fresh *Artemia* (25 nauplii per mysid) was provided daily to avoid cannibalism. Field-collected animals were kept in separate tanks for at least 2

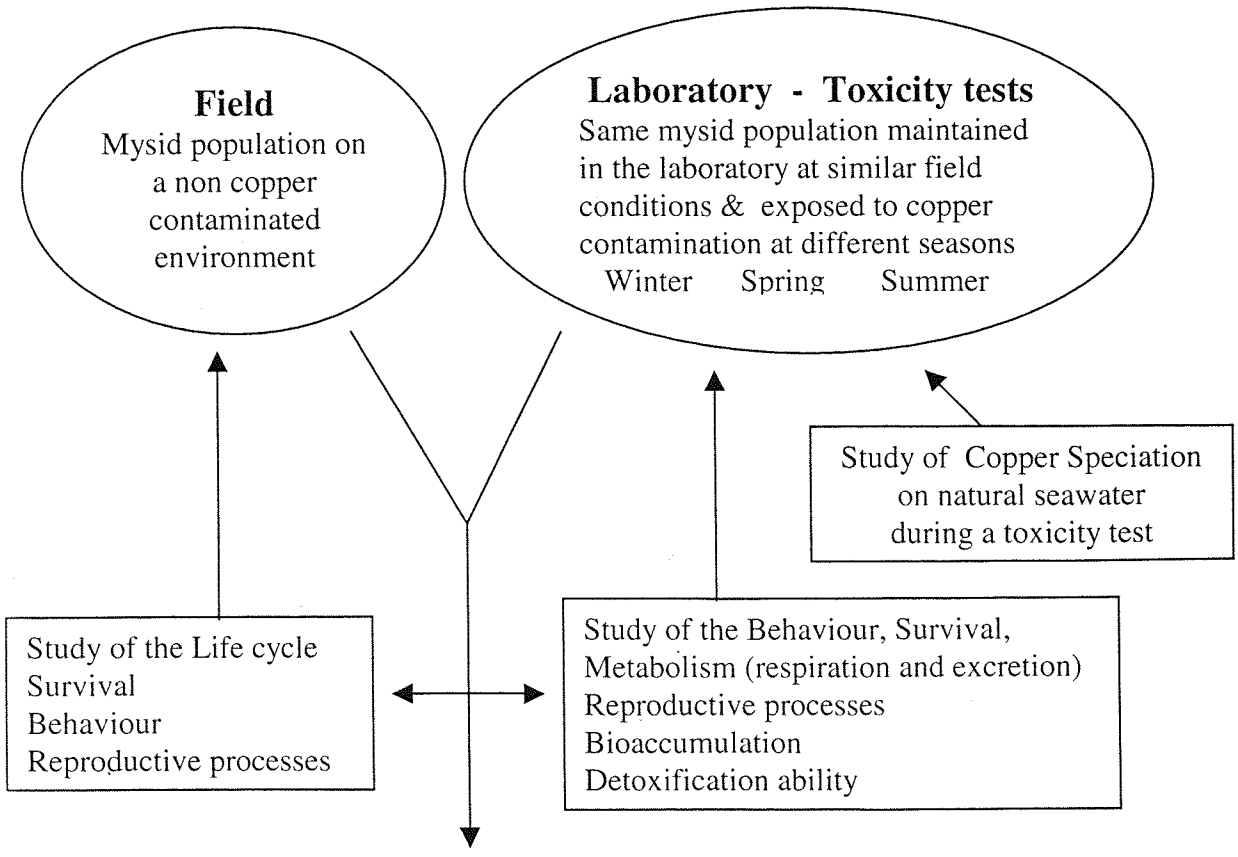
weeks before experimentation, and added to the aquarium system only after careful examination to detect the presence of any pest or disease.

2.3 EXPERIMENTAL DESIGN

Several series of experiments were designed to obtain data on three main topics:

- The biology of the species: life cycle, metabolism and reproduction.
- Lethal and sublethal effects of copper: survival, metabolism, reproduction, behaviour and bioaccumulation.
- Metal speciation and bioavailability of copper in a toxicity test

Specimens collected from the unpolluted site at Keyhaven, were kept in the laboratory in similar conditions to avoid any changes in physiology following the field variations of temperature along the seasons (5 °C to 20 °C) and photoperiod (11L:13D, 9L:15D, 16L:8D). Water quality parameters were controlled, as described in (2.2) aquarium maintenance.



2.4 TOXICITY TESTS

Individuals collected from the field in different seasons were acclimated for one week in the aquarium simulating field conditions of temperature, light and salinity. Individuals were representative of the physiological condition of the population of the field in winter and summer. Individuals were selected for toxicity testing on the basis of life cycle stage, size and animal condition. Toxicity tests were static with seawater and toxicant solutions renewed every 48h. Tanks were cleaned of remaining food and organic material at the same time. Individuals of each life cycle stage of the population were exposed to copper at each test concentration for 10 days or in some cases 1 week. *Artemia nauplii* were provided daily at 20-25 nauplii per mysid. Test chambers comprised glass tanks acid washed in 10 % HCl and rinsed in Milli Q water (18.2 M• cm^{-1}). Tanks were filled with 1µm-filtered, UV filtered and aerated natural seawater. Toxicant metal solution (Analar $Cu(NO_3)_2$) was prepared in Milli Q water. The stock copper solution (100ppm) was added to the seawater in the experimental chambers, to give concentrations of 5, 25, 75 and 200µg l^{-1} copper added (Cu_a). Copper solution additions (µl) to the seawater did not significantly change the pH.

Different biological variables were measured in the toxicity test of the different series of experiments. Seawater and specimen samples were collected during experiments to analyze the copper concentration.

Experimental series 1. Toxicity test performed to detect the lethal and sublethal concentrations of copper on *Praunus flexuosus*. Behavioural responses and survival were recorded.

Experimental series 2. Assessment of normal conditions and winter metabolism and the effects of copper. Respiration, excretion, mortality and behavioural responses to copper were recorded.

Experimental series 3. A study of reproduction and metabolism and responses to copper in spring and summer conditions. Life cycle, reproduction, metabolism, mortality and behavioural responses to copper were recorded.

Experimental series 4. Reproduction responses to copper, detoxification capability and mortality were studied in spring, and summer conditions in the following year.

Experimental serie 5. Copper speciation and bioavailability in a toxicity test. Changes in the form of dissolved copper were studied during a toxicity test, from the beginning of the test to the renewal of the seawater and solutions (48h later).

CHAPTER 3

DISTRIBUTION AND LIFE CYCLE

of *P.flexuosus*

in Southampton Water and the Solent

CHAPTER 3: DISTRIBUTION AND LIFE CYCLE OF *Praunus flexuosus* IN SOUTHAMPTON WATER AND THE SOLENT

3.1 INTRODUCTION

Mysids are very common in coastal and estuarine waters around the British Isles (Tattersall & Tattersall 1951, Makings 1977, Mauchline 1980). *Praunus flexuosus*, was the first species of Mysidacea described (Muller 1776). This species is eurythermic and euryhaline, lives in coastal and estuarine areas of the North Atlantic European Coast and the Baltic Sea, including the British Isles, Scandinavia, Denmark, Iceland, Germany, Holland, France, Spain and Portugal. Although *Praunus flexuosus* is an endemic European species, it has been recorded on the East Coast of the United States at Cape Cod, Massachusetts (Wigley 1963) and its distribution extended along the coastline of Massachusetts, New Hampshire and Maine.

On British coasts *Praunus flexuosus* is probably the most abundant species of mysid. It can be found living in rock pools, *Zostera* beds, marine lagoons and over sandy ground in water of a few cm to 20 meters deep. Populations usually live in swarms.

One distinctive characteristic of *Praunus flexuosus* is its ability to change colour in response to variations in light intensity and alterations in the background. The colour of the body harmonises with the environment. This is effected by means of the expansion and contraction of an elaborate branching system of chromatophores. In *Praunus flexuosus*, chromatophores are sensitive to light, but they are controlled by the eye acting through the nervous system (Mauchline 1980). Chromatophores contain pigments and reflecting substances and, in the case of *Praunus flexuosus*, are segmentally arranged. Chromatophores are closely associated with the nerve ganglia, like branches of the neural centres.

Mysids have an escape mechanism in response to disturbances occurring in the water (Kaartvedt 1993). *Praunus flexuosus* can change the direction of swimming quickly and powerfully. Horizontal and vertical migrations have been observed from coastal shallow water to deeper waters to avoid unfavourable environmental condition, such as intense increases in temperature, food resources or predators (Mauchline 1980, Thetmeyer & Kils 1995). Feeding occurs during active swimming, where individuals ingest diatoms, copepods and organic matter in the water column and from the surface of the sediment. In turn, *P. flexuosus*, as with other mysids are actively preyed upon by fish, shrimps, jellyfish and ctenophores. *P. flexuosus* has also been identified as an important component of the stomach

contents of many commercial fish species including herring, cod, sea bass, flounder, plaice, dab, and sole (Mauchline 1980).

Distribution records of mysids in Southampton Water and the Solent show that specimens of *Neomysis integer* and *Praunus flexuosus* have been collected regularly from different sites in Southampton Water (Totton, Marchwood), and the West Solent (Buckler's Hard, Beaulieu) (Fig.3.1). Between 1964 -1977, populations of mysids were recorded as abundant in those areas (Raymont *et al.* 1964, Austin 1965, Ralph 1965, Seguin 1968, Fergusson 1973, Armitage 1979). Other species recorded in the East Solent, include *Leptomysis gracilis, L. lingura, L. mediterranea, Mysidopsis angusta, Siriella armata, S. clausii, Schistomysis kervielli, S. spiritus, S. ornata, Paramysis arenosa, Praunus neglectus, Gastrosaccus spinifer*. Mysids accounted for 5.7% of the total number of individuals in the sample (Axelsson 1996).

The aim of this study was to characterise the distribution of the mysid populations in the study area and the life cycle of one species mysid population on which to base further studies.

3.2 THE LIFE CYCLE OF *Praunus flexuosus*

Variations in the life cycle of *Praunus flexuosus* are observed with changes in geographic distribution. Early studies of mysids in the British Isles noted a breeding period for *Praunus flexuosus* from February to the end of September (Tattersall & Tattersall 1951), and most authors have recorded a breeding period from spring to autumn.

Praunus flexuosus in Port Erin (Isle of Man), has an over-wintering population that matures and breeds in the early spring and summer. They produce a spring generation of juveniles, which appear in the population in May or June (Liao 1951). The individuals of the previous over-wintering population die in July or August. Some individuals of the spring generation become sexually mature and breed in the early autumn. The progeny of this autumn breeding together with the bulk of the spring generation form the over-wintering population. In Loch Etive (West Scotland) and Millport (Clyde Sea) the life cycle is similar to the one described at Port Erin (Mauchline 1971) (Table 3.2). The reproduction at Millport was described as more active in autumn than in Loch Etive.

In Danish waters *Praunus flexuosus* breeds from May to October (Blegvad 1922). There is no breeding in winter, the overwinter population dies in August after breeding.

Praunus flexuosus on the south-west coast of Iceland has a life cycle where individuals have a one year life span (Astthorsson 1987). The breeding period is during June-August and each female appears to produce three broods and no growth occurs during the winter months of October-March. In Iceland there is one breeding generation per year, whereas in Britain and Denmark there are two. In France, at Roscoff, breeding occurs throughout the year, with a lower intensity in the winter than in the summer (Nouvel & Nouvel 1939). The females live for 12 months. The overwintering generation breed in April-May, whereas the spring generation breed from July until the next spring and females of small size, born in late summer, overwinter and breed in the following April-May.

3.3 MATERIAL AND METHODS

3.3.1 Distribution of mysid populations

Sampling of the population (as described in chapter 2) was performed at the reference sites where collections have been made regularly over the last 20 years, and at new locations (Fig.3.1). The reference sites were located at Southampton Water (Totton, Marchwood), and the West Solent (Buckler's Hard, Beaulieu), (Ralph 1965, Raymont *et al.* 1965, Fergusson 1973, Armitage 1977, Armitage & Morris 1982). Samples were collected (see chapter 2) monthly from the field and twice per month during the reproductive period. Some specimens from the sample were fixed in 5% seawater formalin and the others were maintained alive in the aquarium primarily for behavioural and physiological investigation. Every mysid sampled was identified, using a binocular microscope. Total body length of each individual was measured from the base of the eye-stalk to the posterior end of the uropods with a calibrated eyepiece micrometer. The population was divided into 1mm length classes.

3.3.2 Life cycle

Live specimens and formalin-fixed specimens collected from the field were analysed under a microscope. Life cycle stages were determined for each individual and classified for the following stages:

- breeding female (containing eggs in ovary / marsupium or brooding developing embryos)
- non-breeding female (marsupium and ovaries empty / presence of oostegites or marsupium)
- male (testes and elongated 4th pleopod)
- juvenile (immature individual- absence of sexual characteres described above)

- newborn juvenile (post-released or emerged individual from the marsupium)
- embryo

3. 4 RESULTS

3. 4. 1 Distribution of mysids in Southampton Water and the Solent

The sampling programme carried out in Southampton Water and the Solent showed the distribution of mysids (Table 3.1) at the sites shown in Fig.3.1.

Table 3.1. Mysid species distribution in Southampton Water and the Solent.

Species	**Site**
Mesopodopsis slabberi	Bury Buoy, Cracknore
Neomysis integer	Totton, Pennington, Gilkicker
Praunus flexuosus	Keyhaven
Schistomysis spiritus	Selsey Bill
Schistomysis kervillei	Selsey Bill
Gastrosaccus spinifer	Selsey Bill, Bourne Gap
Siriella armata	Bourne Gap
Mysidopsis gibbosa	Hamstead ledge, Yarmouth
Mysidopsis angusta	Yarmouth
No species collected (no mysid present)	Milwood, Marchwood, Hamble entrance, Sturbridge shoal, Gosport, East Bramble, Porchester, Hillsea, Hayling Island, Calshot, Beaulieu, Buckler's hard, West Lepe, Hamstead ledge, Canford cliff.

Species located with regularity, and therefore suitable for use in the present study, were *Mesopodopsis slabberi, Neomysis integer* and *Praunus flexuosus.*

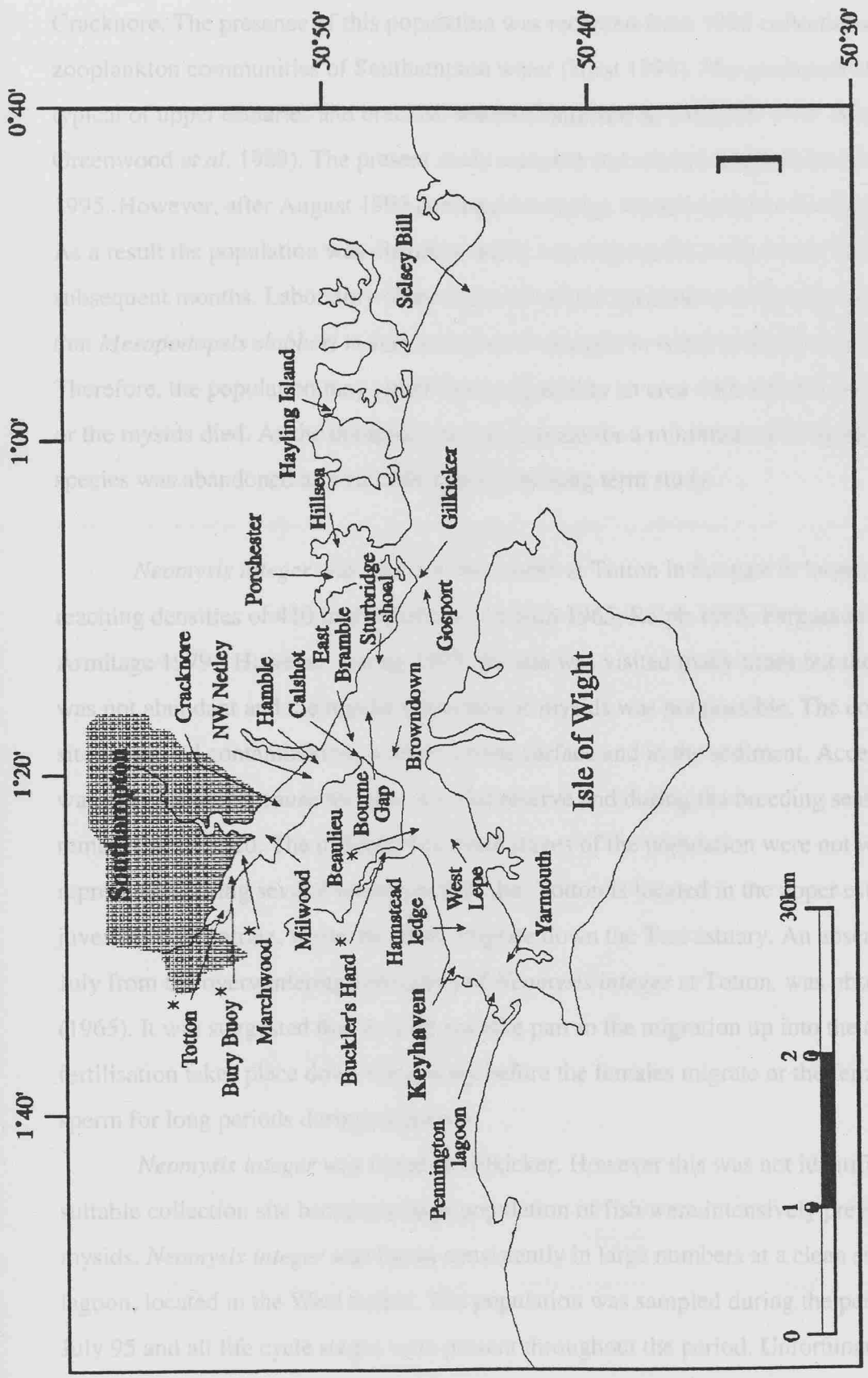


Fig. 3.1 Location of the sampling sites on the study of the distribution of mysids. (✳) reference sites.

Mesopodopsis slabberi was found consistently in Bury Buoy with some individuals at Cracknore. The presence of this population was recorded from 1992 collections as one of the zooplankton communities of Southampton water (Hirst 1996). *Mesopodopsis slabberi* is typical of upper estuaries and brackish waters (Tattersall & Tattersall 1951, Mauchline 1980, Greenwood *et al.* 1989). The present study sampled and studied the population in spring 1995. However, after August 1995 commercial dredge works started in Southampton Water. As a result the population was disturbed and it was impossible to find them during the subsequent months. Laboratory observations from the specimens collected in the field suggest that *Mesopodopsis slabberi* is very sensitive to changes in water conditions (pers.obs.). Therefore, the population may either have migrated to an area with suitable water conditions or the mysids died. As the dredging was to continue for a minimum of 10 months, this species was abandoned as a suitable species for long term study.

Neomysis integer was found in the creeks at Totton in the past in large numbers, reaching densities of 410 individuals m^{-2} (Austin 1965, Ralph 1965, Fergusson 1973, Armitage 1979). However, during 1995, the site was visited many times but the population was not abundant and the regular collection of mysids was not possible. The conditions of the site suggested contamination, with oil on the surface and in the sediment. Access to the site was later denied, because the area is a bird reserve and during the breeding season has to remain undisturbed. The different life cycle stages of the population were not well represented during several months at that site. Totton is located in the upper estuary where juveniles concentrate, while the adults migrate down the Test estuary. An absence of males in July from the overwintering generation of *Neomysis integer* at Totton, was observed by Ralph (1965). It was suggested that they do not take part in the migration up into the creeks and that fertilisation takes place down the estuary before the females migrate or the females retain live sperm for long periods during migration.

Neomysis integer was found at Gilkicker. However this was not identified as a suitable collection site because a large population of fish were intensively preying on the mysids. *Neomysis integer* was found consistently in large numbers at a clean site, Pennington lagoon, located in the West Solent. The population was sampled during the period April - July 95 and all life cycle stages were present throughout the period. Unfortunately, the lagoon received fertilised waters from the farms around the area during August 1995, causing a phytoplankton bloom that liberated toxins, causing the death of all organisms living in the

lagoon. The site was checked 6 months later and although the copepod and the shrimp *Palaemonetes* sp. populations were recovering, mysids were not.

A resident population of *Praunus flexuosus* was found at Keyhaven in 1996, on the West Solent coast (Fig.3.1). The advantage of the marine lagoon at Keyhaven is that the only influx of water to the site comes from the sea with no discharge from farms or rivers. This marine lagoon was found to be diverse, with a biota that consisted of several species of algae (phytoplankton and macroalgae), copepods, amphipods, isopods, crabs (*Carcinus maenas*), shrimps (*Palaemonetes* sp), jelly fish, and several species of fish (gobids, plaice, flounder, bass). The temperature and salinity ranges through the year recorded for Keyhaven lagoon were the following: from 4 to 23.5 °C (Fig. 3.2), and from 28 to 35 p.s.n. (January and August, respectively). Other parameters measured in the seawater presented average levels of dissolved oxygen 7 mg l^{-1}, pH 8, ammonia (0.1 mg l^{-1}), nitrites (<0.05 mg l^{-1}), nitrates (10-15 mg l^{-1}). Surface oil was absent and copper levels in the seawater (see chapter 4) were typical for a non-contaminated coastal water. As a result *P. flexuosus* was selected as the target organism for this study.

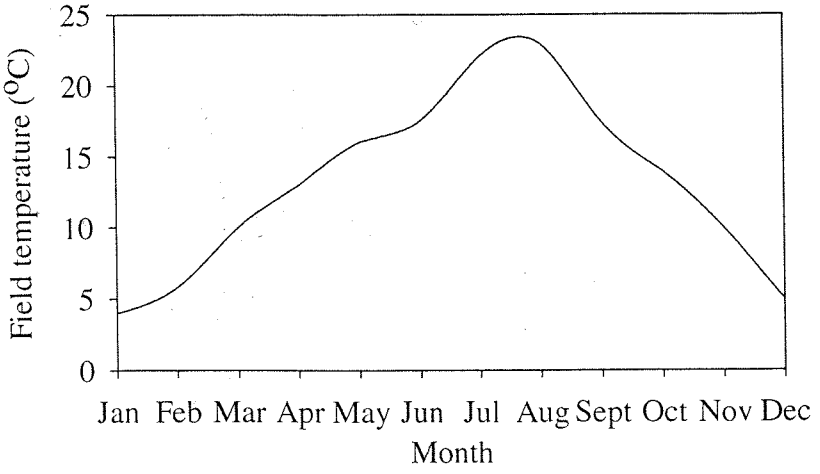


Fig. 3.2. Seasonal variation of temperature (°C) at the field site (Keyhaven)

3.4.2 Life cycle of *Praunus flexuosus*.

Data collected from the analysis of field samples (1996/97) and aquarium surveys allow the identification of the presence of different life cycle stages of brooding females (carrying eggs or developing embryos), non brooding females, males and juveniles throughout the year (Fig. 3.3). From the end of October until the end of March the population overwinters and females do not carry broods. By April the juveniles have matured to adults and all the females found were carrying broods. The population reaches maximum reproductive activity in April. Juveniles born in April/May become adults in only one month, followed by immediate breeding. Juveniles born in July/August will become adults in 2-3 months, by September/October. The females of the spring generation continue breeding from June to September with decreasing intensity. In September females stop breeding and overwinter until the following March/April. Some of the juveniles released in summer breed in September/ October, just prior to entering overwintering condition.

As a result, there are 3 breeding generations in the one year life-cycle of *Praunus flexuosus* at Keyhaven: the overwinter, the spring and the summer generations. The overwinter generation dies after breeding in spring. The spring generation and some individuals of the summer generation breed until autumn. After that, they overwinter, with many of them not reaching sexual maturity until the following spring.

The mean size of individuals remains relatively constant from autumn to spring and increases considerably from March reaching the largest sizes in April (20mm for females, 17mm for males) (Fig. 3.4). The aquarium survey showed a very fast growth of the juveniles released in April. Juveniles reached sexual maturity in May at a size of 11mm (males), and 12mm (females). Reproductive processes are explained in detail in Chapter 8.

3.4 DISCUSSION

Mysid populations were more abundant in Southampton Water and the Beaulieu estuary (West Solent) 20 years ago than at present. Locations where mysid populations were present from 1964 until the late seventies (Austin 1965, Ralph 1965, Fergusson 1973, Armitage 1977) have been visited regularly from 1995 to 1997, without finding any mysids. Mysid populations were found in annual average density of 162 indv.m^{-2} (Fergusson 1973). The populations of mysids have decreased considerably in some areas (Totton and Beaulieu), and in other areas have disappeared completely (Marchwood, Buckler's Hard, Pennington lagoons).

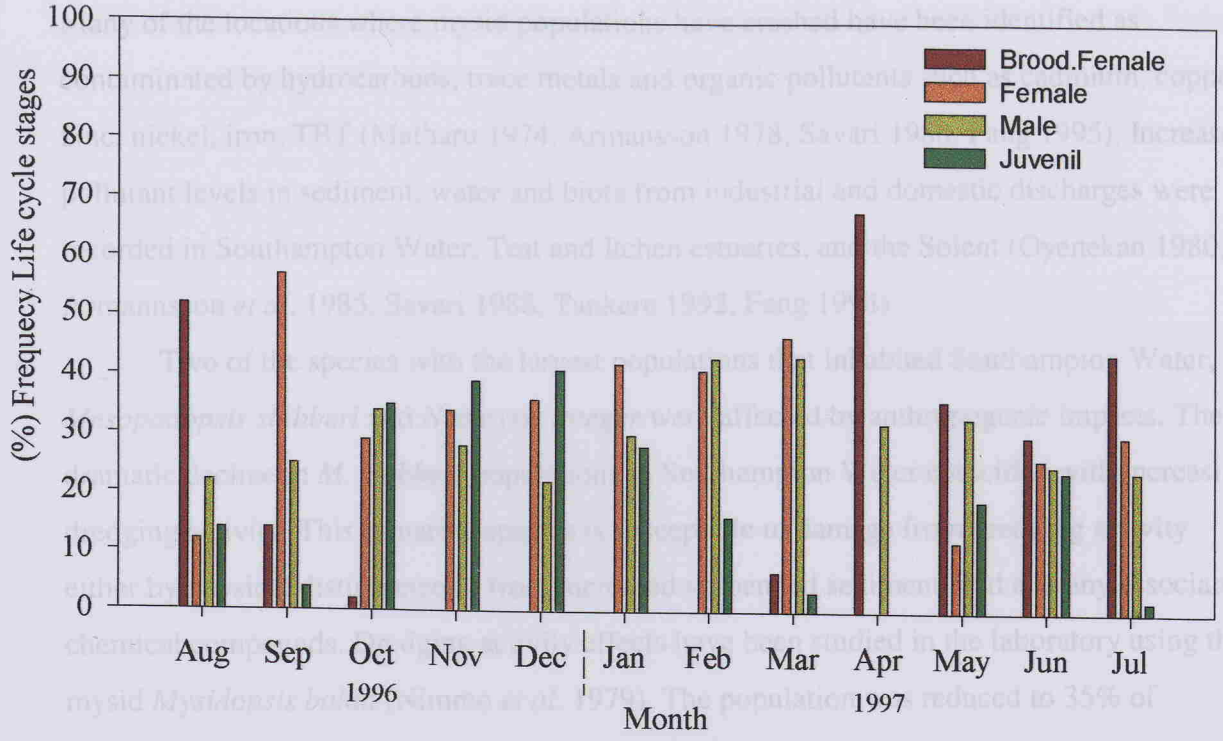


Fig.3.3. Frequency (%) of the life cycle stages of *Praunus flexuosus*: brooding female, female, male and juvenile

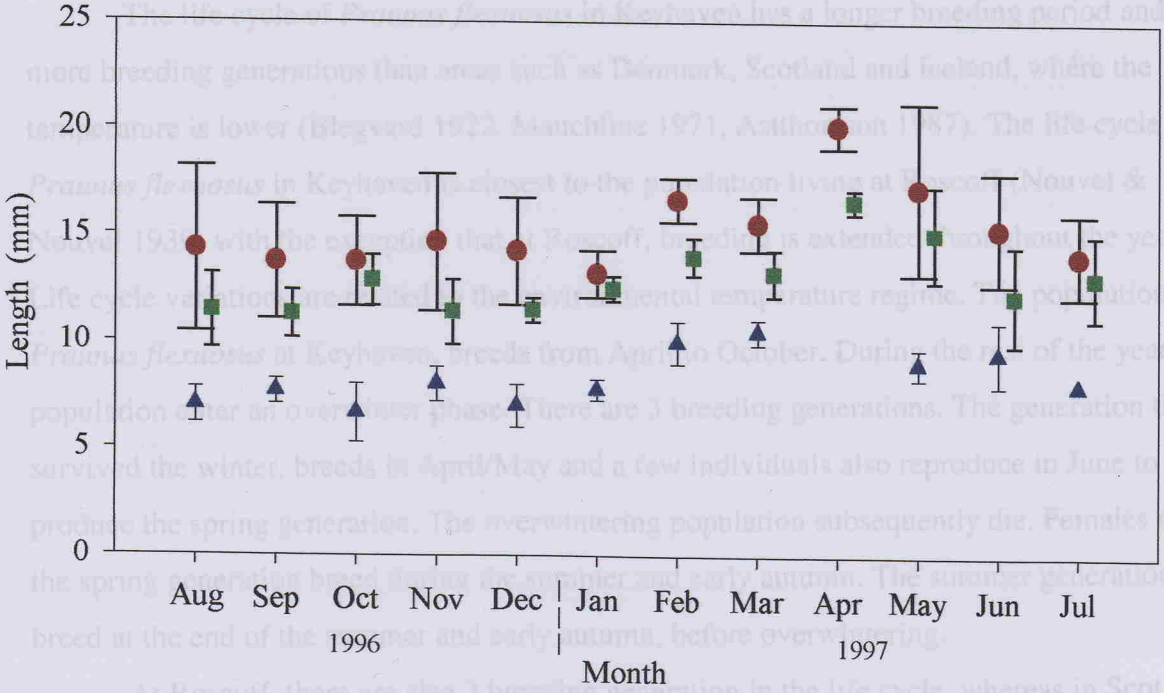


Fig. 3.4. Length (mm) (Mean, SD) of females, males and juveniles of *Praunus flexuosus* population at Keyhaven

Many of the locations where mysid populations have crashed have been identified as contaminated by hydrocarbons, trace metals and organic pollutants such as cadmium, copper, zinc, nickel, iron, TBT (Matharu 1974, Armansson 1978, Savari 1988, Fang 1995). Increased pollutant levels in sediment, water and biota from industrial and domestic discharges were recorded in Southampton Water, Test and Itchen estuaries, and the Solent (Oyenekan 1980, Armannsson *et al.* 1985, Savari 1988, Tankere 1992, Fang 1994).

Two of the species with the largest populations that inhabited Southampton Water, *Mesopodopsis slabberi* and *Neomysis integer* were affected by anthropogenic impacts. The dramatic decline in *M. slabberi* populations in Southampton Water coincided with increasing dredging activity. This estuarine species is susceptible to damage from dredging activity either by physical disturbance or from increased suspended sediment load and any associated chemical compounds. Dredging activity effects have been studied in the laboratory using the mysid *Mysidopsis bahia* (Nimmo *et al.* 1979). The population was reduced to 35% of controls and the production of juveniles decreased to 20% of controls by simulated dredging activity. *Neomysis integer* was also difficult to obtain in areas that are now affected by oil and metal contamination (Totton, Marchwood) or fertilised waters (Pennington lagoons) (pers.obs.).

The life cycle of *Praunus flexuosus* in Keyhaven has a longer breeding period and more breeding generations than areas such as Denmark, Scotland and Iceland, where the temperature is lower (Blegvard 1922, Mauchline 1971, Astthorsson 1987). The life cycle of *Praunus flexuosus* in Keyhaven is closest to the population living at Roscoff (Nouvel & Nouvel 1939) with the exception that at Roscoff, breeding is extended throughout the year. Life cycle variations are related to the environmental temperature regime. The population of *Praunus flexuosus* at Keyhaven, breeds from April to October. During the rest of the year the population enter an overwinter phase. There are 3 breeding generations. The generation that survived the winter, breeds in April/May and a few individuals also reproduce in June to produce the spring generation. The overwintering population subsequently die. Females of the spring generation breed during the summer and early autumn. The summer generation breed at the end of the summer and early autumn, before overwintering.

At Roscoff there are also 3 breeding generation in the life cycle, whereas in Scotland, Port Erin and Denmark there are 2 breeding generations and in Iceland only one (Table 3.2). The over-wintering population dies off at Port Erin, Millport, Loch Etive and in Denmark in July and August when some members of the new generation are starting to breed. However,

at Roscoff the overwintering population dies in May and at Keyhaven in May/June. In Iceland the overwinter population is the only breeding generation, breeding in summer and dying in September. The new spring generation start breeding in June at Keyhaven, July at Roscoff and Millport, August in Loch Etive, September in Denmark and Port Erin, and in the following spring in Iceland. Thus the iniation of breeding is later with increasing latitudes.

Locations at similar latitude such as Denmark and Millport or Loch Etive show differences on the life cycle of *P. flexuosus*, because the intensity or length of season are different. In Denmark the seawater temperature is higher after August than in W. Scotland.

The reproductive characters are tied to intensity of season. In the shortest summer there is one generation per year. As summer length increases 2, and eventually 3 generations per year are possible. This could be related either to physical environmental limitation e.g. temperature, photoperiod, or resource availability.

Table: 3.2. Comparison of the timing of the breeding generations on *Praunus flexuosus* at different locations

Location	**Overwinter generation breeding**	**Overwinter generation die**	**Spring generation breeding**	**Summer generation breeding**
Roscoff	April/May	May	July to April	After overwinter
Keyhaven (Southampton)	April/May	June/July	June/Aug. and overwinter	Sept./Oct. and overwinter
Port Erin (Isle of Man)	Spring/Summer	July/August	Autumn	After overwinter
Millport (Clyde Sea) **Loch Etive (W. Scotland)**	May May	July/August July/August	July to October July to October	After Overwinter After overwinter
Denmark	May	August	Autumn	After overwinter
Iceland	June/August	September	After overwinter	After overwinter

3.5 SUMMARY

The populations of mysids (*Neomysis integer, Mesopodopsis slabberi, Praunus flexuosus*) in Southampton Water and the Solent have decreased considerably or have disappeared completely in some areas, according to existing records. Most of the locations where mysids have declined or disappeared, were defined as contaminated by hydrocarbons, trace metals and TBT. In this study evidence was found of the severe effect on populations of two different species by anthropogenic effects in different locations.

The life cycle of *Praunus flexuosus* in Keyhaven (Solent) has been characterised and compared with the life cycle in different geographical locations. The population of *Praunus flexuosus* at Keyhaven, breeds from April to October. During the rest of the year the population enter an overwinter phase. There are 3 breeding generations. The life cycle of *Praunus flexuosus* in Keyhaven has a longer breeding period and more breeding generations than areas such as Denmark, Scotland and Iceland, where the temperature is lower. The life cycle of *Praunus flexuosus* in Keyhaven is closest to the population living at Roscoff, with the exception that at Roscoff breeding is extended throughout the year.

The variations in the life cycle for this species correlate with the effects of the environmental temperature regime. It is suggested that intensity of the season plays an important role, as controlled by environmental limitation e.g. temperature, photoperiod, or resource availability.

CHAPTER 4

COPPER SPECIATION AND BIOAVAILABILITY STUDIES

CHAPTER 4: COPPER SPECIATION AND BIOAVAILABILITY STUDIES

4.1 INTRODUCTION

Trace metals dissolved in seawater can exist in different chemical forms (species), including free ions, inorganic complexes, and in association with organic material. The speciation of dissolved trace metals in natural seawater is controlled by the interaction of the metals with a complex and varying mixture of inorganic anions, organic ligands, reducible or oxidizable dissolved chemical species, surfaces and organisms. Filterable concentrations of metals may include fine colloidal particles as well as organic and inorganic metal complexes. Both metal speciation and biological availability or toxicity have been postulated as functions of the tendency of the metal to react, as quantified by the free metal ion activity, under pseudoequilibrium conditions (Sunda & Guillard 1976). For metals occurring as organic complexes, pseudoequilibrium conditions among dissolved species may be maintained only if the rates of metal complexation reactions are fast compared with rates of metal uptake. If, however, complex dissociation and ligand-exchange rates are slow compared to biological uptake, the rate of metal incorporation into the biota will be limited by abiotic chemical kinetics (Anderson *et al.* 1982).

The uptake of metals by organisms in contaminated environments, has been recorded in many studies and toxic effects are evident in a large variety of organisms (Phillips 1980a, Rainbow 1997). At present, which form of the metal can be bioavailable has not been fully defined. Although there are studies that correlated the toxicity of metals to the free ion form in phytoplankton, the bioavailability of different chemical species may vary significantly depending on the type of organism. Labile complexes may be bioavailable. In the case of essential metals such as copper, there is a high chemical reactivity with proteins and binding to metalloenzymes. Copper is taken up by the organism both passively and actively, and transported internally to specific organs (Rainbow 1997). Toxicity of a dissolved metal species in organisms is related to the ability of the metal species to react with a biological system.

Advances in analytical chemistry and sample handling have been very important in the last decade in providing accurate measurements for metals in seawater. As regards the understanding of the chemistry of trace elements in seawater, Burton (1979) pointed out the

implications of the physico-chemical limitations in experimental studies on toxicity; when ranges of concentrations, activity and speciation of the metal were not considered.

A metal in natural seawater is distributed between a variety of physico-chemical forms, both dissolved and particulate. The distribution is determined by the properties of the ion or molecule in question and by a number of major variables, including ionic strength, the nature and concentrations of major dissolved elements, particulate matter and organic complexing material, pH and the electron activity (Burton 1979).

A metal such as copper in river water is almost entirely complexed with humic material but these organic complexes become less important as the salinity increases, because of the displacement of copper by calcium and magnesium ions (Mantoura *et al.* 1978). The proportion of cupric ion unassociated is very low in seawater of salinity superior to 10.

Thermodynamic data indicate that temperature and pH strongly influence the chemistry of strongly hydrolysed metals, and metals whose speciation schemes are dominated by carbonate complexation (Turner *et al.* 1981). Copper is dominated by chloride complexation, and it is influenced by pH and temperature only at a small degree (Byrne *et al.* 1988).

It is well known that organic complexation dominates the chemical speciation of many dissolved metals such as copper in seawater (Buffle 1988, Bruland *et al.* 1991, Donat & Bruland 1995, Wells *et al.* 1998)

The organic compounds form complexes with 94-98% of dissolved copper, and therefore constitute the major form of copper in surface water of the Irish Sea (Van der Berg 1984). Calculation of speciation of the inorganic fraction of copper in seawater (at pH 8, 25° C and 33 salinity) reveals that the major complexation is that of $CuCO_3$ (60%), $CuOH^+$ (16%), $Cu(OH)^2$ (16%) and $CuCl^+$ (>1%). The free ion form contributes some 4% to the inorganic copper.

The changes in the form of dissolved copper during a mysids toxicity test were the immediate interest in this study.

There is a wide range of techniques (electrochemical or non electrochemical) available to study the labile chemical species, a fraction of which are considered to be the available species for organisms. The electrochemical approaches are based on voltammetric methods (van den Berg 1988) or ion selective electrodes. A range of non-electrochemical approaches also exist. The main electrochemical approaches are based on complexing capacity titration such as anodic stripping voltammetry (ASV) and on competitive ligand

methods such as cathodic stripping voltammetry (CSV). The advantages of these methods are their sensitivity, small sample volume requirement and speciation determination capability. CSV preconcentrates the trace metal of interest by adsorption of surface-active complexes onto a hanging mercury drop electrode followed by a reductive stripping step producing a cathodic current. The limitation of the voltammetric techniques is the intractability of calibration in complex media containing metal ions and organic ligands.

The non-electrochemical approaches include ion-exchange and chelating resin methods, ligand competition methods, size-based separation techniques (dialysis and ultrafiltration), direct spectroscopic measurements and bioassay procedures. These methods do not measure directly the free metal ion concentrations, but may provide data that enable their calculation. Their ability to measure various forms of metals enable the study of the link between the metal speciation and bioavailability (Apte & Batley 1995). A further approach to speciation is the use of solid phase extraction of naturally occurring metal complexes (Mills & Quinn 1981, Donat *et al.*1986), based on adsorption of metal complexes on a chelating polymer with C18 groups, or XAD (Sugimura & Suzuli 1988). These methods rely on the hydrophobic nature of natural metals complexes, but there will also be polar metal complexes which will escape this separation technique.

Of the non-electrochemical methods used in metal speciation studies, the Chelex-100 chelating ion-exchange technique has most frequently been used (Riley & Taylor 1968, Muller & Kester 1990, Apte & Batley 1995). Chelex-100 has imino-diacetic acid groups bonded to a divinyl-benzene copolymer backbone. The polymer comes in different bead sizes with a pore diameter of about 1.5nm. This pore diameter restricts the size of organic complexes, which can reach the bulk of chelating sites, in the interior of the bead. Chelex resin is efficiently regenerated in dilute acid and operates in basic, neutral and weakly acidic solutions of pH 4 or higher. Metals can be removed from the Chelex resin with acids. The Chelex resin has a high specificity for Cu and a range of other transition metals, over alkali and alkali-earth cations, and has thus found many applications in marine systems. For natural seawater the resin will be expected to collect simple ionic forms and complexes, and labile (or reactive) organic forms that reach the complexing sites of the Chelex-100. There is also a kinetic effect, in that metal complexes which dissociate more slowly than the contact time of the seawater sample with the resin, will not be measured (Muller & Kester 1990).

The Chelex chelating resin method has been chosen here to measure the copper in the toxicity study because of the relative ease of use, which makes it possible to combine it with

the measurement of other parameters needed in a toxicity test, and especially in the case of sublethal effect studies. Chemical speciation can represent a very large study in itself. However, Chelex-available measurements provide an estimate of "labile"(but not necessarily bioavailable) metal concentrations (Apte & Batley 1995). The Chelex measurement is likely to be representative of the availability of the metal to a large range of organisms (and not only to phytoplankton). Mysids (zooplankton - hyperbenthos) were chosen as the experimental animals for the toxicity test of this study, because they are common in coastal and estuarine areas, important food chain components and an excellent experimental organism for toxicology. They are also the organisms used in the sublethal toxicity tests in this thesis.

In this study total dissolved copper and Chelex-available copper have been measured during a toxicity test performed on mysids over a 48h period. The questions addressed in this study were 1) what are the background concentrations of copper initially present in the experimental system, 2) what changes in the form of dissolved copper occur during toxicity testing.

4.2 MATERIALS AND METHODS

The first sections describe the optimisation and testing of the Chelex method, and the last section (4.2.5) describes the measurements of copper in a toxicity test by the developed Chelex method.

4.2.1 Collection of water samples

Seawater samples (1 litre) were collected in polyethylene bottles, which had been soaked in 50% nitric acid for a week, then rinsed with Milli Q. water. Samples collected in the field (Keyhaven lagoon) were taken manually, immersing the sampling bottle below the water surface to a depth of 30 cm approximately and then filled with water. Each sample was rinsed two times before the final fill. The bottle was sealed in a double plastic bag, transported in a cool box and put into a fridge at 5°C and held dark conditions in the laboratory until analysis.

Samples during the toxicity test were collected following the same procedure as mentioned above, except for the immersion of the bottle in the tank or experimental chamber. Samples were poured from the experimental chambers or tanks into the bottles. Exposure of

the bottle and sample to the atmosphere and potential contamination was minimised in all cases.

4.2.2 Sample treatment and Chelex extraction of dissolved copper

Samples taken for the analysis of dissolved copper were filtered through 47mm diameter acid washed 0.4μm Nuclepore membranes, under vacuum (Nalgene filter unit made of polysulfone). Nuclepore membranes were cleaned by soaking for one week in 10% sub-boiled-distilled (SBD) HNO_3, and then rinsed several times with SBD water. Membranes were stored in a plastic Petri dish in Milli-Q water until use.

The Chelex column extraction was performed in a laminar flow cabinet. The Chelex-100 used (Biorad 200 mesh) was held in a plastic Biorad column of 10ml total capacity. Circa 200mg of the sodium form of the resin was loaded into the column, and was supported on the sintered polyethylene fit. For the determination of the Chelex-available dissolved copper, sample solutions were passed through the Chelex column, which had been cleaned and left in the ammonium form, at pH 5.5 or at natural pH~8 and flow rate of 2.5-3 ml min^{-1}. Sample solutions were at pH 5.5 as recommended by Kingston *et al.* (1978), but pH 8 was preferred as less likely to alter the natural speciation. The volumes of samples were determined gravimetrically. Buffered samples (using ammonium acetate buffer) were passed through Chelex for the separation and preconcentration of copper from seawater at a pH of 5.5 . The Chelex resin in the column was cleaned prior to use with 2M nitric acid to remove the metal ions, and Milli Q water to remove any excess acid. To convert the resin into the ammonium ion form 2M ammomiun hydroxide was added. Finally to remove the excess of ammonia, the column was rinsed with Milli Q water. Copper was eluted from the column in 2 x 1 ml aliquot of $2HNO_3$ and the volume of eluate was determined gravimetrically (full details of the procedure are given in Appendix I)

For the determination of the total dissolved copper in seawater, the filtered samples were acidified (1μl of SBD HNO_3 per ml of sample). After a minimum of 48h the sample was transferred into an acid-washed (50% HNO_3) silica ultraviolet (UV) irradiation tube to be UV irradiated for 4 hours. The UV lamp (1kw mercury arc lamp with a silica sheath) was mounted in the centre of an aluminium stand with a cooling fan. When irradiated, samples were cooled to room temperature, and copper extracted as above.

The copper concentration in the dilute nitric acid was determined by graphite furnace AAS. Copper concentration of the samples were calculated from the measurements of the concentrates.

4.2.3 Assessment of accuracy, precision and detection limits of the Chelex technique

The method performance (recovery, detection limit and precision) of the Chelex technique was studied under different conditions. Seawater samples were copper spiked at different concentrations (0, 2, 5, 10, 20, 30, 50 and 70 $\mu g\ l^{-1}$) and (0, 5 and 25 $\mu g\ l^{-1}$) with a working standard and passed through the Chelex column at different pH and treatment of the sample. Samples were analysed at pH 5.5 and 8. The pH was adjusted to the desired value with 2M ammonium acetate buffer. The samples were with no treatment applied, UV irradiation treatment, and acidification plus UV irradiation treatment.

Measurements of samples with different treatments were compared to examine if there were differences in the copper concentration. Acidified and UV irradiated sample concentration is considered as the total dissolved copper present in the sample. When the sample has not received these treatments, copper concentrations measured may not include the part of the metal associated with ligands or organically complexed. The differences between the copper concentrations of the same sample with different treatments are used to estimate the percentage of metal that is associated with ligands.

4.2.4 Storage effects of the sample on the measurement of dissolved copper

The effects of storage of the sample (0.4 µm filtered and stored at 5°C and dark conditions) on the copper concentration measurement were studied by analysing the following subsamples

- Subsample a, did not receive acidification and UV irradiation treatment.
- Subsample b, was acidified in the storage bottle and subsequently UV irradiated.
- Subsample c, was acidified outside the storage bottle and subsequently UV irradiated.

The difference between c and b should indicate if adsorption to bottle was a problem.

4.2.5 Measurement of Chelex-available and total copper during toxicity testing

Toxicity tests with mysids (*Praunus flexuosus*) were performed in a constant temperature laboratory at 10 light:14 dark photoperiod and 5 °C conditions. Acid washed

(50% nitric acid and Milli Q rinsed) glass beakers were filled with 1l of filtered seawater (1μm filtered). Seawater was oxygenated by an airline with an airfilter. A mysid carefully rinsed with Milli Q water, was added to each beaker using a small acid washed polyethylene net. Each beaker was covered with cling film to avoid any contamination. The seawater had a salinity of 32. Test series had duplicates for each condition. Two different types of controls were run in parallel: control (no mysid), which was the system control without mysid, and control, which was the toxicity test control with mysid and no copper additions. Copper additions (5 and 25 $\mu g\ l^{-1}$) were made to beakers containing an experimental animal.

Toxicity tests were run for 48 hours. "Time 0" was defined as the starting time of the experiment when mysids in the chambers were first exposed to the copper additions. "Time 24h" defined as the 24 hours after the initiation of the toxicity test. After 24 hours the animals were fed by adding 20 *Artemia* nauplii (rinsed in Milli Q water) into every chamber containing a mysid. "Time 48h" was the end of the experiment, after the toxicity experiment had run for 48 hours. Samples (25ml) were collected from the chambers at time 0, 24 and 48h. Replicate seawater samples were collected from every chamber at each time and filtered (0.4μm). One of the two samples was directly analysed to measure the Chelex available dissolved copper at time 0, 24h and 48h of the toxicity test. The other sample was kept at 5 °C and under dark conditions for later measurement of the total dissolved copper.

4.3 RESULTS

4.3.1 Method performance

The study of the method performance of the Chelex column extraction used in this study shows that the method was acceptable. The detection limits and recovery of copper (Table 4.1) were calculated from the results of copper spike experiments (Appendix I). It shows that the copper added to the sample is almost fully recovered, in the extraction from the resin under the different conditions of sample treatment. The blank and detection limits are good. The precision of the Chelex method using different sample treatments (Table 4.2) is also acceptable.

Table 4.1. Detection limits and Recovery (%) of copper using the Chelex method, for samples with different treatments.

Blank ($\mu g\ l^{-1}$) Cu	Precision Blank (1σ)	Detection Limit (2σ)	Recovery (%)	Treatment applied to sample	pH of solution
0.096	0.04	0.08	98.9	SW Ac.UV.	5.5
0.07	0.05	0.09	101.2	SW no treatment	5.5
0.056	0.05	0.09	101.7	SW no treatment	8
0.06	0.06	0.09	107.3	SW UV. only	8

Data are calculated from results of copper spike experiments. The treatments applied to samples were acidification (Ac.), photoxidation (UV).

Table 4.2. Precision of the Chelex method using different sample treatments.

Sample copper Concentration ($\mu g\ l^{-1}$)	n	Precision (1σ)	RSD (%)	Treatment applied to sample	pH of solution
6.78	4	0.4	6.1	SW Ac.UV	5.5
10.6	3	0.11	1.0	SW no treatment	5.5
7.9	4	0.37	4.7	SW no treatment	8
10.9	4	1.37	12.8	SW UV only	8

Different seawaters were used for each set of experiments. The treatments applied to samples were acidification (Ac.), photooxidation (UV).
n = number of replicates of samples, RSD = relative standard deviation

Copper concentrations measured in seawater samples vary with the treatment of the sample. The copper concentration of samples not acidified and UV irradiated (2.23 - 2.84 $\mu g\ l^{-1}$) are lower than in those with acidification and UV irradiation treatment (5.7 - 6.78 $\mu g\ l^{-1}$), (see Appendix I). These data suggest that significant complexation processes of copper occur and/or colloidal forms of copper are present in the experimental seawater.

4.3.2 Background concentrations of total dissolved copper in the field and in the toxicity test

There are differences in the total copper concentrations measured in the seawater from the field and from the toxicity test (Table 4.3). Seawater samples collected from the field (Keyhaven) show low copper concentrations (0.6-1.5 $\mu g\ l^{-1}$) whereas the seawater of the toxicity tests show concentrations of between 2 and 8.5 $\mu g\ l^{-1}$. Toxicity test seawater came from the BAS aquarium system except in December 1996 when the water came from Keyhaven. The copper concentration of the BAS seawater (BAS SW) indicates some low

level copper contamination. Seawater was supplied by the MAFF Lowestoff laboratory to BAS from the North Sea. Copper concentrations in that area are quoted as 0.4-0.7 $\mu g\ l^{-1}$ (Burton *et al.* 1994), suggesting that copper contamination occurred at some point during collection from North Sea, transport and use in the recirculating system of the aquarium facilities at BAS. The "contamination" level is generally below any current environmental quality standards.

Table 4.3. Total copper concentration (Mean, [SD]) of samples (acidified and UV radiated) from the field (Keyhaven) and from BAS, as used in the toxicity tests.

Total Copper ($\mu g\ l^{-1}$) dissolved in Sea Water	Field Sea Water (Keyhaven)	Toxicity Test Sea Water (Keyhaven* and BAS SW^)
December 1996	1.5 [0.59]	*1.99 [0.09]
January 1997	0.6 [0.13]	^3.6 (+)
February 1997		^5.9 [0.49]
June - August 1997		^8.5 [0.25]
June - August 1998		^5.8 [0.43]

(+) Data were provided by Dr. L.M. Nair using the Chelex method,
* Data from the field Keyhaven, ^ Data from aquarium system at BAS

In December 1998 the BAS aquarium seawater had the highest concentration of total dissolved copper of 14.2 $\mu g\ l^{-1}$ showing that copper contamination may reach a significant level in a recirculating system.

4.3.3 Storage effects of the sample on the measurements of total dissolved copper

Analysis of the seawater samples stored under dark conditions and at 5 °C temperature showed that a significant effect on copper speciation (i.e. Chelex-available) occurred during storage of the sample (Fig. 4.1.). There were two processes occurring during storage: adsorption of copper onto the walls of the bottle and changes in organic and inorganic complexation. The samples were taken during a toxicity test, where mysids and *Artemia* were present in solution.

The analysis of the subsamples (a - no treatment; b - acidified in the storage bottle and UV irradiated; c - acidified outside the storage bottle and UV irradiated) provided the data (Fig.4.1) to estimate the total copper, the copper adsorbed (b-c) and the copper adsorbed and complexed (b-a) in the sample. Results show that there is a significant fraction adsorbed during storage.

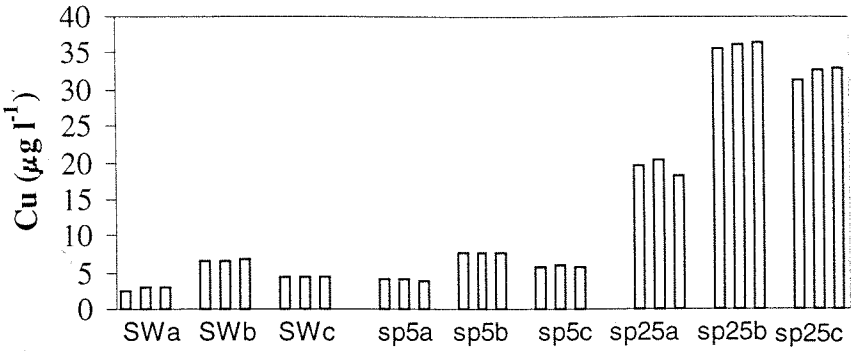


Fig. 4.1. Effects of storage on copper concentration measurements on the samples: control seawater (SW), copper spike of 5 µg l^{-1} (sp5) and 25 µg l^{-1} (sp25). subsample a - no treatment; subsample b - acidified in the storage bottle; and subsample c- acidified outside the storage bottle. Bars represents the replicates of every sample.

The fraction of total copper existing in the samples (SW, sp5, sp25) that was adsorbed onto the bottle during storage ranged from 32.67 % (±0.15) in controls (SW) to 22.30% (±0.18) and 10.53% (±0.6) in copper spike samples (sp5 and sp25).

The fraction of total copper existing in the samples that was organically complexed and/or adsorbed was 56.6 % (±0.35) in controls (SW), 48.02% (±0.20) in sp5 and 46.44% (±1.35) in sp25.

There was a loss of copper available to Chelex during storage because it was adsorbed onto the walls of the storage bottle. Therefore, these factors should be taken into account in the measurement of the copper concentration of a stored sample, in order to avoid an under-estimation of the copper.

From the measurement of the total copper concentration in the stored sample, the overall percentage of the copper that was in an available form to Chelex, and possibly biologically available, is unknown. Immediate measurements of the Chelex-available dissolved copper from the toxicity tests will provide the concentration of copper that is in labile form.

4.3.4 Chelex available and total dissolved copper in the toxicity test.

Available copper was measured in samples immediately after collection without any treatment, and total copper was analysed on samples that had been acidified and UV irradiated. The analysis of the samples without any treatment showed that the Chelex available copper is significantly lower (T=194, p=0.012) than the total dissolved copper concentration (Table 4.4). The recovery efficiency of added copper by the Chelex method was found to be similar for samples with the different treatments, therefore the difference found in copper concentrations could be related to the copper complexation or presence of colloids in the experimental seawater used.

The Chelex-available copper measured in the control (no mysid), increased by 1 $\mu g\ l^{-1}$ from the initial concentration of copper at the experimental seawater (SW) before use in the toxicity test (Table 4.4).

Chelex-available and total copper concentrations were not different between control (with mysid) and control (no mysid), suggesting that the presence of the mysid itself does not increase the dissolved copper concentration.

The fraction of total copper that was in a labile form (chelex-available) in all samples measured at the beginning of the toxicity test was 56.38% (±6.12).

Table 4.4. Total and Chelex-available copper in the toxicity test (5°C, salinity of 32)

Sample	Av.Cu Time 0	Total Cu Time 0	Av.Cu Time 24h	Av.Cu Time 48h	Total Cu Time 48h
SW	6.46 [4]				
Control no mysid	7.97 [6]	14.6 [5]	7.68 [4]	8.86 [5]	15.1 [5]
Control	7.81 [6]	15.7 [2]	6.96 [15]	7.77 [7]	14.0 [8]
5 added Cu	10.9 [4]	19.3 [13]	10.7 [11]	10.3 [36]	15.9 [4]
25 added Cu	20.9 [5]	32.4 [1]	19.1 [1]	18.7 [2]	25.8 [2]

Mean copper concentrations ($\mu g\ l^{-1}$) [RSD (%)] for samples directly analysed (Chelex-available copper: Av.Cu) and acidified and UV irradiated samples (Total Cu). Measurements correspond to different times during the toxicity test. Time 0: initial time (<1h), Time 24: 24h after start of the test, and Time 48: end of test at 48h.

Chelex-available copper concentrations did not differ significantly (H=1.06, p=0.58) during the 48h of the experiment (Fig. 4.2). On the other hand, the total copper measured in the same samples decreased with time significantly at 5 $\mu g\ l^{-1}$ (F=14.2, p=0.0016) and at 25$\mu g\ l^{-1}$ (F=1286, p<0.0001) (Fig.4.3).

Total copper concentration differed significantly from the Chelex-available copper at 0h and 48h of the toxicity test (T=106, p=0.012 and T=110, p=0.023) (Fig. 4.4 and 4.5).

Although the organisms were exposed to a similar fraction of Chelex-available copper over the whole period, the total copper concentrations decreased with time (Fig. 4.6). Comparison of the total copper measured and the total copper concentration expected shows that there is a significant decrease in total dissolved copper (F=14.2, p=0.0016) at the 5 $\mu g\ l^{-1}$ Cu addition at time 48h and at the 25$\mu g\ l^{-1}$ Cu addition at time 0h and 48h (F=1286, p<0.0001), (Fig. 4.4, 4.5). At 48h the total copper concentration became significantly lower for both copper additions (5 and 25 $\mu g\ l^{-1}$) (Fig.4.5).

The background copper concentrations are not low (Table 4.3). This is believed to reflect the background contamination of the seawater in the BAS aquarium recirculating system, which is expected to be a very common feature in recirculating aquarium systems and should be monitored in toxicity studies. Toxic effects were not observed in controls in the toxicity test, but were evident in the organisms exposed to seawater with copper additions, especially at 25$\mu g\ l^{-1}$.

The decrease of total copper recorded over the 48h experiment appeared to indicate that the non-Chelex-available metal was lost to the experimental chamber of the toxicity tests and/or the organism. After 24h of the toxicity test, food was provided to the mysid. The influence of the presence of *Artemia* on the decrease of total copper was not significant in the control where the mysids do not show toxic effects. In the cases where a decrease in total copper was evident the animals were observed (behavioural parameters disrupted) to be under stress owing to toxic effects. In addition, physiological responses at 25$\mu g\ l^{-1}$ copper treatment (Chapters 5, 7, 8) always showed stress and toxic effects on the organisms.

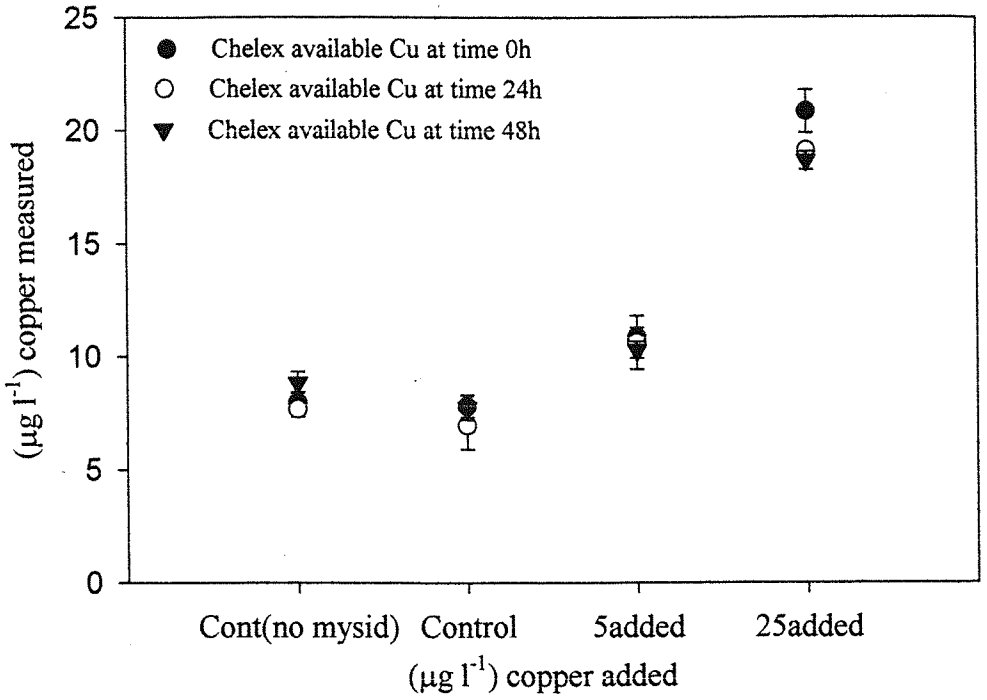


Fig. 4.2. Chelex-available copper (mean, SD) measured in toxicity test at 0h, 24h, 48h.

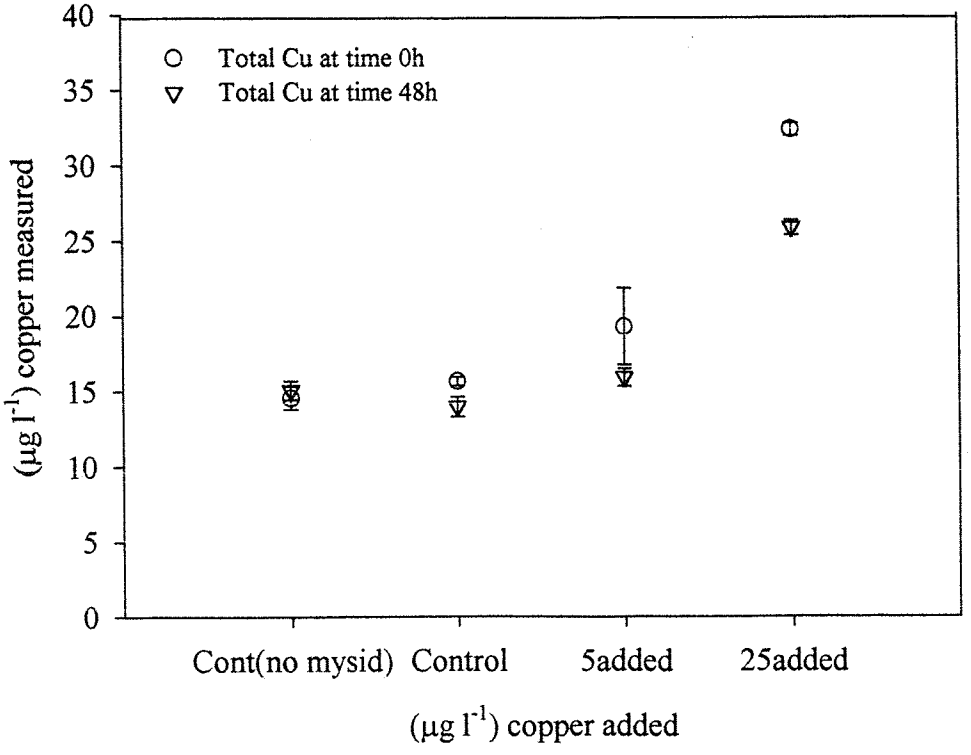


Fig. 4.3. Total copper (mean,SD) measured in the toxicity test at time 0h and 48h

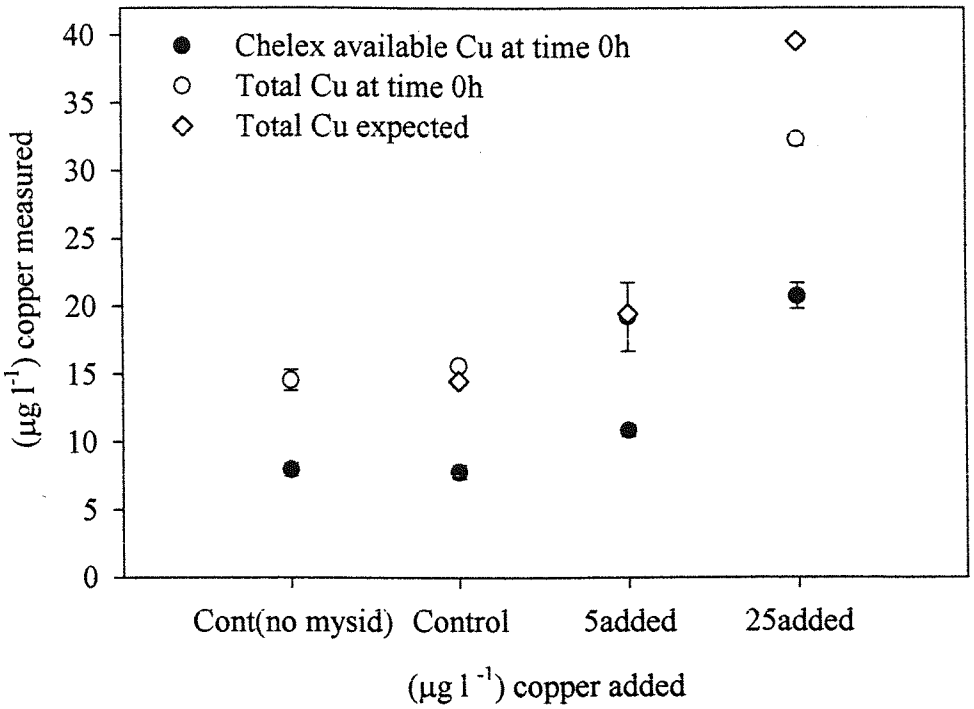


Fig. 4.4. Total and Chelex-available (mean,SD) copper measured at time 0h in the toxicity test, and expected total copper

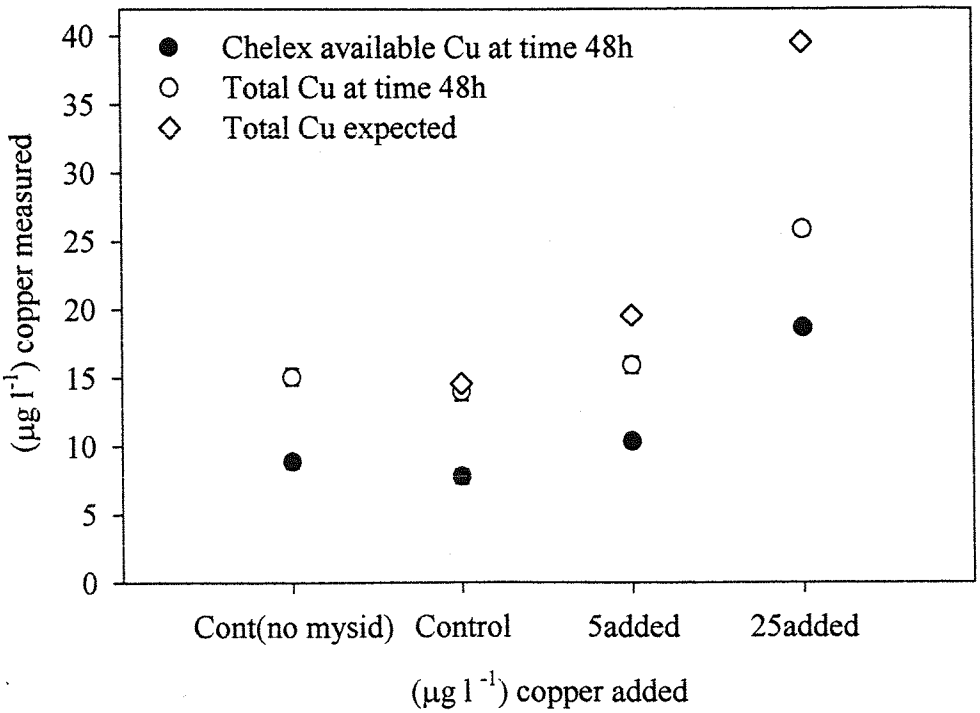


Fig. 4.5. Total and Chelex available copper (mean,SD) measured at time 48h on the toxicity test, and expected total copper.

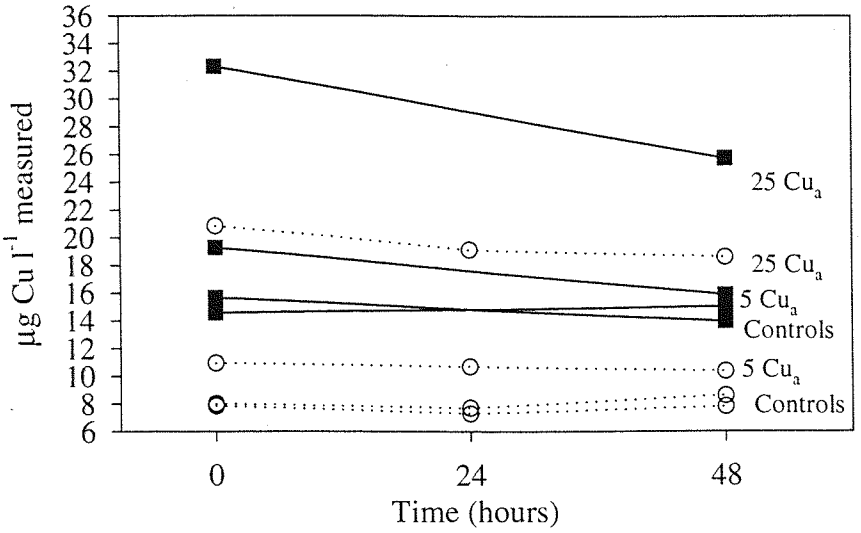


Fig. 4.6. Total and Chelex-available dissolved copper measured during the 48h of toxicity test, at the different treatments (controls, 5 Cu_a, 25 Cu_a)

···⊙··· Chelex-available dissolved copper

—■— Total dissolved copper

Estimation of the fraction of copper that may be taken up by the organism from the decrease in total copper concentration in the toxicity test shows that the decrease in total copper can not be explained solely by the uptake of the mysid. Considering the decrease of 7 µg Cu l^{-1} mysid $^{-1}$ (5mg dry wt) and assuming that bioaccumulation was linear with the time of exposure, the accumulation rate should be 70 µg g^{-1} day^{-1}. However, the accumulation rate calculated from the analysis of the copper concentration in the body of the organism (Chapter 6) revealed that accumulation rates ranged from only 1 to 7.9 µg g^{-1} day^{-1}.

4.4 DISCUSSION

Copper in seawater can be distributed between a wide range of forms and species: free ion, small inorganic and organic complexes, complexes with humic subtances, colloidal and particulate. Detailed metal speciation studies are a major project in their own right, and beyond the scope of this study. The labile dissolved copper, which may be available to the organism in a toxicity test was the target of this study. Certainly the free ion form is not the only bioavailable form of dissolved copper. Dissolved metal complexes may be taken up by the organism through the epithelial barriers. Non-ionic, inorganic species and organic derivates may diffuse into organisms owing to their high lipid solubility (Simkiss & Taylor 1989). The affinity of the metal, such as copper, for organic ligands facilitates a route for uptake by binding with transport ligands in the membrane (Rainbow 1997). The processes of trace metal uptake are affected by the biology of the organism. Active uptake of the metal may occur especially at the respiratory surfaces where absorption and relative permeability of the cells/tissues and metal-binding sites are present (Langston & Spence 1995). Radiotracers studies allowed the comparison of the bioavailability between the free-ionic forms and the complexed forms on several metals, including copper. It showed that complexed forms were available to the shrimp *Peaneus azteum* (Carvalho *et al.*1999).

The Chelex resin method measures weak metal complexes, kinetically available and smaller species, which can penetrate Chelex beads. Some authors have suggested a good correlation with bio-available metal (Buckle *et al.* 1985) and others suggest it may overestimate (Turner 1984). There is a need to establish clear links between speciation and biological responses.

Figura & McDuffie (1979), showed the labile fractions determined by the Chelex method are generally larger than the fractions obtained by differential pulse anodic stripping voltammetry (DPASV), which may be explained by the different time scales of measurement of the two techniques. The Chelex column technique obtains a fraction of moderately labile trace metal species, which can not be detected as labile by the DPASV. Florence (1982) studied trace metal speciation by Chelex resin and anodic stripping voltammetry to determine labile metal. As a result, recommendations were made on the reaction time of the solution with the resin, UV irradiation treatment of the samples and pH of the solution. Apte & Batley (1995) pointed out that the resin conditioning and preparation is critical. Resin pore size varies with solution pH, so metal uptake by the resin is dependent on the pH. The longer the

contact time, the greater the fraction of trace metal measured by Chelex. Therefore, column flow rate has a strong influence on the metal complexation capacity of the samples. Such suggestions concerning the conditioning and preparation of the resin and flow rate were carefully considered in this study.

Given that no single speciation technique has been demonstrated to be an excellent estimation of bioavailable metals, the Chelex technique is an acceptable approach and a more useful measure than the total metal, being closer to the truly biologically available fraction. Thus the application of Chelex -100 separations provides a simple method of estimating labile metal concentrations in water samples. The method performance of the Chelex in this study was acceptable.

The results showed that the total dissolved copper concentration and the Chelex-available dissolved copper concentration were significantly different, with the Chelex available fraction being lower. The added concentration of copper in this type of toxicity experiment can vary substantially with the time of exposure. Total copper concentrations decreased significantly at 48 hours, where the organism was under stress or toxic effects, whereas the Chelex-available copper did not show significant differences. The speciation of copper in seawater during toxicity testing, may also vary. The reduction of total dissolved copper may reflect the complexation and adsorption onto the experimental chamber of non-Chelex available copper. Voltammetric analysis of copper dissolved concentration during toxicity tests of an estuarine copepod showed loss of dissolved copper of 20% to 35%, and indicated that a 99% complexation occurred in all samples of the toxicity test (Hall *et al.* 1999), with complexing capacity increasing with both time and copper concentration. The free cupric ion accounted for only 8% of the dissolved non-complexed inorganic fraction or less that 0.2% of the total copper in solution.

In this study, the difference between total dissolved copper and Chelex available copper concentration in this study is evident from the beginning of the experiment (Time 0), indicating the experimental seawater contains colloidal forms or ligands complexing the dissolved copper. The total dissolved copper concentration is not equivalent to the Chelex-available form. It was estimated that 56.38% (±6.12) of the total copper in all samples of the toxicity was in Chelex-available form.

A high degree of complexation of copper in natural seawater was found in San Diego Bay (Zirino *et al.* 1998), as a result of a local production of organic ligands by macroalgae. Free

copper was three orders of magnitude less than the concentrations of the dissolved, surface bound and tightly bound copper fractions.

The amount of metal in solution is governed by many aspects of the chemistry of the metal in solution. Metal adsorption onto container walls and uptake by organisms are also important factors to consider. Systems normally used in toxicity tests are spiked with the relevant element. The loss of cadmium and zinc from seawater during accumulation experiments was recorded by Henning and Greenwood (1981). A substantial amount of metal was lost (37% Cd lost after 15 days, while 5% Zn lost after 50h) at concentrations of 0.2-3µg ml^{-1}. It was stressed that interpretation of graphs, calculations and deductions could not be made on the basis of added amounts of metal.

The loss of total copper in the 48h toxicity test observed in this study was evident from the beginning of the experiment and increased with time, being most significant in the cases where the organism was under stress and suffering toxic effects.

The Chelex-available copper concentration was constant. The loss of total dissolved copper observed, may be related to the activity of the organism that may release some complexing ligands, and/or uptake of copper by the organism, with subsequent adsorption of copper into the system (chamber, organism and food). The metal fraction involved in such complexation, uptake or adsorption would enable it to be measured and may explain the decrease of total copper observed. The possibility of loss during storage is excluded because samples were acidified in the storage bottle.

Therefore, it is suggested that the organic complexation and adsorption affected more the observed decrease of total copper than the inorganic complexation and adsorption onto the walls of the container. Under stress, mysids increased their excretion rate (see Chapter 7), and under all conditions faeces and remainders of food left by the organism due to inefficient feeding, may also provide adsorption sites. The production of strong ligands by organisms when exposed to copper, affecting the copper speciation in seawater, has been recorded in several studies (Gerringa *et al.* 1995, Moffet & Brand 1996, Zirino *et al.* 1998).

Accumulation rates calculated in this study (Chapter 6) suggest that metal uptake and accumulation by organisms is not the main factor causing the decrease of total dissolved copper, but adsorption and organic complexation appear to be important factors.

The labile copper concentration on toxicity testing showed variations, as occurs in natural seawater where complexation of copper occur significantly in any case. Yoon *et al.* (1999) found that natural seawater (Western Mediterranean Sea) samples contained an excess

of natural organic ligands which are able to complex some added Cu. In the surface layer only 15-40% of complexed Cu was isolated by a C18 Sep-Pak cartridge, and the remainder 60-85% was complexed by hydrophilic organic materials, which could not be isolated. Therefore in order to provide a copper concentration related to the toxic effects, metal speciation studies are necessary for toxicity tests, to provide an assessment of "toxic" metal concentrations and species.

Although bioavailability is a question that requires more investigation, it is shown in this study that the fraction of dissolved copper that was available to the organism in the toxicity test is significantly less than the "nominal" and/or total copper concentration. Chelex-available copper concentration was constant during the 48h of exposure in the toxicity test, whereas the total copper concentration decreased, especially where the organism was under stress. The results of this study confirms that complexation occurs, total copper concentration decrease in a toxicity test, and raise a different question concerning the chemical speciation and the effects on bioavailability during tests; which is to what extent the organisms themselves affect the metal speciation and thus toxicity.

4.5 SUMMARY

The application of Chelex -100 separations may provide a simple method of measuring labile and total metal concentrations in water samples, especially during a toxicity test. The performance of the Chelex technique (accuracy, precision and detection limits) was found to be acceptable.

Total copper concentrations decreased significantly over 48 hours in the toxicity test, where the organism is under stress from toxic effects, whereas the Chelex-available copper concentration did not show significant differences over the same time period.

Analysis suggested that experimental natural seawater contained colloidal forms of copper or ligands, complexing the dissolved copper. Total dissolved copper concentration is not equivalent to the Chelex-available form or labile dissolved copper. The labile dissolved copper and proposed as available to the organism in the toxicity test is inferior to the "nominal" and/or total dissolved copper concentration.

Organic complexation and subsequent adsorption affected more the decrease of total copper than the inorganic complexation and adsorption onto the walls of the container.

The results of this study confirms that organic complexation occurs in the toxicity tests. It suggests that the test organism may affect the metal speciation and therefore bioavailability during toxicity testing.

CHAPTER 5

COPPER TOXICITY

IN *P. flexuosus*

CHAPTER 5. COPPER TOXICITY IN *P. flexuosus*

5.1 INTRODUCTION

The study of the effects of pollutants, at realistic concentrations requires a controlled reliable toxicity test. As *Praunus flexuosus* is a member of the hyperbenthos and plankton, investigations on the effects of contamination could provide a good monitor to estimate levels of toxicity and the ecological consequences.

Trace metals research in Southampton Water and the Solent (Armannsson *et al.* 1985, Fang 1995) has shown an increase of copper concentration in sediment and water around the industrial areas, and the recreational marina of the Beaulieu estuary.

Mysids have been shown to be abundant in Southampton Water and adjacent regions in the past (Raymont *et al.* 1964, Ralph 1965, Fergusson 1973, Armitage 1979). However, recent surveys suggests there has been a decline in species diversity and abundance in Southampton Water and the Solent (see Chapter 3).

Praunus flexuosus (Müller), has been reported as an abundant species on European coasts (Tattersall & Tattersall 1951, Mauchline 1980) including the study area, and thus provides a representative and realistic monitor of the effects of copper on coastal zooplankton populations. The majority of previous toxicity studies have been episodic pollution events, using non-indigenous species (Forbes & Forbes 1994) and pollutant concentrations inappropriate to the natural environment (Luoma 1995). Toxicity testing has been performed frequently at a single temperature, salinity and/or life cycle stage and most trace metal toxicity studies on mysids have focused on cadmium. *Mysidopsis bahia* exhibits different trace metal tolerances. Acute toxicity values (96h LC_{50}) display a toxicity rank of Hg>Cd>Cu>Cn>Ag>Zn>Ni>As>Cr>Pb in *M. bahia* (Lussier *et al.* 1985). Some metals, at combinations of different temperature and salinities including Cr, Ni and Zn, disrupt osmoregulation in *P. flexuosus* (McLusky & Hagerman 1987), suggesting that disruption as the possible physiological cause of lethal toxicity.

Behavioural parameters in toxicity tests are not frequently measured, because of the difficulty in assessing behaviour quantitatively owing to variability in subject and time. Toxic effects on a specific behavioural pattern may be associated with sublethal effects on the organism indicating an inability to deal with the environment; and therefore may, in time,

result in lethal consequences. Behavioural parameters have been surveyed on *Praunus flexuosus* and in relation to responses to copper exposure.

The present study determines the effects of copper pollution at environmentally relevant levels on an indigenous mysid species and in different seasons. The key questions addressed are whether copper exposure (1) affects survival of *Praunus flexuosus*, and whether its effects differ (2) between winter and summer, (3) between juveniles and adults.

5.2 MATERIALS AND METHODS

5.2.1 Experimental animals

Mysid samples used were representative of the population in different seasons. Seasonal samples of *Praunus flexuosus* were collected (described in Chapter 2) from a marine lagoon at Keyhaven, West Solent. To minimise physiological stress, individuals were transported to the aquarium within 1h and maintained at ambient conditions similar to field conditions, including water quality. Regular (every 15d) comparisons of field (Keyhaven) population growth (length), animal condition and behavioural patterns with the population maintained in the laboratory (originally sampled from Keyhaven) show that the experimental animals, including the controls of the toxicity test, were representative of the field population.

5.2.2 Experimental conditions and design

Water quality parameters were maintained (see Chapter 2) well below toxic levels. Water movement and levels of dissolved and particulate organic matter were also controlled. Copper and metal contamination were prevented following Howard & Statham (1993). Individuals collected in December, February and August were acclimated for one week in the aquarium at the experimental conditions, simulating the temperature, light and salinity conditions of the season in the field. Individuals were representative of the physiological condition of the field population in winter and summer. After the acclimation period individuals were selected for life cycle stage, size and good condition for toxicity testing.

5.2.3 Toxicity test

Test chambers comprised glass tanks acid washed in 10% HCl to remove residual contamination and Milli Q water rinsed to remove trace HCl. Tanks were filled with 1µm-filtered, UV irradiated and aerated natural seawater. Toxicant metal solution (Analar $Cu(NO_3)_2$) was prepared in Milli Q water (18.2 m• cm^{-1}). The stock copper solution (100ppm) was added to the seawater in the experimental chambers, to give concentrations of 5, 25, 75 and 200 µg l^{-1} copper added (Cu_a).

Static toxicity tests were performed on ten individuals of each life cycle stage (N=30) at each test concentration for 10 days (except in December where 5 individuals were used). *Artemia* nauplii were provided daily at 20-25 nauplii per mysid. All test seawater and toxicant solutions were renewed every 48h, when tanks were cleaned of remaining food and organic material. Toxicity tests were performed on juveniles (immature individuals), non-brooding females and males at 0, 5, 25, 75 and 200 µg l^{-1} copper added for 10 days at the following temperature, salinity and light conditions, 10(±1)°C December, 5(±1)°C February, and 20(±1)°C August with 9L:15D, 11L:13D, 16L:8D respective light:dark photocycles and salinity regimes of 30(±1) (winter) and 33(±1) (summer).

Mortality was recorded every 12 hours. Behavioural parameters were recorded twice per day, in the morning and in the evening, at feeding time. The normal pattern of behaviour was determined through observations and video recording comparison of behaviour in the field and in the aquarium. The behavioural parameters recorded were the following: sensory capacity as ability for detection of food or objects; swimming capacity; equilibrium as maintenance of normal orientation; feeding; daily activity as routine swimming and interactions with other mysids; morphological changes as modifications in body shape and colour; and cannibalism as predatory activity between specimens. The responses were classified to 3 levels of disruption. Responses were defined as normal, abnormal, disruptive (very weak response) and severely affected (no response).

Background total copper measured in control seawater (acidified and UV radiated samples) for experiments in December, February and August were <2, <6 and <8 µg l^{-1} copper respectively. This measure of total copper concentration is not equivalent to the concentration of copper available to the organism (see Chapter 4).

5.3 RESULTS

Survival in controls was 100 % in all tests, in both winter (December and February) and summer (August). In winter there was very low mortality in experimental animals. There was only 0 % to 1.33 % mortality (Fig.5.1) with increasing copper exposure, in both December (10 °C) and February (5 °C) after 10 days of exposure. In August, however, (at 20 °C) copper produced significant mortality (17 %) at 5 $\mu g\ l^{-1}$ Cu_a and mortality increased with copper concentration, reaching 93 % and 90 % at the higher concentrations (75 and 200 $\mu g\ l^{-1}$ Cu_a). Summer mortality at 96 hours of exposure was 50 % in the 25 $\mu g\ l^{-1}$ Cu_a treatment, however, when 96h mortality were regressed against copper concentration the 96h LC_{50} was 30.8 $\mu g\ l^{-1}$ (Fig.5.2).

Mortality was significantly different (Fig. 5.3) between the life cycle stages (female, male and juvenile) ($H=43.6$, $p<0.0001$). Mortality was significantly different with duration of copper exposure on the life cycle stages (males $H= 15.5$, $p=0.0038$; females $F= 7.91$, $p=0.0002$; juveniles $H= 26.6$, $p<0.0001$). Significant mortality of females (20 %) occurred at 12h at both 75 and 200 $\mu g\ l^{-1}$ Cu_a (Fig. 5.3A) and at all enhanced copper levels after 24h. Mortality increased with copper concentrations, and after 96h of exposure, mortality was 30 % at 5 $\mu g\ l^{-1}$, 60 % at 25 $\mu g\ l^{-1}$ 80 % at 75 $\mu g\ l^{-1}$ and 90 % at 200 $\mu g\ l^{-1}$ Cu_a.

The mortality of males in summer was lower in response to copper than in females. No lethal effects were observed during the 10 days of exposure to 5 $\mu g\ l^{-1}$ Cu_a (Fig. 5.3B). Lethal effects after 24h of exposure were recorded only at the highest exposure level (200 $\mu g\ l^{-1}$ Cu_a). At 25$\mu g\ l^{-1}$ Cu_a, the mortality increased to 40 % after 48h. At the highest concentrations, mortality was 80 and 90 % following 10 days of exposure.

Juveniles in summer showed a very low tolerance to copper (Fig. 5.3C). Lethal effects occurred at every copper concentration over 5 $\mu g\ l^{-1}$ Cu_a within 24 hours. Mortality was 60 % at 200 $\mu g\ l^{-1}$ Cu_a after only 12h of exposure which increased to 100 % within 24h. At 75 $\mu g\ l^{-1}$ Cu_a the mortality was 100 % after 72h of exposure. Mortality at 25 $\mu g\ l^{-1}$ Cu_a was 50 % after 48h of exposure and at 5 $\mu g\ l^{-1}$ Cu_a was 20% within 7 days.

The summer 96h LC_{50} estimated for juveniles (immature individuals) was 21 $\mu g\ l^{-1}$ Cu_a, for females 22.7 $\mu g\ l^{-1}$ Cu_a and for males 61.4 $\mu g\ l^{-1}$ Cu_a. Mortality levels recorded after 96h of exposure did not increase considerably over the following 6 days of exposure.

The specimens exposed to copper that were in the process of moulting died within 24h pre- or post-moulting. The image (Fig. 5.4) compares a control specimen and an individual exposed to copper for 10 days. At the beginning of the experiment, both

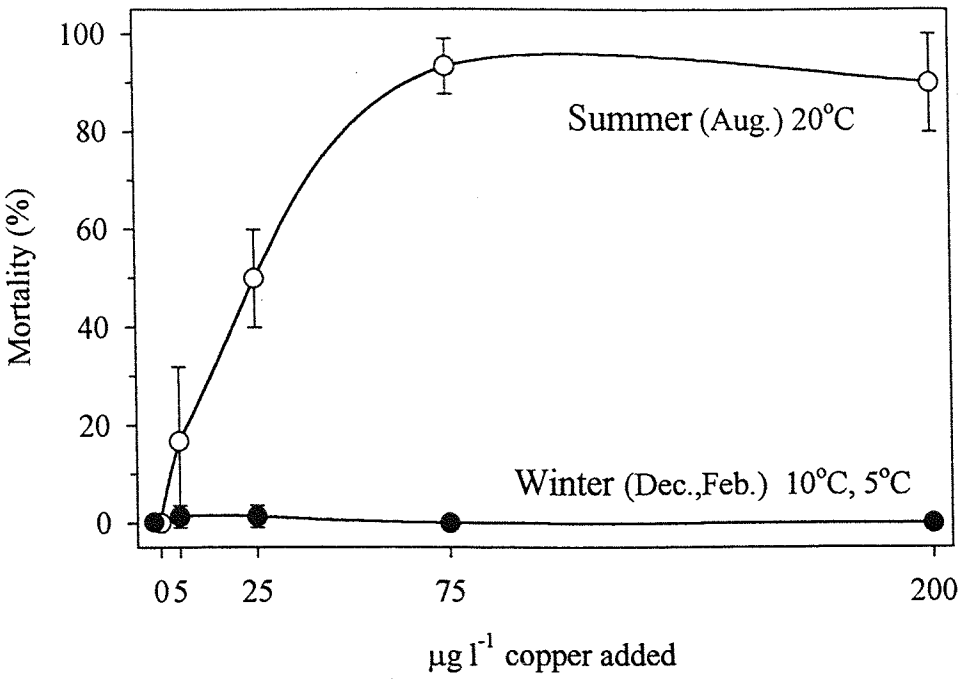


Fig. 5.1. ***Praunus flexuosus*** **mortality (Mean, SD) of the population (females, males and juveniles) in winter (December, February) and summer (August) exposed to copper for 10 days. No mortality was observed in winter and summer controls.**

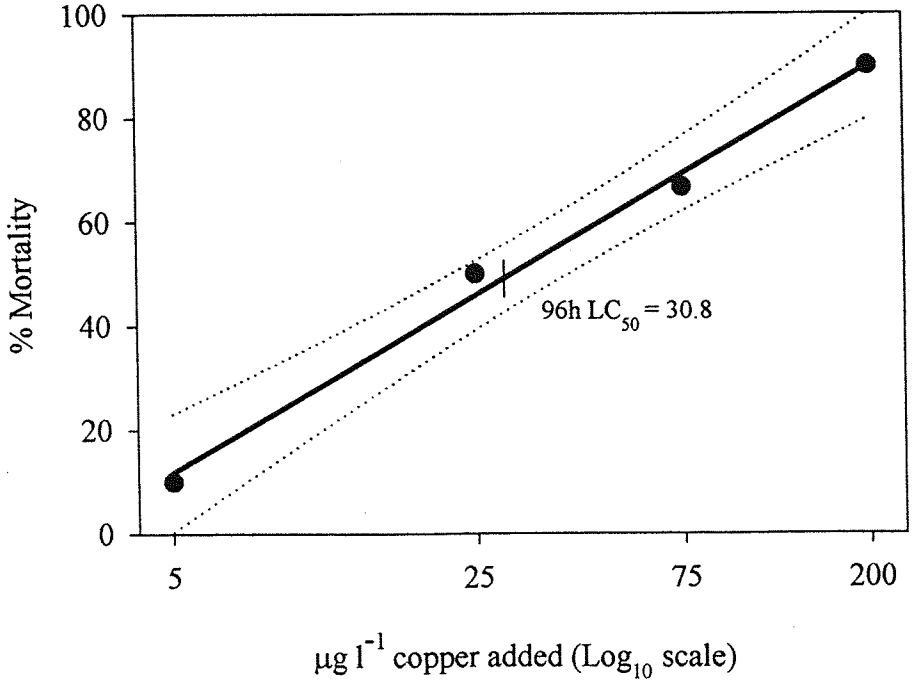


Fig. 5.2. Mortality (%) of the population in summer after 96h of copper exposure. Regression analysis Mortality % = -22+48.4 $\log_{10}$[Cu]; r^2=0.992; P<0.05; 95% C.I. calculated 96h LC_{50}

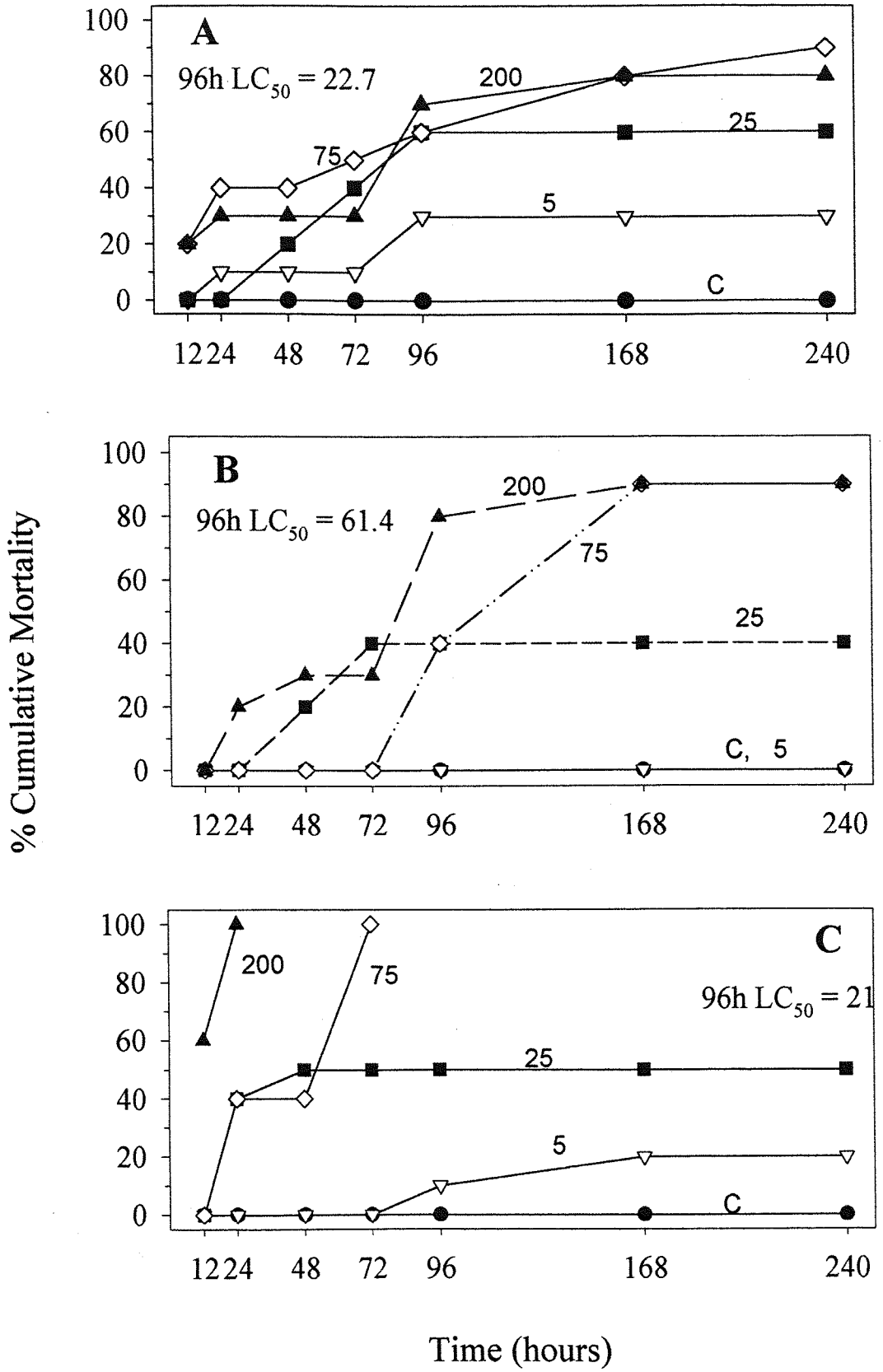


Fig. 5.3. ***Praunus flexuosus*** **cumulative mortality (%) for females (A), males (B), juveniles (C) of the population in summer exposed to copper for 10 days. No mortality was observed in controls. Value on plots are in μg l^{-1} Cu_a. 96h LC_{50} was calculated for every stage.**

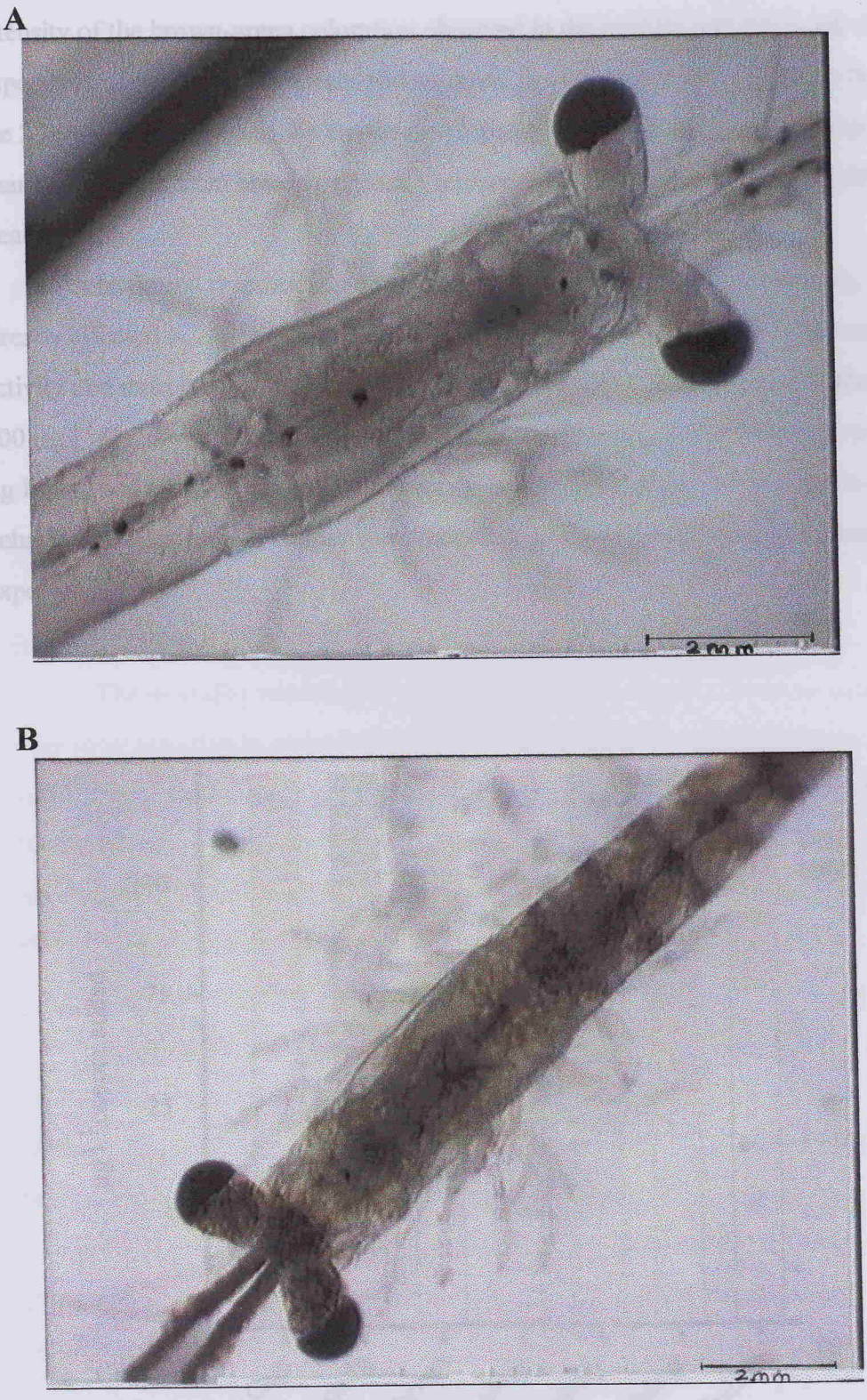


Figure 5.4. *Praunus flexuosus* specimens control (A) and exposed to copper (B) after 10 days at 75 µg l^{-1} Cu_a in winter.

specimens had the same clear cream colour (Fig. 5.4A.). After being exposed to copper for 10 days, the exposed specimen (Fig. 5.4B) showed an intense brown-green coloration. The intensity of the brown-green coloration observed in the toxicity test increased with the level of exposure to copper in both winter and summer. Brown colour was visible on the 5th day in the 25 μg l^{-1} treatment and the colour developed to green on 9th day of the exposure. The change of colour from brown to green occurred within 6 days at the 75 and 200 μg l^{-1} copper treatments.

Behavioural responses to copper after 96h of exposure in summer (Fig. 5.5) were already affected at 25 μg l^{-1} Cu_a , showing abnormal responses in swimming capacity, daily activity and morphological changes. At 75 μg l^{-1} Cu_a those responses were disruptive and at 200 μg l^{-1} Cu_a were severely affected. All behavioural responses monitored were affected at 75 μg l^{-1} Cu_a and all were disrupted or severely affected at 200 μg l^{-1} Cu_a . In winter similar behavioural responses to copper were recorded, at the same concentrations but after 7 days of exposure.

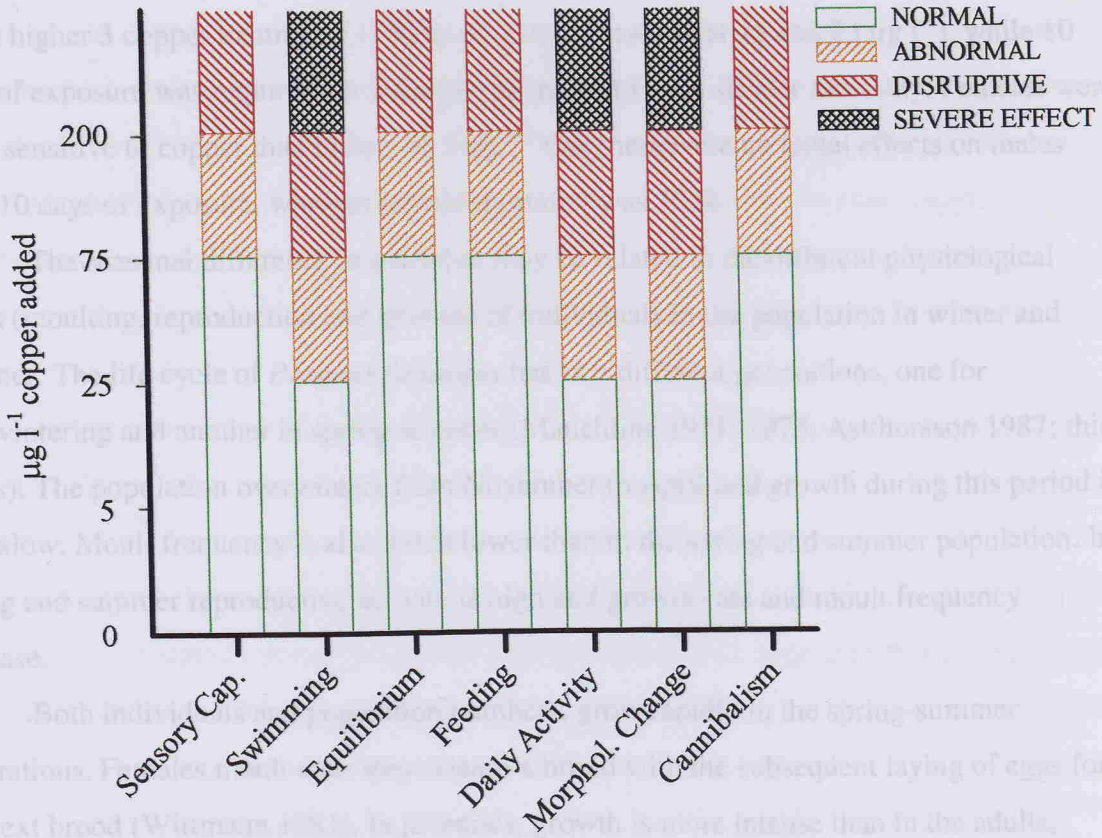


Fig. 5.5. *Praunus flexuosus* behavioural responses to copper after 96 hours in summer

5.4 DISCUSSION

There are pronounced seasonal and ontogenetic differences in copper toxicity in *P. flexuosus*. While there was no significant mortality of the population in winter (no mortality at the highest level of exposure after 10 days, 200 $\mu g\ l^{-1}$ Cu_a), in summer the 50 % of mortality occurred at 25 $\mu g\ l^{-1}$ Cu_a after only 72h. The population in summer showed lethal effects at every copper exposure level after 24 hours. The 96h LC_{50} for copper (observed at 25 $\mu g\ l^{-1}$ in experiments, calculated from regression analysis 30.8 $\mu g\ l^{-1}$ and including the different life cycle stages 21, 22.7, 61.4 $\mu g\ l^{-1}$) found in summer for *Praunus flexuosus* in this study, showed a lower level of tolerance compared with other mysids. Lussier (1985) reported a 96h LC_{50} of 181 $\mu g\ l^{-1}$ copper for *Mysidopsis bahia* and Cripe (1994) found a 96h LC_{50} of 153 $\mu g\ l^{-1}$ copper. Brandt *et al.* (1993) demonstrated a 96h LC_{50} of 150 $\mu g\ l^{-1}$ copper in *Neomysis mercedis*. In *Praunus flexuosus* mortality increased with copper concentrations and time of exposure. Mortality levels did not increase significantly between 96h of exposure and the levels found at 10 days of exposure.

The mortality recorded for different life cycle stages in summer showed that juveniles were most sensitive to copper. There was high juvenile mortality after only 24h of exposure to the higher 3 copper treatments (100 % at 200 $\mu g\ l^{-1}$; 40 % at 75 and 25 $\mu g\ l^{-1}$), while 10 days of exposure was required for females and males to have similar mortality. Females were more sensitive to copper than males. At 5 $\mu g\ l^{-1}$ Cu_a there were no lethal effects on males after 10 days of exposure, whereas female mortality was 30 %.

The seasonal difference in mortality may be related to the different physiological states (moulting, reproduction and growth) of individuals in the population in winter and summer. The life cycle of *Praunus flexuosus* has two different generations, one for overwintering and another in spring-summer (Mauchline 1971, 1973; Astthorsson 1987; this thesis). The population overwinters from November to April and growth during this period is very slow. Moult frequency is also much lower than in the spring and summer population. In spring and summer reproductive activity is high and growth rate and moult frequency increase.

Both individuals and population numbers, grow rapidly in the spring-summer generations. Females moult after they release a brood with the subsequent laying of eggs for the next brood (Wittmann 1981). In juveniles, growth is more intense than in the adults, which is enhanced by a need to reach maturity in order to start reproduction within the same

season. Data and observations from the toxicity tests in this study showed that, in all the cases where specimens were moulting, no animals survived and mortality occurred within 24 hours pre- or post-moulting. Saroglia & Scarano (1979) showed an increase in sensitivity to contaminants during moulting in the shrimp *Penaeus kerathurus*. White & Rainbow (1984) found that moulting increased accumulation of Zn by *Palaemons elegans*. Cripe (1994), comparing the sensitivity of shrimp postlarvae and mysids to three metals (cadmium, copper and zinc), and suggested that the increase in sensitivity during moulting may explain the differences in sensitivity found between adults and larvae. The data of the present study confirm that there is an increase in sensitivity to contaminants during moulting, and therefore it is a crucial time for toxic effects.

Euryhaline crustaceans may be able to vary their apparent water permeability (Mantel & Farmer 1983, Campbell & Jones 1990) and any such intraspecific variability would represent a possible physiological control over metal uptake by the crustaceans, independent of the effects of varying physicochemical factors.

Although the rate of trace metal uptake is governed by the physico-chemistry of the metal, chemical speciation, and binding of the metal to transport ligands or organic ligands of the membrane (Rainbow 1997), the physiology of the invertebrates affect significantly metal uptake rate, as in the case of active calcium pump. White & Rainbow (1984) recorded an increase of labelled zinc uptake rate on moulting individuals. It may represent an increased flux of zinc from solution into the body as a result of an increased permeability of the cuticle. There are several interactions between calcium regulation and trace metals, suggesting that may be a number of shared pathways (Wright 1995). The increase on calcium uptake and therefore in metal uptake could result in different biochemical responses related to metal metabolism, celullar responses and oxidative stress.

Environmental contaminants may enhance oxidative stress in aquatic organisms (Winston 1991), and when antioxidants defenses are overcome by prooxidant forces may be the basis for many physiological disfunctions. For example, glutathione peroxidase can act as an antioxidant and bind a number of metals including Cu, Cd, Cr, Fe, Hg, Ni, Pb and Zn and therefore can potentially detoxify (Christie & Costa 1984). It is suggested that glutathione peroxidase can reduce the toxicity of metals such as Cu and Fe by assisting in the removal of toxic species (e.g. superoxide ions and peroxides) which react with these metals to form hydroxyl radicals (Hanna & Mason 1992). Some enzymes are rapidly induced following exposure to environmental xenobiotics, to transform the compounds in more excretable or in

metabolites (Livingstone 1993). In the other hand, factors such as temperature, seasonal and sexual cycles affect enzyme activity (Collier *et al.* 1995).

The mysids that survived the higher levels of copper exposure in this study showed a marked change of colour (to brown-green), with increasing level of copper and time of exposure. A similar change of colour pattern with copper exposure was observed in winter and summer. Focused Ion Beam analysis confirmed that the change in colour was associated with copper accumulation in tissues (see Chapter 6). Hang & Hung (1990) observed a green colouration in oysters living in a highly copper-polluted areas. Thus the change in colour with copper may be used as an immediate diagnostic of organisms exposed to relatively high levels of copper and may indicate both bioaccumulation and subsequent detoxification processes by the organism.

Behavioural responses were affected, and in some cases disrupted by copper in both seasons. Although no mortality occurred in the winter toxicity tests, it is evident that specimens with disrupted behaviour would have reduced capabilities to survive in the real environment. Behavioural parameters were a very sensitive indicator of toxic effects.

McLusky *et al.* (1986) emphasised the importance of environmental variables on metal toxicity, with maximal toxicity at low salinity and high temperature. McLusky & Hagerman (1987), tested *Praunus flexuosus* with chromium, nickel and zinc at different salinities and temperatures. It was shown that the increase in temperature and salinities above or below the isosmotic point, led to reduced median survival at a given metal concentration. They suggested that death of *Praunus flexuosus* in metal solutions may be related to a progressive decrease in the ability of the individuals to osmoregulate. However, McLusky & Hagerman. (1987) were unable to show whether death was related to a progressive decrease in the ability of the animal to osmoregulate or whether the loss of osmoregulation ability is a secondary effect of metal poisoning. A clear pattern of osmotic regulation in *Praunus flexuosus* has been reported, though no seasonal (winter vs summer) or temperature (5 to 20 ^{o}C), effects were observed on osmotic concentration in the blood (McLusky 1979).

The present study did not consider salinity variations (range winter-summer was 30-33 ±1), but still found significant mortality from exposure to copper. Euryhaline crustaceans may be able to vary their apparent permeability (and metal uptake), representing a possible physiological control over metal uptake, independently of variations in physicochemical factors, including salinity variation (Rainbow & Kwan 1995). Crustaceans with calcified

exoskeletons have a high activity calcium pump, which affects metal uptake, especially at moulting (Rainbow 1995).

The seasonal difference in toxicity observed in this study is related to the different physiological state of the population in summer and winter. Seasonal variations are a combination of multiple parameter changes and the physiological response to these changes by the organism. Growth, moulting and reproduction are all synchronised with seasonal variables, creating specific physiological states. This study focused on these seasonal effects rather than the role of temperature or salinity at different levels from those characteristic of the season. To test the effects of different temperatures and salinities on the toxicity of an organism, without causing an important stress on their physiology, is difficult and such tests would be very different from a real situation in the natural environment. The study of sublethal effects, such as metabolism, growth, reproduction and biochemical mechanism may explain more specifically the reasons for the observed seasonal variation in copper toxicity.

5.5 SUMMARY

The results of this study show that there are pronounced seasonal and ontogenetic differences in copper toxicity. While the mortality of the population in winter was insignificant after 10 days of copper exposure, in summer lethal effects occurred at every copper exposure level after 24 hours.

The population (males, females and juveniles) in summer showed a 96h LC_{50} for copper of 30.8 $\mu g\ l^{-1}$, which indicated a low level of tolerance for *Praunus flexuosus* compared with other mysid species.

The mortality recorded for the different life cycle stages of the population in summer shows that juveniles were most sensitive to copper. Both individuals and population numbers, grow rapidly in the spring-summer generations, and in juveniles growth is more intense than in the adults.

Results shows that there is an increase in sensitivity to contaminants during moulting, and therefore it is a crucial time for toxic effects. For all the cases where specimens were moulting, mortality occurred within 24 hours pre- or post-moulting.

Behavioural parameters were very sensitive indicator of toxic effects. The change in colour described in this study with copper may be used as an immediate indicator of organisms exposed to copper contamination and may indicate both bioaccumulation and subsequent detoxification processes by the organism.

Although mortality was not recorded in winter toxicity tests, disruption in behaviour created very low probabilities for the organisms to survive in the real environment.

The seasonal difference in toxicity observed in this study is related to differing physiological states of individuals in the populations in winter and summer. Several physiological functions are more active in summer than in winter, such as moulting, growth, reproduction and specific biochemical mechanism related to these functions.

CHAPTER 6

COPPER BIOACCUMULATION

in *P. flexuosus*

CHAPTER 6. COPPER BIOACCUMULATION IN *Praunus flexuosus*

6.1 INTRODUCTION

Bioaccumulation of trace metals or any other contaminant in an organism affects the cycling of the contaminant in the ecosystem. Copper is an essential trace metal in crustaceans, and is taken up, metabolised, accumulated or excreted. Metal concentration in the body tissues of an organism living in a copper contaminated environment may increase to toxic levels, or the organism is able to detoxify.

Metal bioaccumulation may be studied by several methods. The most commonly used is via acid digestion of the body tissues followed by atomic adsorption spectrometry (Bryan 1968, Phillips 1976, Yamamoto *et al.* 1987, Bebiano & Machado 1997). Furthermore the use of radiolabelled metals were sometimes included to provide more specificity to the study (White & Rainbow 1984). Other approaches use biomarkers such as metallothionein measurements, cytochemical responses (Benson *et al.* 1990, Depledge *et al.* 1995), and microanalytical techniques have been also employed (Chassard-Bouchard *et al.* 1992).

Microanalytical techniques are based on scanning electron microscopy (SEM). The advantages of these techniques is the determination and distinction of the elements on a micro scale, and the requirement of only a small sample and relatively rapid analysis. The most commonly used technique with biological specimens is energy-dispersive X-ray (EDX) microanalysis or electron microprobe, and secondary ion mass spectrophotometry (SIMS) (Chassard-Bouchard 1991). Recent developments of techniques are improving limits of resolution and accuracy, but there are surface analysis techniques and internal reservoirs of metal that will not be detected.

The uptake and accumulation of the metal by the organism depends on many factors, including the chemical speciation and bioavailability of the metal, and the physiology of the organism (Rainbow 1997). Kinetics of metal uptake and loss in relation to the weight of the organism may cause inter-individual variability in body metal concentration (Depledge 1990). Bioaccumulation is influenced by biological variables such as permeability of external surfaces, efficiency of excretory systems, activity of ligands in the cytosol and changes in the body weight which could be modified by growth, reproduction development, nutrition and season (Brown & Depledge 1998).

The aims of this study here were to determine whether or not *Praunus flexuosus* accumulates copper when exposed to dissolved-copper contaminated seawater, and whether or not there are seasonal effects on the copper content and accumulation.

6.2 MATERIALS AND METHODS

6.2.1 Microscope analysis for detection of copper accumulation in mysids

Specimens exposed to copper in the toxicity test were analysed with 3 different microscopy techniques in order to detect accumulation of copper in the organism. The techniques applied were the following:

1) EDX and WDX (wavelength dispersive X-ray analysis) spectrometer on a SEM platform

The specimens from the toxicity test were rapidly frozen in liquid nitrogen and analysed using liquid nitrogen cryomicroscopy techniques (freeze-dried and carbon-coated in an Emitech K950 coating unit) on a Leica-Cambridge S360 SEM. Mysids were imaged and surface (cuticle) copper contamination was assessed via Oxford Instruments ISIS 300 EDX and Microspec WDX X-ray spectrophotometers.

2) X-Ray Analytical Microscope (electron microprobe)

Specimens of mysids from the toxicity test were frozen at -20°C. Mysids were placed in an Oxford Instruments XGT-2000W X-Ray analytical microscope and scanned by a 100 μm X-ray beam having a 15kV source and rhodium (Rh) target. Fluorescent X-ray analysis was performed on the specimens without any pre-treatment. Simultaneous analyses were carried out for Ca and Cu, mapping the elements in the organism.

3) Focused Ion Beam Secondary Ion Mass Spectrometry system.

Specimens from the toxicity test were freeze-dried, mounted on aluminium stubs and gold-coated (in a Biorad SC502 sputter coating unit). They were placed on a FEI FIB 200 focussed ion beam workstation (prototype), to be imaged in the S360 SEM via secondary electrons at low magnification and precision ion milled (Garnacho *et al.* 2000).

This was the first biological application of a SIMS mass spectrometer harnessed to a FIB milling/imaging system (Garnacho *et al.* 2000). The process involves using the ion beam

to ablate material from the observed specimen and analysing it on a mass spectrometer. The advantage is the very small area that it is possible to analyse (20x20μm). The SIMS detector was tuned to quantify 63AMU positive (Cu^+) ions, as a single depth profile output. Signals from 20x20 μm sample boxes milled into carapace and membrane targets were recorded.

6.2.2 Analysis of the mysids whole body copper content

Mysids exposed to copper in different experiments were collected to determine their total copper content (see Chapter 2) at the end of the 4 following experiments:

1) Specimens exposed to 20, 35, 50, 75, 100, 200 and 300 μg l^{-1} Cu_a for 7 days at 10°C in December
2) Specimens exposed to 5, 25, 75 and 200 Cu_a for 10 days at 5°C in February
3) As previous experiment but at 20°C in August
4) Brooding females exposed to 5, 25, 75 and 200 Cu_a at 17°C in June for one week and then transferred to clean conditions for two weeks.

Samples (living mysids) were collected from the field and toxicity tests described above, rinsed in Milli Q water avoiding any metal contamination, placed in a polyethylene tube and frozen at -20 °C. All equipment used was acid cleaned to avoid contamination. Total copper content of the mysids was analysed by acid digestion. Specimens were dried at 60 °C to constant weight. Each specimen was weighed on an electro balance (micrograms). Specimens were grouped to reach a minimum dry weight sample of 5mg and then digested in concentrated nitric acid (Aristar grade, BDH) using a gradual heating programme to 160°C. The acid digestion tubes were covered with loose-fitting glass stoppers and digestions were made up to 1ml with HCl solution. Standard reference material shrimp (IEAST) was used for calibration. Samples were analysed for total copper content by flame atomic absorption spectrometry on a Varian AA157 spectrometer with deuterium background correction. Concentrations are given as μg g^{-1} dry weight (dry wt) of mysid.

6.3 RESULTS

6.3.1 Microscopy analysis of copper in the mysids

Copper was detected by the EDX and the WDX spectrometer on a SEM platform in mysids exposed to copper, but concentrations were below the levels needed for accurate measurements.

XGT-2000W X-Ray analysis mapped the organism for elements and copper emissions were detected. Quantification of copper by the prototype used in 1997 was limited when mapped as 100 μm pixels. It was possible to localise maximum concentrations of copper in cuticular segments, but not in pleopods or at the membranous intersegmental area between the carapace and first abdominal segment.

The SIMS system on a FIB 200 workstation analysis of the control and copper exposed specimens showed profile differences, that confirmed copper accumulation in the copper exposed organisms.

Sample boxes milled into carapace and membrane targets gave two output signal types: (A) low signal having 3-15 counts per second (Hz), indicating a uniform background of typical control carapace, control membrane and copper exposed membrane; (B) a high signal (15-50 Hz) showing copper accumulation in the copper exposed carapace (Fig.6.1).

The exoeskeletal structure in crustacea is basically organic material (nitrogenous polysaccharide chitin) strengthened by calcium salts and or proteins bound by quinone. There are four main regions (Lockwood 1968): epicuticle (protein and lipid material tanned by quinone), pigmented layer (calcified chitin and tanned protein), calcified layer (untanned chitinous layer) and a membranous layer (uncalcified) immediately above the hypodermis cells. The copper accumulation measured by FIB-SIMS system shown at (Fig 6.1) was located on the calcified layer of the exoskeletal estructure.

Readings (3 replicates) represent depth profiles where increasing sputter time equals to increasing penetration of the organism. The high signal (B) showed 3 phases: Low level, peak and a decline to background. Assuming a sample box depth of 1.8 μm, the low level phase between 0 and 0.47 μm contains 17.5% of the total above background signal and the last phase between 1.3 and 1.8 μm an additional 12%. The peak level phase between 0.47-1.37 μm represents 70% of the accumulated copper with a peak concentration reaching 5 times the background level. This implies that very little copper was adsorbed onto the cuticle surface and most was selectively accumulated sub-surface. Total body copper content

analysis confirmed that copper is accumulated in the copper-exposed animals and the data here provide evidence that some is absorbed and deposited into the carapace.

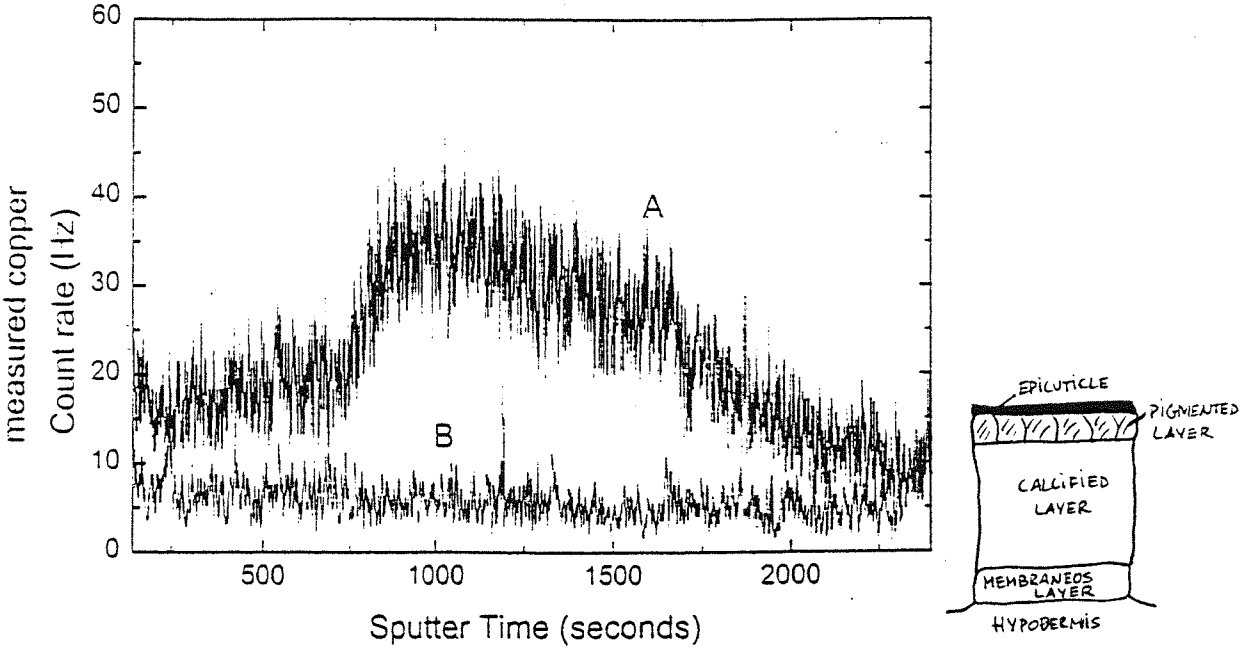


Fig. 6.1 SIMS-FIB signal of copper in the cuticle (within the calcified layer) of *Praunus flexuosus* exposed to 200µg l^{-1} Cu_a for 10 days. Sputter time equates to depth through the cuticle (2250 seconds = 1.8 µm). A: carapace of copper exposed mysid. B: inter-segmental membrane of copper exposed mysid, which coincides with the control carapace and membrane signal.

6.3.2 Total body copper content in *P. flexuosus*

Total copper content in the organism increased with increasing copper concentration in solution, and at different rates depending on the seasonal condition.

The total copper content in copper exposed *P. flexuosus* in December at 10°C (Fig 6.2) increased significantly ($F=5.2$, $p=0.04$) with respect to controls (42.5 µg g^{-1} dry wt) to maximum values of 105.7µg g^{-1} dry wt after 7 days of exposure. In February at 5°C copper accumulation showed a similar pattern to the one observed in December, increasing significantly ($F=32$, $p<0.001$) with respect to controls (41.7 µg g^{-1} dry wt) to 104.2µg g^{-1} dry wt at 200 µg l^{-1} Cu_a, after 10 days (Fig. 6.3). The high variability of copper content observed at 200 µg l^{-1} Cu_a indicates stress interference on the accumulation rate. In summer (August) at 20°C copper accumulation was higher (Fig 6.4) than in December or February. The copper

content of the body mass increased significantly (F=8.06, p=0.029) with respect to controls (50.2 $\mu g\ g^{-1}$ dry wt) to 25 $\mu g\ l^{-1}$ Cu_a (108.9 $\mu g\ g^{-1}$ dry wt). Similar values to the maximum levels observed in winter at 200 $\mu g\ l^{-1}$ Cu_a are found in summer at 25 $\mu g\ l^{-1}$ Cu_a. In summer there was a high mortality and the copper content shown at 75 and 200 $\mu g\ l^{-1}$ Cu_a came only from the 10% of mysids that survived in the trials (see Chapter 5). Those data were therefore, excluded from the statistical analysis.

The copper accumulated by females exposed in June at 17 °C for one week had decreased after two weeks in recovery conditions, but not to control levels specimens exposed to the highest copper concentration (Fig 6.5). Recovery of behavioural parameters to normal response was observed at the specimens exposed to 5 and 25 $\mu g\ l^{-1}$ Cu_a and from disruptive to abnormal response at 75 and 200 $\mu g\ l^{-1}$ Cu_a.

Controls and field specimens had similar copper contents. Comparison of the total copper content in controls with specimens of similar dry weight showed that winter controls contained 8 $\mu g\ g^{-1}$ dry weight less than summer controls. Differences are significant between winter and summer controls (t=-6.16, p=0.01), but not between February and December controls (t=-0.63, p=0.55). Copper accumulation in mysids is also significantly lower (H=9.67, p=0.022) in winter than in summer.

If we assume that copper accumulation is linear with time, estimations based on the data described above give different accumulation rates for the seasons considered (winter and summer). The accumulation rate in December at 10°C was 2.12 $\mu g\ g^{-1}$ dry wt day^{-1}, but decreased to minimum rates in February at 5°C (1.1 $\mu g\ g^{-1}$ dry wt day^{-1}), and these rates increased in summer (August) at 20°C to the maximum rate (7.9 $\mu g\ g^{-1}$ dry wt day^{-1}).

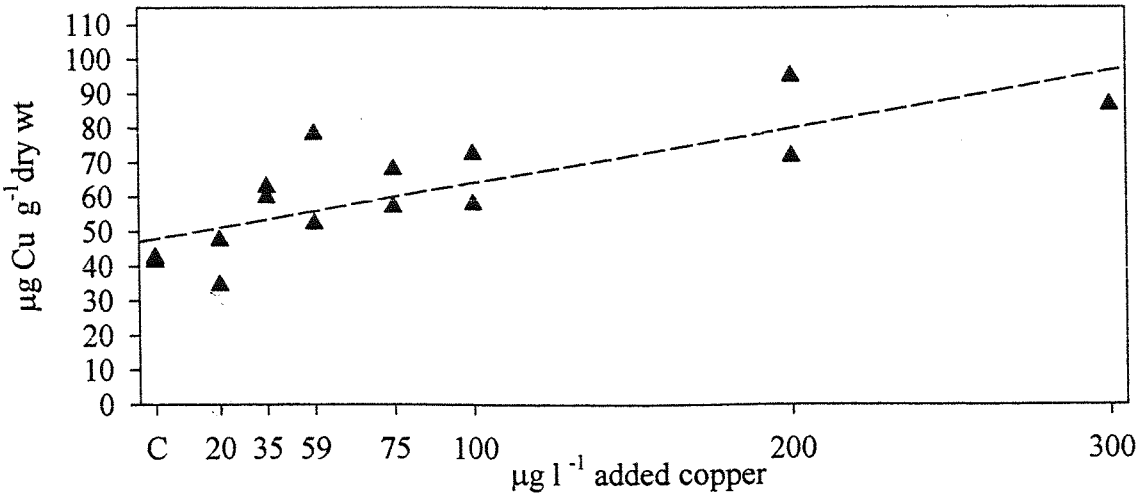


Fig. 6.2. *Praunus flexuosus* total copper content in body mass after 7 days of copper exposure at 10°C in December. (Regression calculated with 95% C.I.)

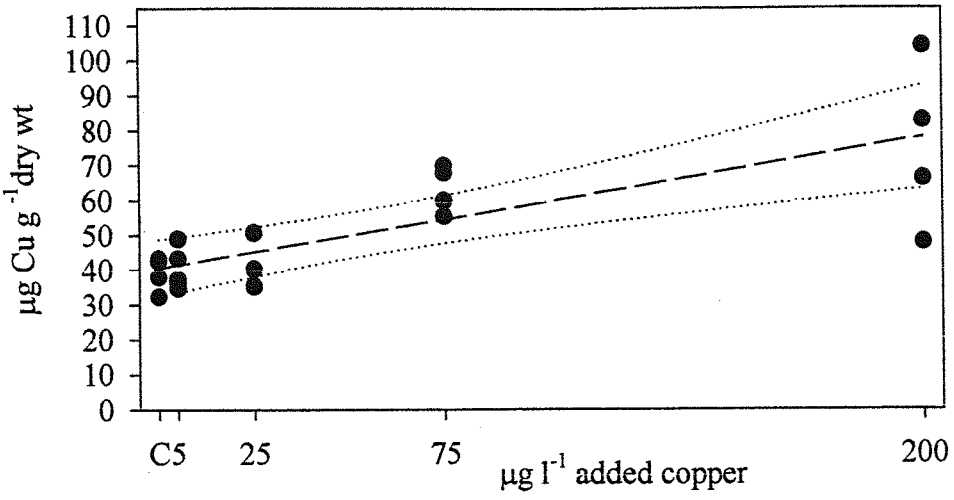


Fig. 6.3. *Praunus flexuosus* total copper content in body mass after 10 days of copper exposure at 5°C in February. (Regression 95% C.I.)

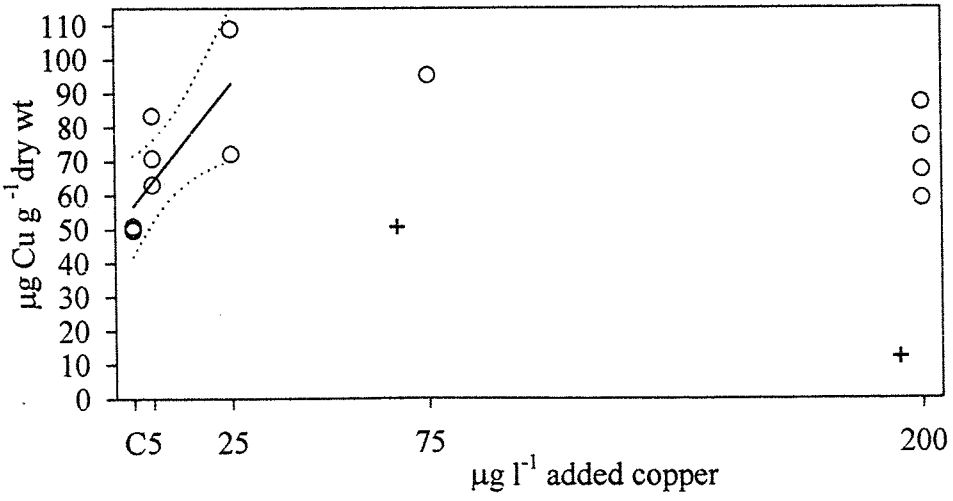


Fig. 6.4. *Praunus flexuosus* total copper content in body mass after 10 days of copper exposure at 20°C in August. (Regression 95%C.I.), (+) moribund animals. Values at 75 and 200 Cu_a correspond to the 10% survivors of the population

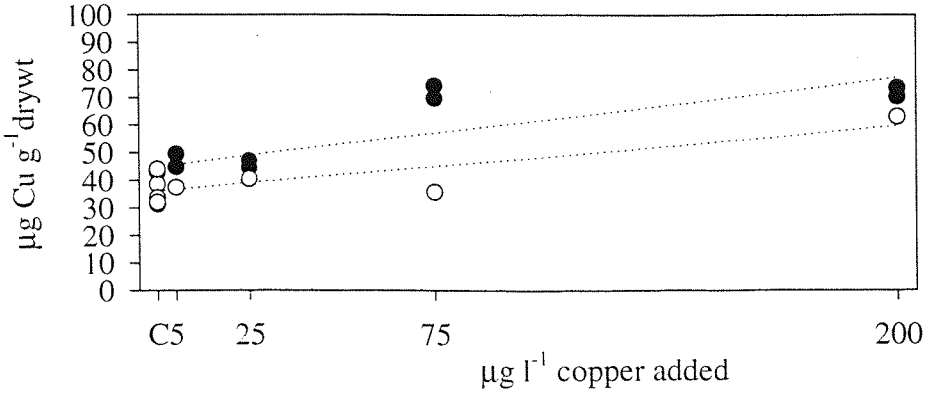


Fig. 6.5. Copper content in body mass of *Praunus flexuosus* females in June at 17°C maintained at two different conditions: copper exposed for one week, and under recovery (control seawater) for two weeks after the week of copper exposure.

- ● copper exposed for one week
- ○ 2 weeks in control seawater after 1 week of copper exposure
- ····· Regression (95% CI)

6.4 DISCUSSION

Praunus flexuosus accumulate copper when exposed to copper contaminated seawater. Copper accumulation was detected in the carapace and as suggested by X-ray mapping in the cuticle of other body segments, but neither in the pleopods nor the membranes between segments. The FIB/SIMS system was applied for the first time to biological material in this study; and detected and semi-quantified copper at contamination levels below resolution limits for EDX and WDX spectrophotometers. This system located copper accumulated within the cuticle, at the calcified layer of the carapace, suggesting that it was not adsorbed onto the surface but accumulated within the carapace.

Phillips (1980) and Langston & Spence (1995) found that body concentrations were maximum at pre-moult while post-moult concentrations of discarded cuticle were high. The results from the FIB/SIMS analysis corroborate that some copper is accumulated and accumulated within the cuticle and as suggested Lasenby & Van Duyn (1992) can be effectively off-loaded at moulting.

Analysis of the total copper content of *Praunus flexuosus* showed that copper was accumulated at a higher rate in summer (August) at 20°C than in winter at 10°C and 5°C (December and February). The copper content of controls as well as copper exposed mysids was higher in summer. Variations in the metal content in the organism with season were recorded on collections of Antarctic krill (*Euphasia superba*), showing elevated metal concentrations during January to mid February (Yamamoto *et al.*1987) i.e. the Austral summer. Metal concentrations measured in samples of the cumacean *Diastylis rathkei* in Kiel Bay also showed seasonal variations in metal concentrations. High concentrations of Cu, Cd, Pb, and Zn occurred during the summer months, which corresponds to the main growth period of the species (Swaileh, 1995). It was suggested that the increase of copper concentration with increasing weight observed on *D. rathkei*, was related to the increase in the concentration of haemocyanin (copper-containing respiratory pigment) with age. A similar trend in copper concentration was observed by White & Rainbow (1987) in the crustacean *Systellaspis debilis*, reporting that young organisms contained little or no haemocyanin.

Control copper concentrations for *Praunus flexuosus* found in this study ranged from 41.7µg Cu g^{-1} in winter to 50.2 µg Cu g^{-1} in summer, being within the range of total copper content found in other peracarids at uncontaminated waters. In oceanic waters, where the concentrations of dissolved trace metals are generally much lower that in coastal waters, mesopelagic species of mysids at the NE Atlantic Ocean showed values ranging from 12.6 to 44.3 µg Cu g^{-1} (Ridout *et al.* 1989). Other peracarids in coastal waters where copper background levels increase, such as cumaceans in the Western Baltic showed concentrations from 61.7 µg Cu g^{-1}, and an average of 102 ± 19.9 µg Cu g^{-1} (Swaileh & Adelung 1995). Decapods in copper contaminated areas such as the prawn *Penaeus monodon* on the Malaysian Coast showed concentrations from 32 to 99 µg Cu g^{-1} (Patimah & Dainal 1993) and from 12.8 to 159 µg Cu g^{-1} (Awaluddin *et al.* 1992). Decapods crustaceans need 26.3 µg Cu g^{-1} to meet enzymatic requirements for copper with a further 57.4 µg Cu g^{-1} (total 83.7 µg Cu g^{-1}) necessary to provide a typical decapod copper load of the respiration pigment haemocyanin (White & Rainbow 1985).

The maximum copper concentrations in body mass found in copper exposed *Praunus flexuosus* were lower than those found in amphipods and decapods. The maximum copper concentration in winter was 105.7 µg Cu g^{-1} (when exposed at 200 µg Cu l^{-1} for 10days) and in summer 108.9 µg Cu g^{-1} (when exposed to 25 µg Cu g^{-1} for 10 days). There are no

available data for summer at 75 and 200 μg Cu l^{-1} of exposure because of the low survival at those concentrations. The decapod *Palaemon elegans* when exposed to a range of dissolved copper up to 100 μg Cu l^{-1} for 28 days, regulates body copper concentrations to a constant level of 129 μg Cu g^{-1} (Rainbow & White 1989). The amphipod *Corophium volutator* showed a copper body concentration of 100 μg Cu g^{-1} when exposed to 100 μg Cu l^{-1} for 96h and 300 μg Cu g^{-1} when exposed to 1000 μg Cu l^{-1} (Bat *et al.* 1998).

Seasonal changes may occur in the availability of metals in the environment and in the physiology of the organisms. Seasonal variations in copper accumulation can be caused by a combination of parameters including growth, reproduction and moulting cycles, food supply and environmental conditions acting directly on uptake or indirectly on growth curves (Brown & Depledge 1998). Seasonal fluctuations in metal concentration on marine algae have been reported and are considered to be a result of variations in growth during the year and the requirement of metabolic energy for the uptake of metals (Lobban & Harrison 1994, Phillips 1994). When the concentration of metals in crustaceans decreases with increasing body size (indicating that a significant proportion of metals may be surface-adsorbed, since smaller specimens have high surface area to volume ratios that larger ones), metals are not under metabolic influence (White & Rainbow 1987). In this study, there was no significant difference on the relation length and weight between the winter and summer specimens exposed to copper. The increase on total copper content observed in summer could be related to metabolic factors. The pattern of metal accumulation observed in this study reflects differences on the physiology of the organisms. The increase of body copper content in *P. flexuosus* during summer may be strongly affected by the physiological state. Comparison of winter and summer controls in *Praunus flexuosus* with similar dry weight showed a higher copper content in summer than in winter and for copper exposed mysids the accumulation rate was also higher in summer than in winter. The metal uptake could be higher in summer. In summer moulting frequency metabolism and growth increased. Biochemical responses involved in metal metabolism could be different with the season. Physiological changes with season are related to growth and reproduction. Crustacean metal content can decrease at moulting and in general organisms exhibit alterations of whole-body metal level during reproduction. White & Rainbow (1984) found that moulting of crustaceans is associated with a temporary increase in concentrations of metal inside their bodies, which could be due to an increase in the permeability of the body surface prior to tanning and/or calcification of new

cuticule. Moulting cycles, particularly in decapods contribute significantly to variations in tissue metals concentrations, even in unpolluted sites (Nugegoda & Rainbow 1998).

An experimental study on Zn and Cd accumulation by the mysid *Mysis relicta* showed that little or none of the metal taken up was assimilated and that most of the metal taken up was egested (73-99%) via faecal pellets (Lasenby & Van Duyn 1992). Metal concentration in moults were 5 to 8 times higher in Cd and 4 to 13 times higher in Zn than in the whole body concentrations.

The slight decrease of copper accumulated observed in females after two weeks of recovery suggest that a small fraction of the copper accumulated was excreted and the rest was detoxified in some way as recovery of behavioural parameters were observed.

If trace metal bioaccumulation occurs, detoxification of the trace metal ion in the body of the invertebrates is always required (Rainbow 1997). Detoxification may be transitory, when the metal is passed on to another biomolecule to play an essential metabolic role, such as copper from metallothionein to haemocyanin (Brouwer *et al.*1986) or it may be long term, when the metal is passed to an insoluble deposit or granular form (Taylor & Simskiss 1984, Viarengo 1989, George 1990).

Phillips (1980) pointed out the importance of the dichotomy between adsorbed and absorbed metals and those elements attain their maximum level in the exoskeleton just prior to moulting. The results from the FIB SIMS analysis confirmed that *P. flexuosus* absorbed and accumulated copper within the cuticle.

The loss of metal at moulting depends on the uptake route. If the metal is taken up from food the loss of metal at moulting could be insignificant, but if the uptake occurred across the cuticle the metal may be accumulated mainly into the carapace. In the case of *P. flexuosus* copper accumulation may occur in internal tissues and in the carapace. The loss of the metal accumulated in the carapace at moulting may be significant, but the mysids did not survive after moulting (see Chapter 5).

6.5 SUMMARY

Microscopy analysis of the copper exposed mysids detected and located copper accumulations. Microscopy analysis gave important information that could not be provided by other methods. X-ray fluorescence mapping showed that copper was accumulated in the cuticle of body segments but not in the pleopods or the membranes between segments. The FIB/SIMS system was applied for the first time on biological material. It detected and semi-quantified copper at contamination levels below resolution levels of EDX and WDX instruments. This system located copper accumulated within the cuticle (calcified layer) of the carapace, suggesting that copper was not adsorbed onto the surface but accumulated within it.

Analysis of the total copper content of *Praunus flexuosus* showed that copper was accumulated at a higher rate in summer (August) at 20 °C than in winter at 10 °C and 5 °C (December and February). In this study it is shown that seasonal changes in the physiology of the organisms are the cause of the variations in the metal content with the season. The pattern of metal accumulation reflects differences on the physiology of the organisms. The availability of the metal was similar in both seasons but the physiological state was different. The copper concentrations in body mass and accumulation rates were higher in summer when individuals show their higher growth rate, metabolic rate and moulting frequency, which will also increase the rate of metal uptake. Biochemical mechanism involved in metal metabolism could explain the different bioaccumulation with the season.

Copper concentrations of individuals in the recovery experiment suggested that a small fraction of the copper accumulated was excreted and the rest was stored and detoxified in some way, as recovery of normal behavioural parameters was observed.

P. flexuosus copper accumulation may occur in both internal tissues and in the carapace. As copper accumulation was detected by FIB/SIMS analysis within the cuticle, the loss of the metal at moulting may be significant, but no individuals survived after moulting in copper exposure trials. It suggests that rapid copper uptake through the body surface occurred, probably at the same time as the calcium uptake was occurring during the moulting process; or mobilisation of the copper through internal tissues occurred at moulting caused lethal effects.

CHAPTER 7

METABOLISM in *P. flexuosus* AND EFFECTS OF COPPER EXPOSURE

CHAPTER 7. METABOLISM IN *Praunus flexuosus* AND EFFECTS OF COPPER EXPOSURE

7.1 INTRODUCTION

Mysids are distributed in freshwater, estuarine and marine environments. They are a highly adaptive group of crustaceans. Although their behaviour is very active, the energetic demand for swimming and position maintenance is not high. For example, *Neomysis mirabilis* in swimming consumed 1.3 times the rate of standard metabolism (Klyashtorin & Kuzmicheva 1975). *Praunus flexuosus*, as in other Mysida, do not have gills and obtain oxygen through the cuticle. Hemocyanin is the carrier of oxygen in the hemolymph and copper is required by the hemocyanin molecule. Ammonia is the principal product of excretion in mysids (Jawed 1969).

Analysis of oxygen to nitrogen atomic ratios described the metabolism of mysids as protein based (Fergusson 1973, Chin 1974, Gaudy *et al.* 1980). Their biochemical composition is very high in protein content (80-90%) with a very low content in carbohydrates (2-3%) in *Neomysis integer* (Raymont *et al.* 1964). Similar ratios have also been found on a wet weight basis for *Praunus flexuosus* biochemical composition, with protein 21.8%, lipids 3.6%, carbohydrates 0.9%, ash 1.9%, organic carbon 1.72%, and water 71.8% (Seguin 1968).

Mysid oxygen consumption has been studied by several authors, who showed that respiration is dependent on several factors, such as temperature, salinity, season, weight, age and reproductive status (Kinne 1970, 1971, Simmons & Knight 1975, Newell & Branch 1980, Burggren & Roberts 1991, Schmidt-Nilesen 1997).

Although mysids have been used extensively in toxicity studies (see Chapter 1), studies on the effects of contaminants on metabolism are not frequent. An increase in metabolic rates (respiration, excretion and O:N ratio) with increasing contaminant concentration (pesticides and hydrocarbons) have been described (Reitsema 1981, McKenney 1982, 1985, Carr *et al.* 1985, McKenney & Matthews 1990). Increasing respiration with increasing temperature and toxicant concentration were also observed in mysids by Laughlin & Linden (1983) and Smith & Hargreaves (1985). The experiments showed synergism between temperature and toxicant (naphthalene or hydrocarbons), however there is an absence of data on other biologically important variables such as seasonal effects.

The aim of this investigation was therefore to characterise seasonal variation in metabolism and study the effect of a contaminant (dissolved copper) on a natural population of mysids (*Praunus flexuosus*) in different seasons.

7.2 MATERIALS AND METHODS

Specimens collected from the field were maintained in the aquarium under conditions described in chapter 2. Metabolism was assessed under the following conditions: Natural winter, spring and summer conditions, and at different temperatures in spring. The effects of a range of copper exposures were tested on the population in winter and summer.

Oxygen consumption and ammonia excretion were measured in adults and juveniles of the population in winter, spring and summer. Effects of temperature were studied on the different life cycle stages available (brooding female, female, male, juvenile, and newborn juvenile) in the spring population. Two groups of the overwinter population collected in March, were maintained at a constant temperature (10°C) and the other at field temperatures (rising from 10°C to 20°C) as the spring progressed, to measure their metabolic rates in June.

Effects of copper on metabolism were studied in the winter (February, 5°C) and summer (August, 20°C). Respiration and excretion were measured for individuals (female, male and juvenile) exposed to copper (0,5,25,75, 200 $\mu g\ l^{-1}$ Cu_a) at 24h, 96h and 10 days of exposure.

7.2.1 Experimental protocol

Preliminary experiments were carried out to determine the period necessary for the animals to acclimate to the experimental chamber. A second series of experiments was carried out to determine the effects of feeding on the metabolic rate. Metabolic rate was measured at regular intervals during day and night time, prefeeding, and 4 and 10 hours postfeeding. The specific dynamic action (SDA), causes a rise in metabolic rate after feeding (Chapelle & Peck 1995, Peck 1998) and was confirmed for *Praunus flexuosus*. In order to avoid any overestimation of the metabolic rate of the animal, respiration and excretion measurements were performed after allowing for an acclimation period and SDA effect.

As a result of the preliminary measurements an experimental protocol was designed to obtain a representative measure of the routine metabolic rate of the mysids. Experimental specimens were fed in the holding tanks before being transferred into the respirometers, where they were incubated for 17 hours. Mysids were placed in the respirometers during an

evening and trials were allowed to run through the night to the following morning. Therefore, the SDA effect, the following decrease in metabolic rate, and day/night rates were also included. This protocol was used because it was representative of daily routine metabolism, as mysids in the field usually feed several times per day (Tattersall & Tattersall 1951, Mauchline 1980, pers.obs.).

7.2.2. Metabolism measurements: respiration and excretion

Five animals were used per experimental treatment. Oxygen consumption and ammonia excretion were measured for each individual. Mysids maintained at the different experimental conditions were transferred from the holding tanks to the respirometers in order to measure the metabolic rates. The respirometers were hollow perspex cylinders of 198 ml volume (Fig.7.1), with a septum in the lid through which small samples were taken with a syringe. The chambers were sealed with a concave lid allowing air bubbles to be expelled. The experimental chambers were maintained at a constant temperature in winter of 5±0.1 °C by submersion in a jacketed water bath connected to a thermocirculator, which is located in a temperature controlled room. Spring and summer respirometers were maintained in an incubator at 10 and 20±1 °C respectively. Photoperiods in the experiments were as in the field (see Chapter 2). Each respirometer contained one mysid and control respirometers contained no mysid. Oxygen concentrations in chambers during the experiment were not allowed to fall below 70% saturation, to avoid oxygen stress effects on measurements.

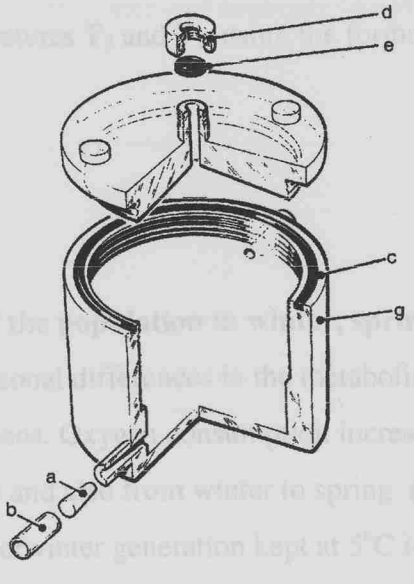


Fig. 7.1 Respirometer used in this study (a rubber bung, b silicon tubing, c rubber O-ring, d cap, e rubber septum, g perspex). (Modified figure from Chapelle & Peck 1995).

After the incubation period samples (25 µl) were taken for analysis of oxygen from the chamber through the septum in the lid of the chamber using a gas-tight syringe. The oxygen content of the seawater at experimental and control respirometers was measured using a couloximeter (Peck & Uglow 1990, Peck & Whitehouse 1992). The technique is based on a fuel cell and accuracy was estimated at ±1% (Peck & Uglow 1990). Animal movements and respiratory currents generated by the mysids kept the contents of respirometers well mixed. After oxygen was measured, 10 ml samples were taken from the same respirometers to measure ammonia content. Animals were observed for stress during experiment trials and measures were only made under conditions of low stress. Ammonia excretion was measured using the phenol-hypochlorite method of Solorzano (1969) as modified by Catalano (1987) and Clarke *et al.* (1994) for use with 10 ml seawater samples. The minimum detection limit for ammonia was ~ 0.5 µmol (Clarke *et al.* 1994). Respiration and excretion rates were obtained from differences between experimental and control respirometers. Rates were calculated as µg of oxygen and µM of ammonia per hour per mg. dry weight of mysid. At the end of the experiments each mysid was removed from the chamber after the final measurements and rinsed briefly in Milli Q water to remove salt before placing in an oven at 60°C to dry for 24 hours. Dried specimens were transported in a desiccator and weighed to the nearest 0.001mg using a Cahn Electrobalance.

O:N ratios was calculated as the atomic ratio of oxygen consumed to nitrogen excreted for every individual in each of the different experimental conditions. Temperature effects were evaluated using the Q_{10} coefficient for specific physiological rates (V_2 and V_1) at the respective temperatures T_2 and T_1, using the formula $Q_{10} = (V_2 / V_1)^{10 / T_2 - T_1}$

7.3 RESULTS

7.3.1 Metabolism of the population in winter, spring and summer.

There are seasonal differences in the metabolism of mature and immature individuals under natural conditions. Oxygen consumption increases significantly from winter to summer ($t = -5.72$, $p<0.0001$) and also from winter to spring ($t = 4.47$, $p=0.0003$) (Fig. 7.2A). Individuals of the overwinter generation kept at 5°C in (February) showed the minimum rates of oxygen consumption (0.97-1.82 µg $O_2 \cdot$ mg.drywt$^{-1} \cdot h^{-1}$).

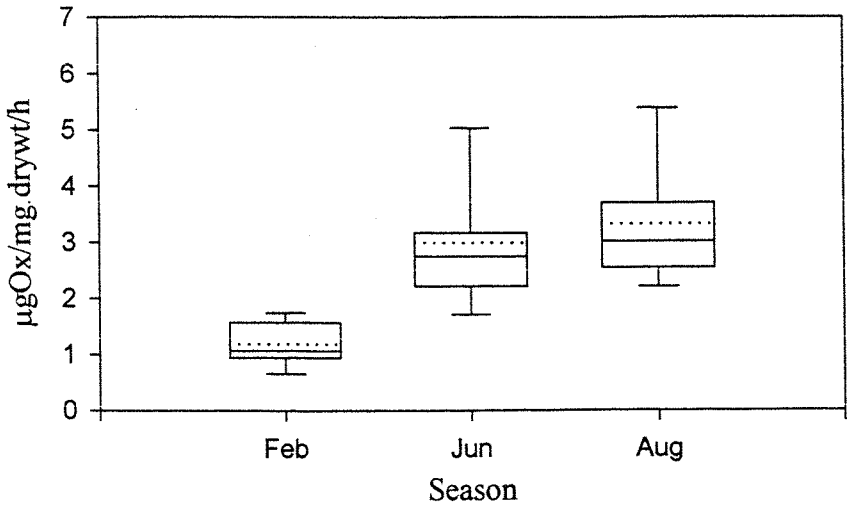


Fig. 7.2A Respiration rates of the population in winter (Feb), spring (June) and summer (Aug).

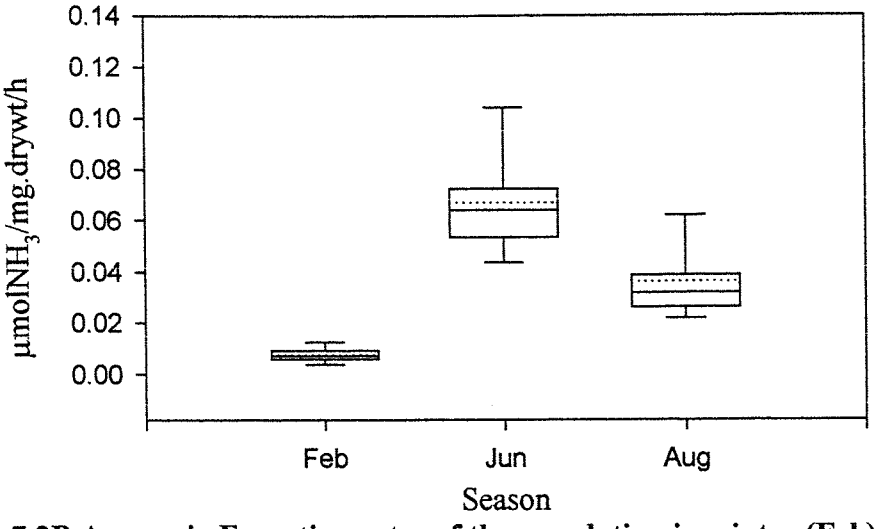


Fig. 7.2B Ammonia Excretion rates of the population in winter (Feb), spring (Jun) and summer (Aug)

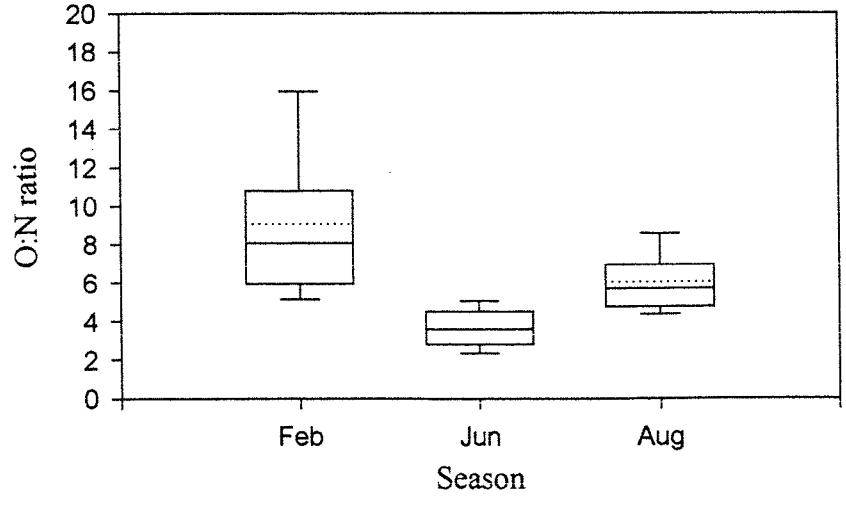


Fig. 7.2C O:N ratio in the winter (Feb), spring (Jun) and summer (Aug)

The respiration rate increased in the spring generation (June) at 20°C to 3µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$ and the maximum (5.6µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$) was observed in the summer (August) at 20°C.

Ammonia excretion rates also differed significantly between seasons (Fig. 7.2B), with bigger differences between winter and spring ($t = -9.90$, $p<0.0001$), and summer ($t = -3.54$, $p<0.0001$) than between spring and summer ($t = -3.14$, $p=0.005$). The minimum rates of ammonia excretion were in winter (0.01µM NH_3 · mg.drywt $^{-1}$ · h $^{-1}$) and the maximum (0.08µM NH_3 · mg.drywt $^{-1}$ · h $^{-1}$) in spring (Fig. 7.2B). Summer ammonia rates (0.04µM NH_3 · mg.drywt $^{-1}$ · h $^{-1}$) were around half the spring rates.

The minimum O:N ratio (3.7) was in spring, increasing in the summer (6.3) to reach the maximum (9.2) in winter (Fig.7.2C). O:N ratio differences of the population between winter, spring and summer were significant ($H=15.9$, $p=0.003$).

Metabolic rates were also measured for the different life cycle stages in spring. Respiration rates in immature individuals (6µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$) are higher than in mature individuals (2.5µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$). Mass specific oxygen consumption decreases with age. For the spring population, respiration rates (Fig.7.3) are significantly different ($H=20.9$, $p=0.0003$) between the life cycle stages.

Respiration rates of the population maintained at a constant temperature (10°C) from March to June were significantly lower from those held at field temperatures (20°C) in June (Fig. 7.3), in males ($t = -3.97$, $p= 0.004$) and females ($t = -3.51$, $p= 0.008$). For juveniles and brooding females the 20 °C metabolic rates were higher than at 10 °C, but differences were not significant ($t=0.863$, $p=0.411$ and $T=33$, $p=0.310$) (Fig.7.3). In contrast, for newly released (newborn) juveniles, oxygen consumption was highest at the lower temperature. It may indicate greater activity or temperature stress for the newborn at 10 °C. At both temperatures juveniles had higher mass specific metabolic rates than adults.

Ammonia excretion rates varied with temperature and life cycle stages. At 20 °C excretion decreased significantly ($H=18$, $p=0.0012$) from 0.15 to 0.035 µM NH_3 · mg.drywt $^{-1}$ · h $^{-1}$ with the life cycle stage, from newborn juveniles to brooding females (Fig. 7.4). At 10 °C the decreasing pattern of excretion was less clear, with variations across the life cycle stages and the decrease only occurred in males and newborn juveniles (Fig. 7.4). Interestingly ammonia excretion did not appear to differ with temperature for brooding or non brooding females ($t=0.829$, $p=0.4$) and newborn juveniles ($t= -2.06$, $p=0.078$), but it was significantly lower at 10 °C for juveniles ($t =5.59$, $p =0.0005$) and for males ($t = -6.58$, $p=0.0002$)

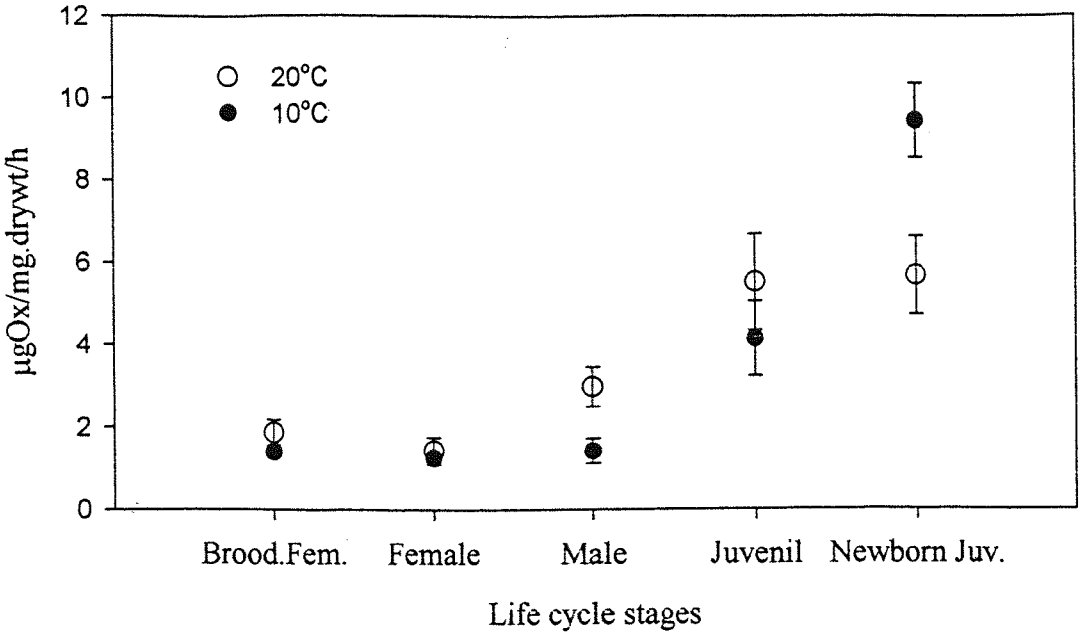


Fig. 7.3. Respiration rates (mean, SE) for the different life cycle stages of the population in June at 10°C and 20°C

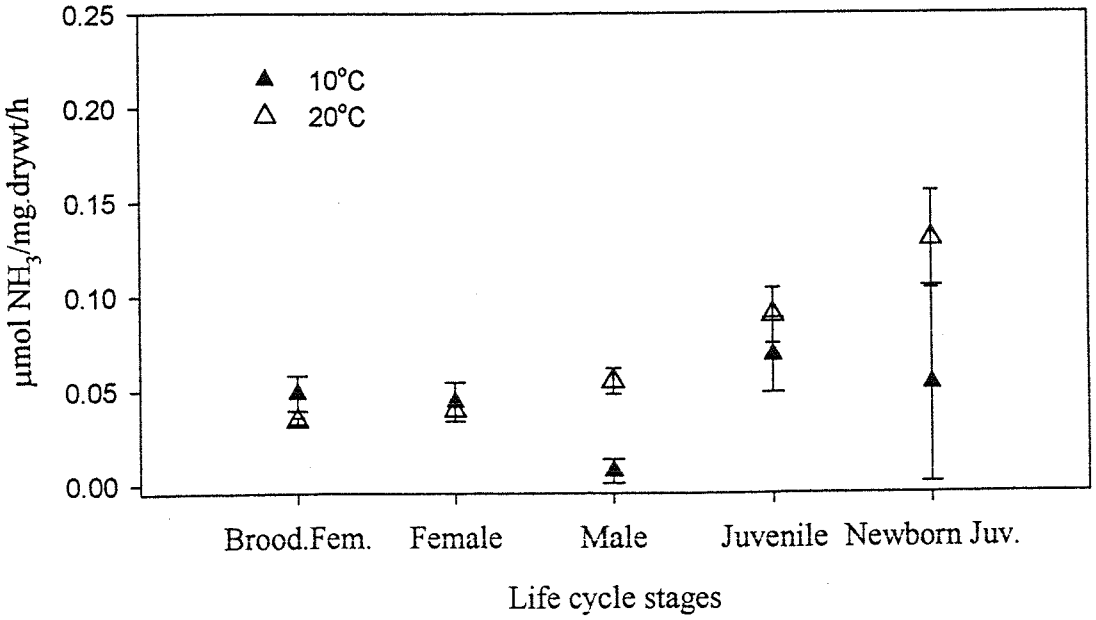


Fig. 7.4. Ammonia Excretion rates (mean,SE) for the different life cycle stages of the population in June at 10°C and 20°C

The O:N ratio of the population maintained at a constant temperature (10 °C) was lower than the one of the population maintained at the field temperature, except for males and newborn juveniles which increased significantly (T=30, p= 0.015 and T=30, p =0.01) (Fig. 7.5). The strong increase in O:N ratio for males at 10 °C probably indicates a significant metabolic use of stored lipids at low temperatures.

The Q_{10} coefficient for respiration of the population at 5 and 20 °C (winter and summer) was 1.75 and for ammonia excretion was 1.95. These effects are also dependent on other seasonal factors. The Q_{10} coefficient calculated from the respiration and ammonia rates of the population at the same season (in June), but maintained at two different temperatures (10 and 20 °C) are minimal (ranged from 0.03 to 1.4) for brooding females, females and juveniles, which indicates a small dependence on temperature. Only newborn juvenile excretion (2.45) and male respiration and excretion (2.18 and 7.7), had values above 2.

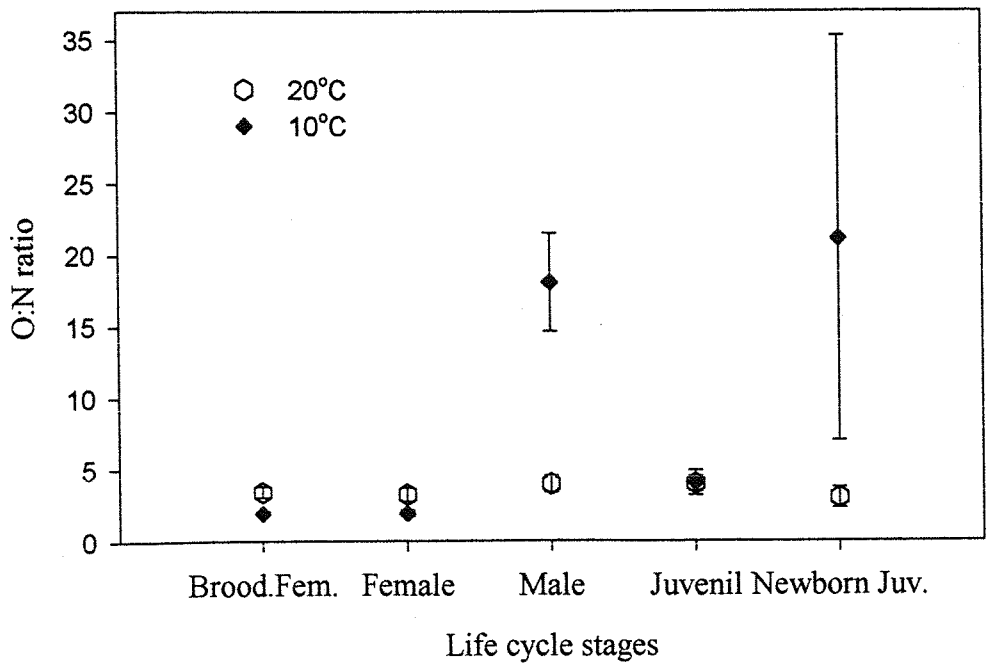


Fig. 7.5. Atomic O:N ratio (mean,SE) for the different life cycle stages of the population in June at 10°C and 20°C.

7.3.2 Metabolic responses to copper

7.3.2.1 Oxygen Consumption

7.3.2.1.1 Winter conditions

Respiration rates of individuals of the population in winter exposed to copper show a clear response with increasing copper concentration and time of exposure. Winter respiration rates after 24 hour of copper exposure (Fig. 7.6A) are significantly different from controls at 75 and 200µg $l^{-1}Cu_a$ ($F=5.81$, $p=0.0028$). At 25µg $l^{-1}Cu_a$ respiration increased from 1.2 to 1.5 µg $O_2 \cdot mg.drywt^{-1} \cdot h^{-1}$ followed by a decrease to 0.5µg $O_2 \cdot mg.drywt^{-1} \cdot h^{-1}$ at higher copper concentrations. Respiration rates after 96 hours of exposure increased from the 24h values at 75 and 200 µg $l^{-1}Cu_a$ (Fig. 7.6B). Individuals in the process of moulting showed an increase of oxygen consumption by 2 to 3 times the standard (no moulting) rate (Fig.7.6C). This reflects the increased mass specific metabolism during moulting combined with the copper effects. Values for moulting animals are excluded from the analysis of respiration responses to copper. Respiration rates decreased significantly ($H=14.7$, $p=0.005$) with copper exposure after 10 days, being significantly different from control ($p<0.05$), at concentrations of 25µg $l^{-1}Cu_a$ and above (Fig. 7.6C). Mortality rates were low or zero in winter trials.

7.3.2.1.2. Summer conditions

During summer respiration responses to copper resulted in a decrease from 3.5 to 1.8 µg $O_2 \cdot mg.drywt^{-1} \cdot h^{-1}$ at concentrations of 25µg $l^{-1}Cu_a$ and above, and at 24 hours of exposure (Fig. 7.7A). At 5µg $l^{-1}Cu_a$ respiration increased in 24 hours in a similar fashion to the increase recorded at 25µg $l^{-1}Cu_a$ in winter. Copper caused a high mortality in summer and at the higher copper levels (75 and 200 µg $l^{-1}Cu_a$) the respiration rates recorded were representative only of the surviving individuals of the population (Fig. 7.7). Moribund individuals show a significantly lower respiration. These data were excluded from the analysis of respiration responses to copper. Respiration rates at 96 hours were not significantly different from controls ($F=0.388$, $p=0.814$), except at 200µg $l^{-1}Cu_a$ (Fig. 7.7B). Respiration decreased with copper concentration after 10 days of exposure to the lowest levels recorded (1.4 µg $O_2 \cdot mg.drywt^{-1} \cdot h^{-1}$ at 25 and 75µg $l^{-1}Cu_a$) (Fig. 7.7C). However, values shown at 75 and 200µg $l^{-1}Cu_a$ are only representative of the few survivors (7 and 10% of the initial population). These values are, therefore, clearly not representative of the population as a whole. Moulting individuals show an increased respiration rate in all

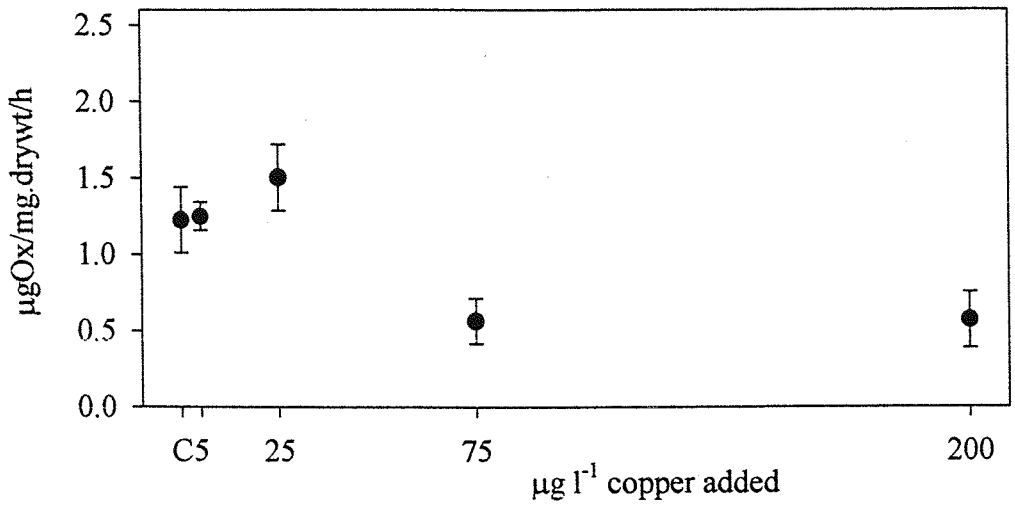


Fig. 7.6A. Winter Oxygen Consumption (mean,SE) after 24h of copper exposure

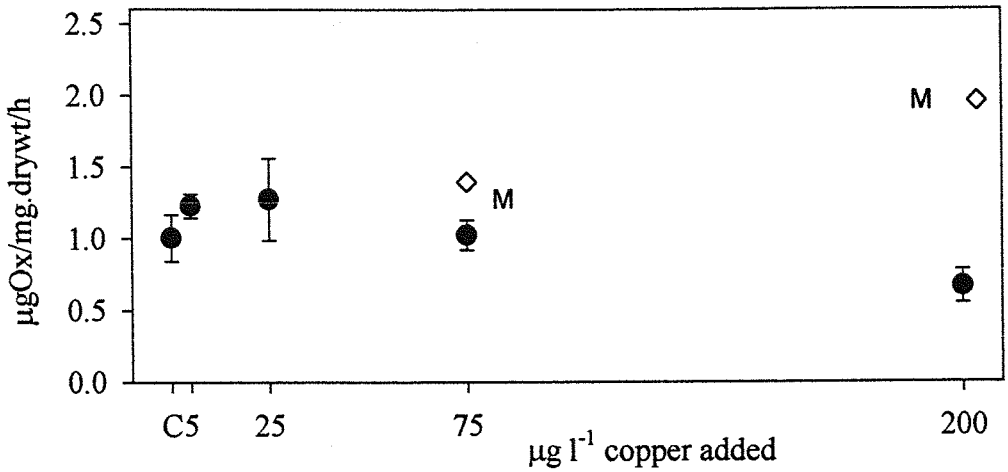


Fig. 7.6B. Winter Oxygen Consumption (mean,SE) after 96h of copper exposure

● Respiration rates (mean,SE) excluding moulting animals

◇ Respiration rate of moulting animals

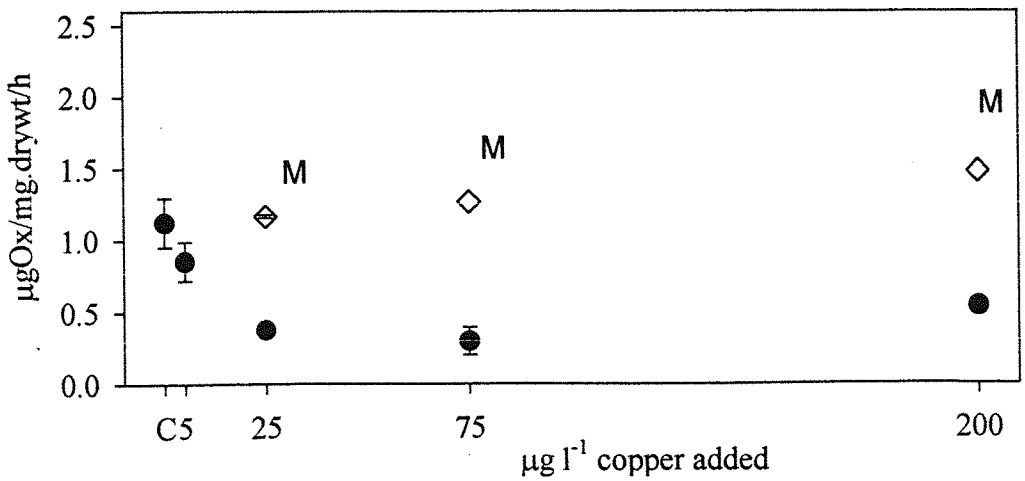


Fig. 7.6C. Winter Oxygen Consumption (mean,SE) after 10 days of exposure to copper

● Respiration rates (mean,SE) excluding moulting animals

◇ Respiration rates of moulting animals

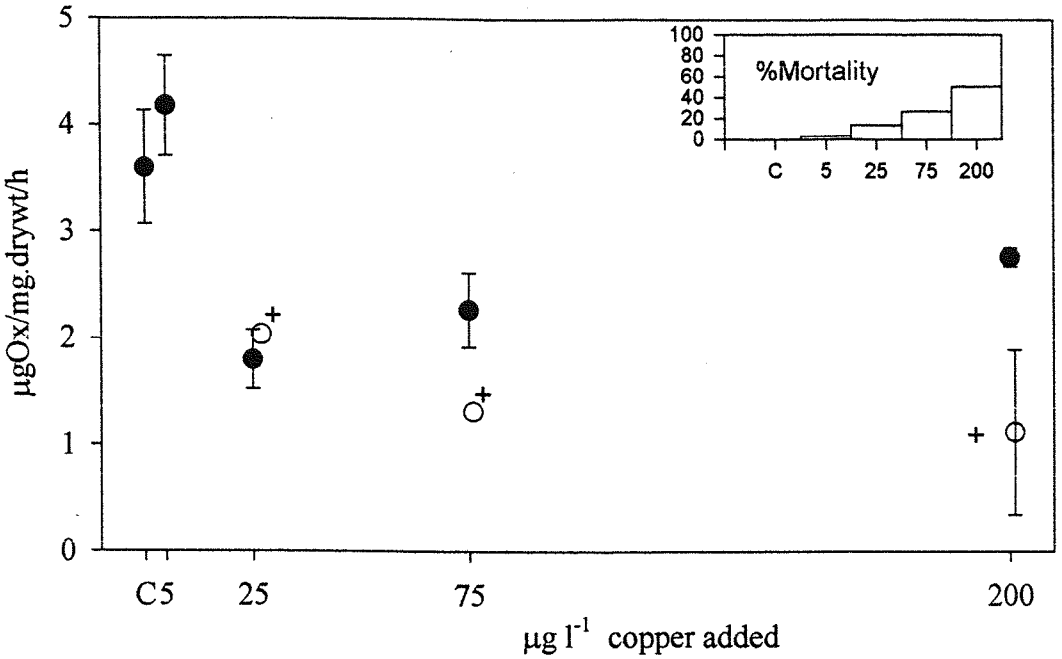


Fig.7.7A. Summer Oxygen Consumption after 24h of copper exposure

● Respiration rates (mean,SE) excluding moribund animals
○ + Respiration rates of moribund animals

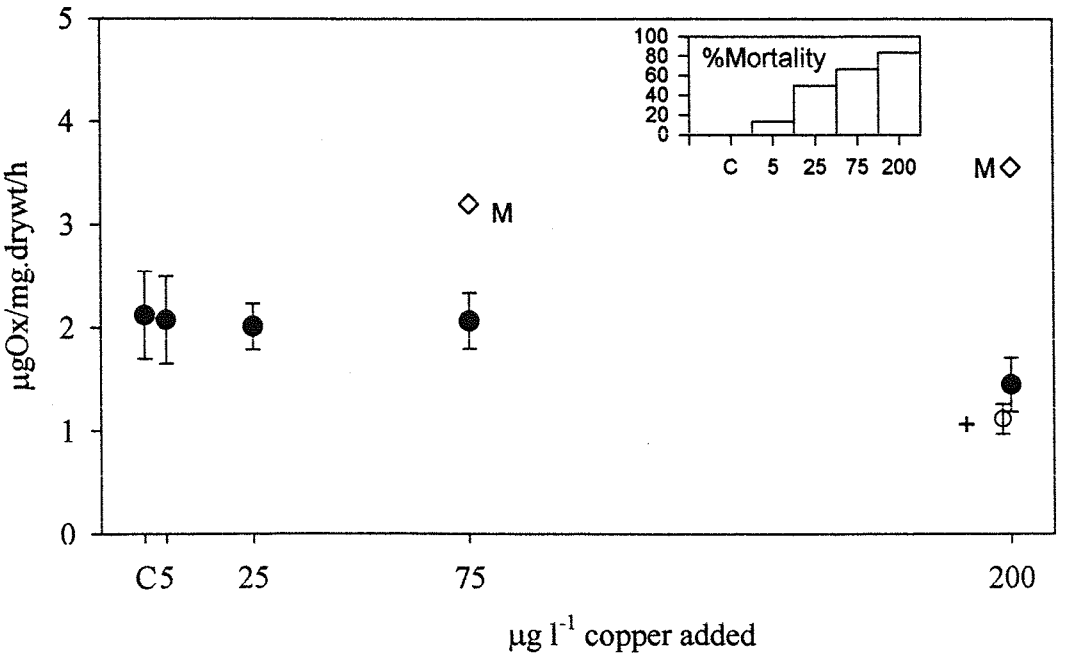


Fig.7.7B. Summer Oxygen Consumption after 96h of copper exposure

● Respiration rates (mean,SE) excluding moulting and moribund
◇ M Respiration rates of moulting animals
○ + Respiration rates of moribund animals

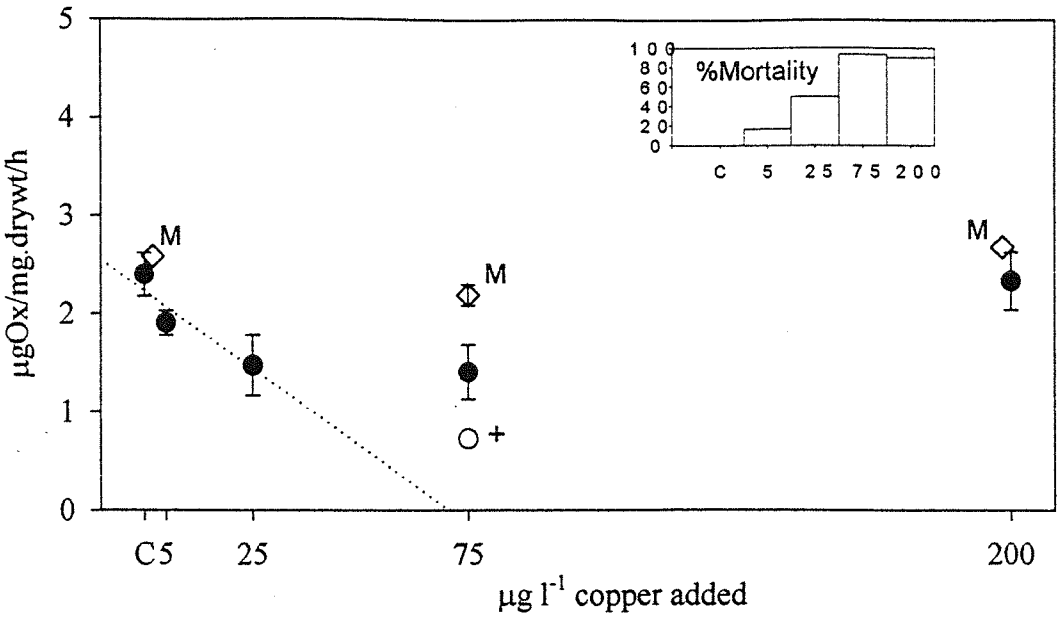


Fig. 7.7C. Summer Oxygen Consumption after 10days of copper exposure
Values at 75 and 200 Cu_a were representative of few survivors of the population, which were excluded from the regression line.

● Respiration rates (Mean,SE) excluding moulting
◇ M Respiration rates of moulting animals
○ + Respiration rates of moribund animals
····· Regr, y=-4.62x+2.85, r^2=0.41 (95%CI)

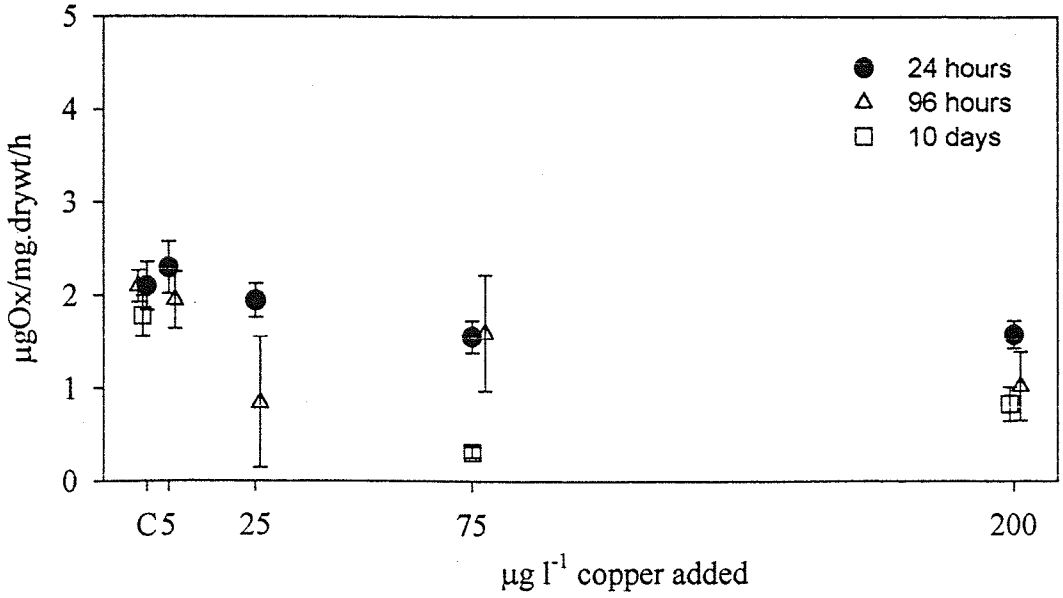


Fig. 7.8. Brooding females Oxygen Consumption in summer after 24h., 96h. and 10 days of copper exposure.

treatments. An additional stress with copper occurs at moulting. Individuals died after moulting in all the copper exposure trials in the summer.

Respiration rates of the brooding females exposed to copper were lower than in controls. Oxygen consumption of brooding females decreased from 2 to 0.7 $\mu g\ O_2 \cdot mg.drywt^{-1} \cdot h^{-1}$ or less with increasing copper concentration at 25$\mu g\ l^{-1}Cu_a$ and above, after 96 hours of exposure (Fig.7.8) . Brooding females were not available for comparison after 10days of copper exposure at 5 and 25 $\mu g\ l^{-1}Cu_a$.

7.3.2.2 Ammonia excretion

Ammonia excretion responses to copper are different in winter and summer (Fig.7.9). Ammonia excretion increased with copper concentration and exposure time in both seasons. However, in summer the increase in excretion with respect to controls was significant ($F=13.9$, $p=0.0008$) from the lowest concentration (5$\mu g\ l^{-1}Cu_a$) at 10 days of exposure, whereas in winter the increase was less pronounced (Fig.7.9). In winter ammonia excretion responses to copper varied significantly between treatments ($F=17$, $p<0.0001$), but only the 200$\mu g\ l^{-1}Cu_a$ treatment was significantly above ($p<0.05$) control values. Winter excretion responses with copper (Fig. 7.9A) ranged from 0.006 in controls to 0.022 $\mu M\ NH_3 \cdot mg.drywt^{-1} \cdot h^{-1}$ (at 200 $\mu g\ l^{-1}Cu_a$ and 10 days of exposure) and in summer from 0.025 or 0.038 in controls to 0.11 $\mu M\ NH_3 \cdot mg.drywt^{-1} \cdot h^{-1}$ (at 25 $\mu g\ l^{-1}Cu_a$ and 10 days of exposure) (Fig.7.9B). Summer excretion rates were higher than in winter in all treatments. Excretion rates at the higher copper levels in summer were only representative of the small percentage of survivors (7 and 10% of the population).

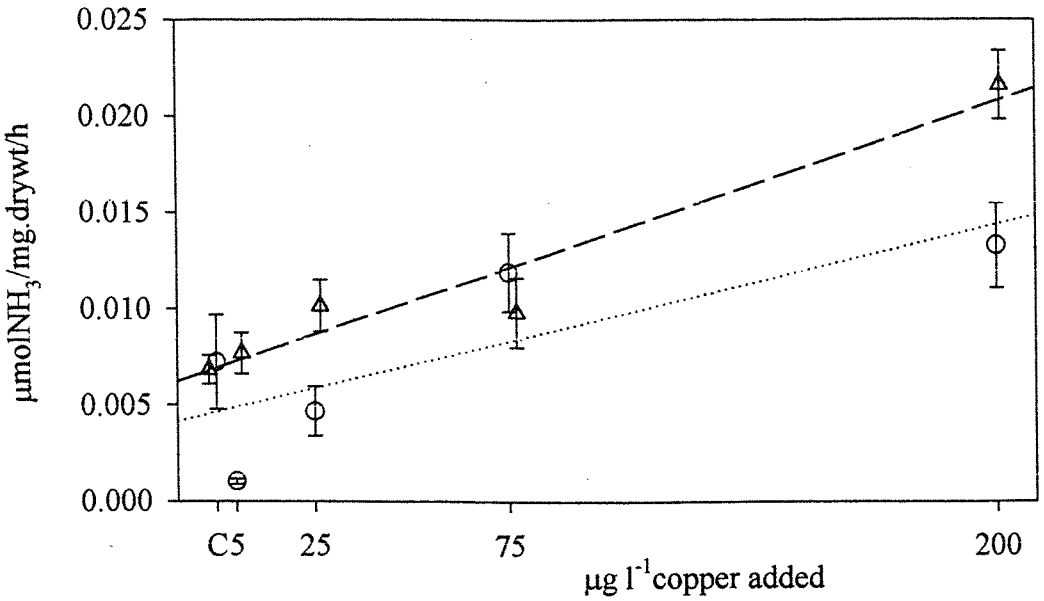


Fig. 7.9A. Ammonia Excretion rates (mean,SE) on the population in winter after 24h and 10d of copper exposure.
There was no mortality in winter in these trials.

○ 24 hours
····· 24h Regr
y=0.0047x+0.00005, r^2=0.39
△ 10days
— — 10days Regr
y=0.0069x+0.00007, r^2=0.74

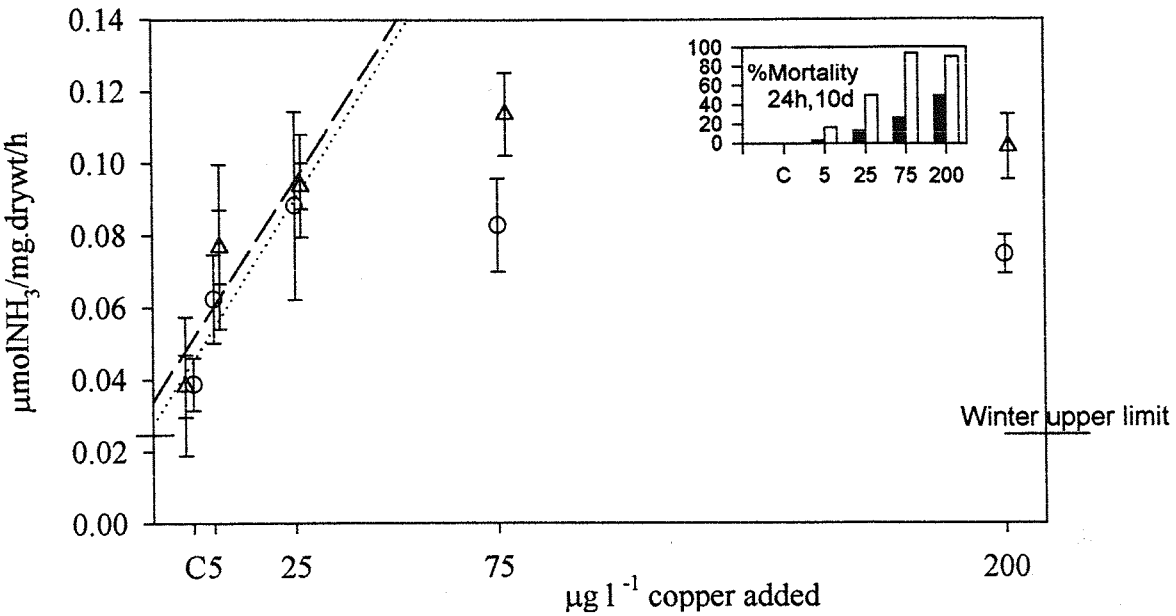


Fig. 7.9B. Ammonia Excretion rates (mean,SE) of the population in summer after 24h and 10d of copper exposure.
Values at 75 and 200 Cu_a are representative only of the few surviving individuals.

○ 24 hours
△ 10days
○
····· 24h Regr (95%CI)
y=0.045x+0.0018, r^2=0.23
△
— — 10days Regr (95%CI)
y=0.051x+0.00175, r^2=0.53

7.3.2.3 O:N ratio

O:N ratios of the population exposed to copper in winter show a significant decrease ($H=17.6$, $p=0.0014$) from 11 to 2 with increasing concentration (Fig. 7.10). At 24h of copper exposure a decrease occurred at the highest copper concentrations. After 10 days of copper exposure the decrease became evident at all concentrations.

High O:N ratios measured in winter at 24 hours and low copper concentrations may reflect the initial stress caused by copper, evidenced by a strong decrease in metabolism through prolonged exposure (Fig. 7.6C, 7.10).

The population response in summer to copper is faster (Fig.7.11) than in winter. The decrease in O:N ratio with increasing copper exposure in summer was significant ($F=13.3$, $p<0.0001$) for all concentrations and time of exposure. The reduction of O:N ratio at 24 hours from 7 to 1.8 observed in summer, was followed by significant mortality at 96 hours and 10 days of exposure.

A decrease in lipid and carbohydrate metabolism with copper exposure occurred in both seasons as shown by a strong reduction of the O:N ratio (Fig.7.12). O:N ratios were higher in winter than in summer in controls and copper treatments. The reduction of O:N ratios to extremely low levels in both winter and summer, reflects the powerful suppression of metabolic rate after 10 days of copper exposure. O:N ratios in summer and in winter were decreased by copper to levels below the minimum of the normal population metabolism.

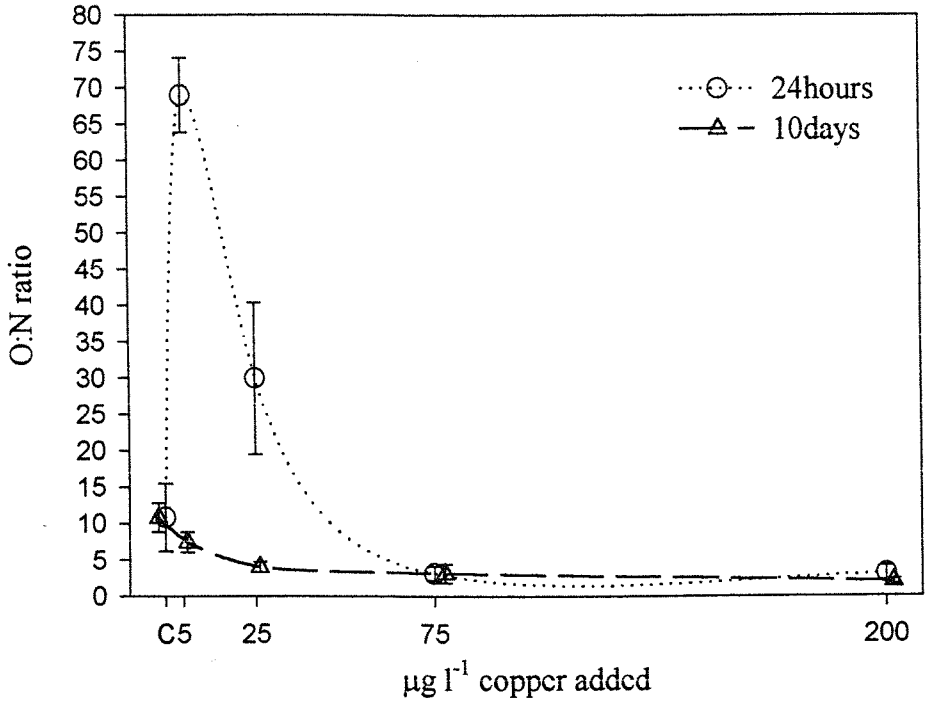


Fig. 7.10. O:N ratio (mean,SE) of the population in winter after 24h and 10 days of copper exposure

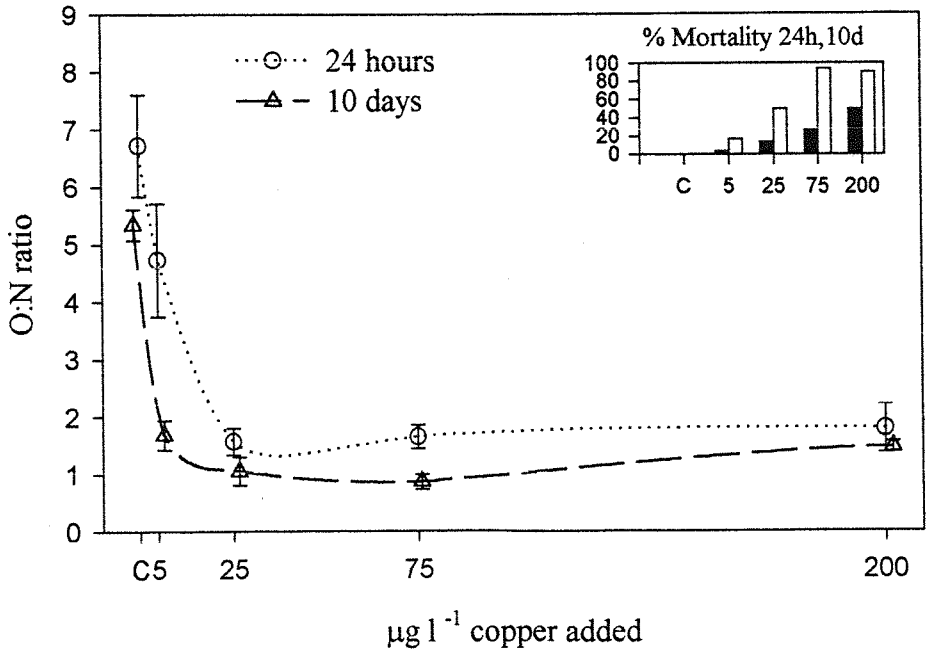


Fig. 7.11. O:N ratio (mean,SE) of the population in summer after 24h and 10d of copper exposure

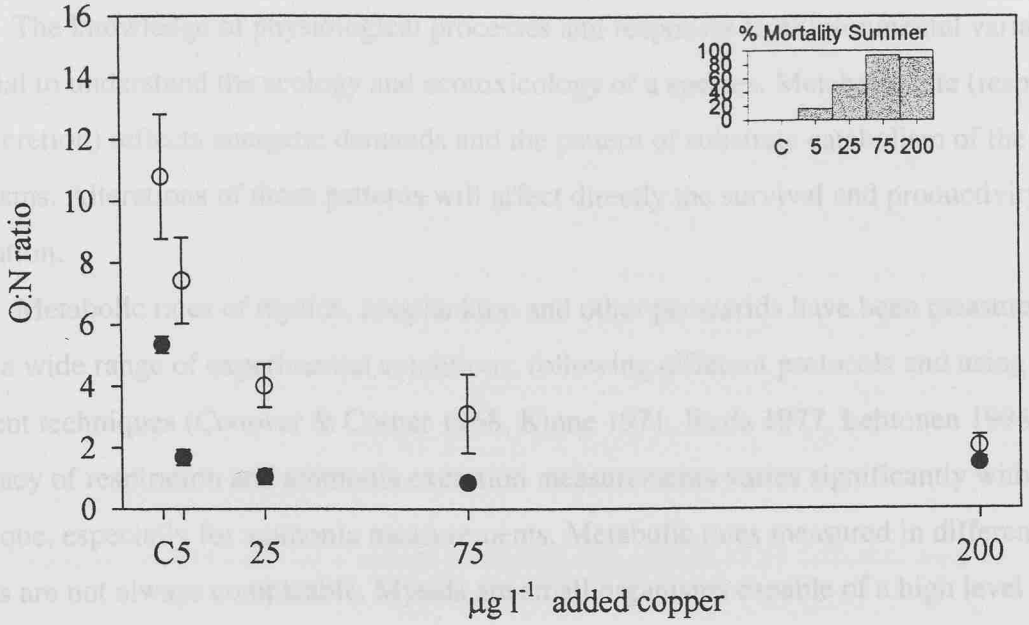


Fig. 7.12. O:N ratio (Mean, SE) in winter and summer after 10days of copper exposure

○ Winter - 10days exposure
● Summer - 10days of exposure

7.4 DISCUSSION

7.4.1 Routine metabolism

The knowledge of physiological processes and responses to environmental variables is essential to understand the ecology and ecotoxicology of a species. Metabolic rate (respiration and excretion) reflects energetic demands and the pattern of substrate catabolism of the organisms. Alterations of those patterns will affect directly the survival and productivity of a population.

Metabolic rates of mysids, zooplankton and other peracarids have been measured under a wide range of experimental conditions, following different protocols and using different techniques (Conover & Corner 1968, Kinne 1971, Ikeda 1977, Lehtonen 1994). Accuracy of respiration and ammonia excretion measurements varies significantly with technique, especially for ammonia measurements. Metabolic rates measured in different studies are not always comparable. Mysids are small organisms capable of a high level of activity. Oxygen and ammonia levels to be measured are low and thus high resolution measurement techniques are required. Density of the organisms in experiments can also have a strong effect. Grouping mysids in the same chamber or respirometer is not an accurate way to measure metabolic rates because of interactions between individuals which may affect the measurement of metabolism itself. In mysids these interactions can include cannibalism (Tattersall & Tattersall 1951, Mauchline 1980, pers. obs.). In this study metabolic rates were measured on individuals. Preliminary experiments were performed in order to design an experimental protocol to measure representative values of routine metabolism. Several factors, such as the post-feeding rise in metabolism (SDA) and duration of incubation, are the cause of significant variations in metabolic rate measurements. Other factors have also been shown to be important including diet and starvation (Mayzaud 1976, Seale & Boraas 1982, Lehtonen 1994, Chapelle & Peck 1995, Peck 1998), duration of incubation (Le Borgne 1979), pH and ammonia in seawater, and physiological state of the animals (Conover & Corner 1968, Campbell 1973, Jawed 1973, Seale & Boraas 1982).

Praunus flexuosus populations in the West Solent show different metabolic rates between seasons. Respiration rates were 2-3 times lower in winter at 5°C (0.97 -1.82 $\mu g\ O_2 \cdot mg.drywt^{-1} \cdot h^{-1}$) than in summer at 20°C (1.9 - 5.6 $\mu g\ O_2 \cdot mg.drywt^{-1} \cdot h^{-1}$). Respiration rates measured on other species of mysids at 5°C were similar to those measured for *Praunus*

flexuosus. *Mysis relicta* consumed 1.2-1.3 µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$ (Lasenby & Langford 1972) and 0.91µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$ (Sadenman & Lasenby 1980).

Seasonal changes in respiration rates were demonstrated in *Neomysis integer* with minimum levels in autumn followed by monotonically increasing levels to March (Raymont *et al.* 1966). The lipid content was found to decrease through the summer in *Neomysis integer* (Morris 1971). Respiratory responses of *Neomysis mercedis* to temperature were found to be influenced by season (Simmons & Knight 1975). In the present study, respiration rates in *Praunus flexuosus* population decreased with age. Newborn juveniles in June consumed 10µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$ whereas for adults it was 1.7µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$. Similarly for *Mysidopsis bahia* at 25°C mass specific respiration rates decreased with age, from 9.1µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$ for newborn juveniles to 5 µg O_2 · mg.drywt $^{-1}$ · h $^{-1}$ for juveniles 4 day old (McKenney & Matthews 1990).

Ammonia excretion for *Praunus flexuosus* was significantly different with season, being minimum in winter (0.01µM NH_3 · mg.drywt $^{-1}$ · h^{-1}) and maximum in spring (0.08 µM NH_3 · mg.drywt $^{-1}$ · h^{-1}). Summer ammonia excretion rates (0.04µM NH_3 · mg.drywt $^{-1}$ · h^{-1}) were intermediate. The maximum ammonia excretion was in June and was significantly higher than in August. Seasonal variations in respiration and nitrogen excretion recorded for 4 species of copepods, showed high rates in spring, which decreased gradually through summer to a minimum in winter (Conover & Corner 1968). However, the O:N ratios were different for each species, but not different between season, and only slightly different between life cycle stages.

O:N ratios measured in this study for *Praunus flexuosus* varied significantly with season with a minimum in June (3.7), increasing over summer (6.3) and reaching a maximum in winter (9.2). O:N ratios showed that *Praunus flexuosus* has a protein based metabolism all year and that lipid metabolism although only at low levels, is most active in winter. There was little or no fueling of metabolism by lipid in spring. Low O:N ratios indicated a high use of protein. The rates found for *P. flexuosus* were all low and close to the minimum possible (~ 3) (Mayzaud & Conover 1988), which indicates a sole use of protein to fuel metabolism. Even the highest value recorded here are well below the range 25-30, which indicates that protein metabolism support 50% of the metabolism costs (Ikeda 1974). Respiration and excretion increased with temperature and decreased with age, but the O:N ratio of different life cycle stages of the population in June at 20°C did not show significant differences.

The temperature dependence of respiration (Q_{10}=1.76) and ammonia (Q_{10}=1.95) is moderate, and typical of values for mysids. Toda *et al.* (1987) found Q_{10} of 1.9-2.1 for respiration in *Neomysis intermedia*. For *Mysis relicta* Q_{10} ranged from 1.9 to 2.3 (Lasenby & Langford 1972). Rudstam (1989) reported Q_{10}= 2.1. For other mysid species Q_{10} ranged from 1.6-2.5 (Clutter & Theilacker 1971, Mauchline 1980, Toda *et al.* 1987, Weisse & Rudstam 1989, Roast *et al.* 1999). However, the Q_{10} values found in this study may not be solely a temperature effect, as other physiological factors vary seasonally (e.g. feeding, growth, reproductive activity). There is evidence that metabolism in *P. flexuosus* is relatively independent of temperature. The Q_{10} for respiration in the spring population held at 10 and 20 °C was 1.36 showing a minimal effect of temperature.

Another factor showing seasonal effects on metabolism was that when spring and summer metabolic rates were measured at the same temperature, respiration rates in summer were higher than in spring and excretion was higher in spring than in summer. O:N ratios were higher in spring than in summer, indicating a slightly higher use of lipid and/or carbohydrate.

7.4.2 Copper toxicity effects

Copper effects on the metabolism of *Praunus flexuosus* resulted in decreased respiration and increased excretion with increasing copper concentration in winter and summer. Consequently, O:N ratios decreased with copper, and metabolism shifted to an ever greater reliance on protein catabolism with copper exposure. Responses were faster in summer, when *P. flexuosus* metabolism is already based predominantly on protein catabolism.

The metabolic responses to copper observed in this study are different to other toxicity studies. Exposure to pesticides or oil resulted on an increase in oxygen consumption, reduction of ammonia excretion and increase of O:N ratio in *Mysidopsis bahia* (McKenney 1985, McKenney & Matthews 1990). O:N ratios increased from 15 in controls to 48 after pesticide exposure and from 25 in controls to 51 after oil exposure. It was suggested that pesticides and oil increased energy costs, which raised the utilisation of metabolic energy reserves and shifted metabolism to a greater catabolism of lipids.

In *Mysidopsis bahia* exposure to cadmium resulted in an increase of O:N ratio at low exposure concentrations (4 $\mu g\ l^{-1}$) and a decrease at higher concentrations (64 $\mu g\ l^{-1}$) after 4 days of exposure (Carr *et al.* 1985). After 10 days an increase of O:N ratio from 15 in control

to 70 at the highest cadmium concentration was recorded, and after 18 days O:N ratios were reduced considerably from 45 in control to 15.

The observed effects of copper on *Praunus flexuosus* in this study suggest that copper may interfere differently with different physiological processes, such as calcium metabolism, ionic regulation, respiration, excretion or enzymatic processes that may be specially critical during the moulting cycle. In trials of copper exposure mortality was highest during moulting. Metabolic costs of moulting animals were also greatly enhanced. This suggests that in *P. flexuosus* copper may interfere with moulting.

A series of morphological malformations in *Mysidopsis bahia* exposed to cadmium suggested that the metal interfered with the calcification process of the new molt (Gentile *et al.* 1982). Metal competition with calcium and with zinc in metallo-enzyme glutamate dehydrogenase may occur, affecting enzymatic processes involved in moulting and osmoregulation of cell volume (Hochachka & Somero 1973). It may be possible that copper contamination of the external environment and subsequent increase in metal uptake interferes with calcification and osmoregulation processes during moulting in *Praunus flexuosus*, being a critical phase for toxic effects.

Brooding females survived longer than non-brooding females, males, and juveniles when exposed to copper. The longer survival may be related to different rates of moulting. Brooding females will not moult until the brood is released, whereas in non-brooding females and males moulting is more regular and for juveniles moulting occurs at the highest frequency, especially in summer. In this respect the observation that mortality was highest in juveniles (see chapter 5) is important. The metabolic rates measured in moulting individuals were higher than in non moulting individuals in both controls and copper exposed conditions. Alterations in metabolism were recorded during the moulting process in the isopod *Idotea balthica*, as an increase of 2 to 3 times of the standard rates in oxygen consumption (Bulnheim 1974).

At moulting the concentration of circulating hemocyanin, the copper-containing respiratory pigment in crustaceans, decreases dramatically (Hagerman 1983). It has been observed that degradation of hemocyanin releases significant amounts of copper into the cytosolic metal pools (Engel 1987). Metal metabolism changes were recorded during moulting. Changes in the metals bound to metallocyacin (MT) occurred over a period of 90 min. after ecdysis in the blue crab. Data suggested that copper is stripped from hemocyanin in the digestive gland after ecdysis, displacing zinc from MT in the cytosolic pool (Engel &

Brouwer 1991). Ionic regulation is crucial between the water and the organism and between cell and haemolymph, and calcium is taken up by the blood from tissues during moulting. As for calcium, copper uptake from the seawater is greater during the moulting cycle.

There are many physiological and biochemical processes involved in metal metabolism and regulation, that are critical to the impact of metal contamination on the organism, and most of these are as yet undefined. As seasonal differences occur in those processes, copper toxicity may be affected. Seasonal differences are recorded in enzyme activity (Collier *et al.* 1995), including enzymes involved in the metabolism and excretion of xenobiotics (Rotchell *et al.* 1999). At the present there is lack of information on biochemical responses to copper for mysids.

O:N alterations with copper exposure observed in this study in winter and in the early days of copper exposure in summer were a rapid indicator of sublethal stress, which later resulted in a high mortality. Alterations of O:N ratio are a very sensitive and early indicator of stress and could possibly be used as a tool for screening for metal pollutant effects.

7.5 SUMMARY

The study of the metabolism of the mysid population of *P. flexuosus* shows different metabolic rates between seasons. Respiration and ammonia excretion rates are lower in winter than in summer. O:N ratios also varied significantly with season, being minimal in spring increasing in summer and maximal in winter. The metabolism is predominantly based on protein catabolism. The Q_{10} found in this study showed that the temperature dependency of respiration and ammonia is low to moderate, and there was evidence that metabolism in *P. flexuosus* is relatively independent of temperature. Therefore, variations observed in metabolic rates between seasons are not only dependent on temperature.

The metabolic responses to copper observed in this study are different to other mysid toxicity studies. Copper effects on the metabolism resulted in decreased respiration, increased excretion and decreased O:N ratio with increasing copper concentration, in both winter and summer. Consequently, metabolism was shifted to an even greater reliance on protein catabolism by copper exposure. The metabolic responses to copper are faster in summer than in winter. The effects of copper observed in *P. flexuosus* suggest that copper may interfere with different physiological processes such as calcium metabolism, ionic regulation, respiration, excretion or enzymatic processes that may be specially critical during moulting and other physiological functions occurring with increased activity in summer.

Alterations of O:N ratio found in this study show it to be a very sensitive and early indicator of stress and sublethal toxicity, that resulted in lethal effects with prolonged exposure.

CHAPTER 8

REPRODUCTIVE BIOLOGY of *P. flexuosus* AND EFFECTS OF COPPER EXPOSURE

CHAPTER 8. REPRODUCTIVE BIOLOGY OF *Praunus flexuosus* AND EFFECTS OF COPPER EXPOSURE

8.1 INTRODUCTION

Growth and reproductive success affect abundance, age structure and the survival within a population. Alterations in reproductive processes may be a suitable criteria for detecting ecological damage, and disruptions in structure of functional communities in the ecosystem.

Reproductive processes are adaptive to environmental conditions, and therefore may vary with species and geographic location. Reproduction on mysids is adaptive to factors such as temperature, light regime (Mauchline 1980) and food availability (Wittmann 1984).

The aim of this study is the description and evaluation of the reproductive processes of a mysid population (*Praunus flexuosus*) under natural conditions and the quantification of the effect of copper contamination in the seawater.

A review of different aspects on mysid reproduction processes is presented. Mysids, as do all peracarids, carry embryos in a marsupium, formed by lamellae or oostegites, in which the entire embryonic development takes place.

8.1.1 Production of eggs and fertilisation

The ovaries consist of two tubular organs connected by a slender bridge. The ripe ovary fills the posterior thorax and extends into the abdomen. The germinal area is located centrally and ventrally within the ovary. Eggs are continuously invested with yolk (Mauchline 1980). The ovarian tubes expand and the eggs are passed directly from the oviducts into the marsupium. Fertilisation proceeds in the marsupium during egg laying. First, two transparent egg sacs appear. After fertilisation two pronuclei become visible as diffuse dark spots near the centre of the egg (Wittmann 1981). At this time the egg sacs break down and the egg membrane becomes more rigid and resistant to malformation.

8.1.2 Embryonic development

The embryonic stage continues from oviposition and fertilisation to hatching. Eggs extruded into the marsupium, are immediately fertilised. There are 3 moults within the marsupium:

- the egg develops and moults to an eyeless embryo (embryo developing abdomen)
- the eyeless embryo moults to one with stalked eyes (eyed embryo with thorax and abdomen: nauplioid stage)
- nauplioid stage embryo moults to the fully developed free-living juvenile (embryo with all appendages: postnauplioid stage). The third moult synchronises with the emergence of the juvenile from the marsupium and the statoliths are excreted into the statocyst.

The statocyst is necessary to achieve competence in swimming. In some species the final embryo moult takes place as the newborn juveniles emerge from the marsupium (Berril 1971), while in other mysids, such as *Mysis stenolepsis,* the final embryo moulting occurs 3 hours after release (Wittmann 1984).

8.1.3 Synchronisation of brooding

The liberation of fully developed young is followed by ecdysis of the parent, copulation, and subsequent extrusion of the eggs of the next brood into the brood pouch (usually within 24 or 36h). The moulting period of the breeding female is synchronised with marsupial development in the embryos (Wittmann 1981). Observations on *Leptomysis* species suggest that if insemination does not occur shortly after moulting, egg laying is retarded (Wittmann 1981). When the mysid does not mate until 20-60 min after ecdysis, egg laying is initiated immediately after copulation. Deposition takes about 40 minutes for broods of 20-25 eggs (Wittmann 1981).

8.1.4 Marsupium control

Brooding females have been observed to ventilate the marsupium with rhythmic movements of the oostegites (Mauchline 1980, Wittmann 1981, pers.obs.)

P. flexuosus is believed to regulate the ionic composition of the marsupial fluid both hyper- and hypo osmotically with a notable degree of control (McLusky & Heard 1971). These authors also found a linear correlation between the osmotic concentration of the blood and the marsupial fluid. However, the degree of regulation was much stricter in the case of blood. Consequently, embryos within the marsupium of species living in fluctuating salinity

environments may be protected from extreme variations in salinity. The range of salinity within which the embryo can develop is more restricted than the total range in which the female can survive (Vlasbom & Elgershuiezen 1977).

Wittmann (1978) has shown that under laboratory conditions the females of *Leptomysis* are able to adopt embryos of other females of the same species. Females of *Schystomysis parkeri* from a coastal environment, were also recorded carrying adopted embryos (San Vicente & Sorbe 1993). The adoption and recognition of embryos was examined in *Anysomysis mixta, Paramesodopsis rufa* and *Tenagomysis tasmaniae* under field and laboratory conditions, and all species practised intraspecific adoption of prematurely liberated embryos (Johnston pers.comm.). The phenomenon of adoption decreases the potential mortality of embryos arising from their accidental loss from the marsupium.

8.1.5 The reproductive cycle and adaptations to the environment

Analysis of reproduction of different mysid species from different environments shows a correlation between increasing body, egg and brood sizes and incubation periods on one hand and increasing latitude and decreasing environmental temperature on the other hand (Wittmann 1984). Mysid eggs are larger at higher latitudes.

In temperate climates, important seasonal changes can take place in the number of embryos per brood. Mean brood size can increase or decrease by 50% over a period of 4 weeks. The eggs carried in spring and summer by females are small and appear to have fewer and smaller oil globules than eggs carried by overwintering females. It has been suggested that increased lipid storage may be responsible for the large size of winter eggs (Mauchline 1973).

The number of embryos per brood increases with body size of species in epipelagic mysids but no such correlation exists among mesopelagic and bathypelagic species (Mauchline 1973). However, all invest the same fraction of body volume in reproduction. The volume of the eggs in mysids is a cubic function of body length (Mauchline 1973, 1980).

8.1.6 Reproductive strategies in relation to the life cycle

Small crustaceans can increase the overall number of eggs brooded by decreasing egg size and production of successive broods. In mysids, increased egg size contributes less to increased incubation times than the decreased environmental temperatures to which large eggs are generally subjected (Wittmann 1984). The increased lipid store in large eggs requires

an increased egg size to accommodate it, but may not contribute proportionally to increased incubation time.

Growth patterns are a consequence of the effects of genetic, physiological and environmental factors on organisms. The primary constraint on brood size within and between species is body size; the size and number of eggs within a brood are dependent variables (Mauchline 1985). The size of the resultant embryo initially reflects egg size but is subsequently influenced by environmental factors, as is the number of successive broods produced.

8.1.7 Temperature effects

Mysids growing in high temperature seasons usually attain sexual maturity at a smaller body size than those growing at low temperature seasons. Thus, not only do mysids grow faster at higher temperature but they have an abbreviated development period. In western Scotland *Neomysis integer* matures sexually at a length of 9-10 mm in summer and at 11-12 mm length in spring (Mauchline 1971). Growth curves of *Neomysis integer* estimated in the laboratory at 9 °C and 16 °C indicated that growth factors were not significantly different (Astthorsson 1980). Conversely, the relationships between intermoult period and length were significantly different. Astthorsson (1980) suggested that increased growth rate at higher temperature was mainly achieved through higher moulting frequency rather than increased growth increment at moulting. Thus, summer generation mysids will achieve sexual maturity faster than in other seasons in littoral environments through an increased frequency of moulting.

The temperature effect on incubation period was suggested to be a key factor in understanding variation in length and timing of the breeding season, variations in age at maturity, frequency of broods, numbers of young per brood and adult body size (Wittmann 1984). Incubation is, in most species, well synchronised with the maternal moult cycle and with the development of the eggs in the ovarian tubes (Mauchline 1980). Therefore, changes in incubation time should produce effects on both reproductive and population biology.

Reproduction in mysids can be affected at the intraspecific level by temperature, body size (consequently egg size), chemical factors and nutrition. Kinne (1955), found in *Neomysis integer* at 18.5 °C, an incubation period of 14.5 days and an egg diameter of 0.5 mm. At 15 °C in the same species, Vlasblom & Elgershuizen (1977) found an incubation period of 21.3 days, with an egg diameter of 0.5 mm. Johnston *et al.* (1997) determined, in a laboratory

experiment, the incubation time in relation to temperature in three Tasmanian coastal mysid species. The results showed similar values to the model proposed by Wittmann (1984) where egg diameter and incubation time are related to temperature. Toda *et al.* (1983) studied *Neomysis intermedia* in conditions of no food limitation and found that the daily specific reproductive rate (brood size/brood interval) increased exponentially with temperature between 10 and 25 °C.

8.1.8 Effects of season

The effects of nutrition on reproduction result in differences in growth, age and attainment of sexual maturity, and numbers of juveniles in relation to parental size (Wittmann 1981). Semi-starvation produced prolonged intermoult periods and reduced the number of eggs brooded which were also slightly smaller than eggs in the control animals.

The strong seasonal variations observed in mysid reproduction in temperate climates are supposed to be mainly a direct response to variations in food availability (Wittmann 1984). Clearly both factors (temperature and food supply) are important. When food supply is not limited, temperature is the main control. Energetic studies on ormers (*Haliotis tuberculata*), showed that growth is limited, not directly by temperature, but by the effects of temperature on energy acquisition and energy losses (Peck 1989).

8.1.9 Pollution effects

Pollution effects on reproductive processes of mysids have been studied in *Mysidopsis bahia*. Acute (96h) and chronic (17 days) toxicity of Cd and pesticide (Kepone) resulted in a 48h delay in the formation of broods and a decrease in brood size and growth (Nimmo *et al.* 1977). Reductions in brood size were also observed as a long-term effect of suspended particles (Nimmo *et al.* 1979). Cadmium affected the growth and development of gonads (Gentile *et al.* 1982). A 7 day toxicity test showed that fecundity and sexual maturity were affected by cadmium (Khan *et al.* 1992). Mercury exposure for 35 days produced delays in sexual maturation and was subsequently reflected in a delay of the appearance of embryos in the marsupium and in release of juveniles (Gentile *et al.* 1983).

Reduction in brood size (number of juveniles released), and delays and reductions in sexual maturation were recorded for mysids exposed to different pesticides such as Endrin (McKenney 1982), Fenthion (McKenney 1986) and Dimilin (Nimmo *et al.* 1980).

8.2 MATERIALS AND METHODS

Reproductive processes were studied under natural conditions and also under laboratory controlled variations in temperature, ammonia and copper exposure. Specimens were collected from the field and maintained in the aquarium-laboratory conditions (described in Chapter 2) to allow acclimation to the different experimental conditions.

8.2.1 Reproduction under field and laboratory conditions

Experiment 1: Mysids were collected from the field every two weeks during the reproductive season (April to October). Individuals were transported to the laboratory and maintained alive in the aquarium at similar conditions of temperature, light and water quality to those in the field. They were fed *Artemia* nauplii *ad libitum*, and surveyed daily. Brooding females were held individually in 1 litre tanks until they released their young. During this process they were examined under the stereo microscope and video-recorded. After release of juveniles, females were transferred to the rest of the population to mate. Marsupiums of these females were examined to detect the presence of fertilised eggs, and to initiate the study of embryo development. Comparison of live aquarium-laboratory and field individuals was made from measurements of female size (mm length), number of embryos brooded, newborn juvenile size (mm length), reproductive effort (ratio of embryos brooded vs female size) and behaviour on both populations. To complete the study of reproduction processes the number of newborn juveniles released, newborn juvenile size (mm length), fertilisation of the eggs, embryo stage and incubation time was also measured on the population collected in the field and maintained in the aquarium.

8.2.2 Temperature effects

Experiment 2: Mysids collected from the field in March (10 °C) were maintained at two different conditions in the aquarium. One group was maintained at a constant temperature of 10 °C until June, whereas the other group of mysids was kept in the aquarium at the same temperature as in the field and followed a gradual temperature increase through March (10 °C), April (12 °C, 14 °C). May (15 °C, 17 °C) and June (18 °C and 20 °C) (Fig.3). In June, reproductive parameters were compared between these two groups (then at 10 °C and 20 °C).

Experiment 3: This experiment was performed with brooding females, in order to examine the effects of an increase in temperature on incubation time. Brooding females collected in May were held at 16 °C (field temperature), and a second group was maintained at 21 °C (experimental increased temperature) during the incubation period.

8.2.3 Effects of ambient ammonia, nitrate and nitrite levels on reproduction.

Experiment 4: Breeding females and males (n=30) were kept in two different conditions of nitrogen compounds for one week to survey the production of broods. Control seawater levels were ammonia 0.1 mg l^{-1}, nitrates 10 mg l^{-1}, nitrites <0.05 mg l^{-1} and experimental levels were ammonia 0.3 mg l^{-1}, nitrates 20 mg l^{-1}, nitrites 2 mg l^{-1}. Experimental specimens were transferred to control seawater on the 7th day of exposure and surveyed for another 7 days.

8.2.4 Effects of copper exposure on reproduction

Experiment 5: Groups of 10 brooding females and 4 males were exposed to different copper concentrations (0, 5, 25, 75, 200 µg l^{-1} Cu_a) in 1 litre beakers (acid cleaned), aerated and fed daily with *Artemia* nauplii (as toxicity test methodology, chapter 2). Brooding females were incubating embryos at nauploid stage. Toxicity testing was performed for 10 days at 20°C in August 1997, for 8 days at 17°C in June 1998 and for 8 days at 20°C in August 1998. Individuals were examined twice per day. Water was renewed and tanks cleaned every 48h, at which time animals were video recorded using a JVC video camera attached to a Wild M8 stereo microscope.

8.2.5 The ability for recovery from copper effects

Experiment 6: Individuals exposed to copper for 8 days in the experiments in June and August 1998, were transferred to control seawater (without copper additions) for 14 days to evaluate the capability for recovery from the copper effects. Mortality, behaviour and reproductive processes were surveyed daily for every specimen. The experiment performed in August also included some male specimens never exposed to copper to mate with females previously exposed to copper.

8.3 RESULTS

8.3.1 Reproductive biology of *Praunus flexuosus* under natural conditions.

Experiment 1

Analysis of *P. flexuosus* population samples collected from Keyhaven and the survey of live animals in the aquarium during the reproductive periods of 1996 and 1997 show that reproductive activity increases rapidly in March, after the overwintering period.

Reproductively active females are those in which egg production is occurring in the ovary or eggs, or embryos are being incubated in the marsupium. The first reproductive maximum occurs in April when 70% of the females in the population are reproductively active (Fig. 8.1A). Reproductive activity decreases slightly in May and June and reaches the maximum for the year in July (Fig.8.1A). From August reproductive activity decreases rapidly to <5% in October, by which time the population is overwintering.

Examination of the percentage of brooding females in relation to the whole population in March shows a percentage of less than 10% of brooding females (Fig. 8.1B). By April, however, brooding females were a 70% of the population. In May and June breeding activity is a mixture of egg production and brooding.

The proportion of females brooding decreased to 35% in June, as a result of the death of overwinter females after the release of their broods. The percentage of brooding females in the population increased in July but steadily declined until October.

Examination of the portion of females brooding in relation to all females reinforced the pattern observed before. Less than 20% of the females were brooding in March but this increased to 100% in April, and remained above 45% until August (Fig. 8.1C). In July the percentage of brooding females included the breeding activity of the new spring generation. After August the percentage of females brooding declined rapidly on all measurements.

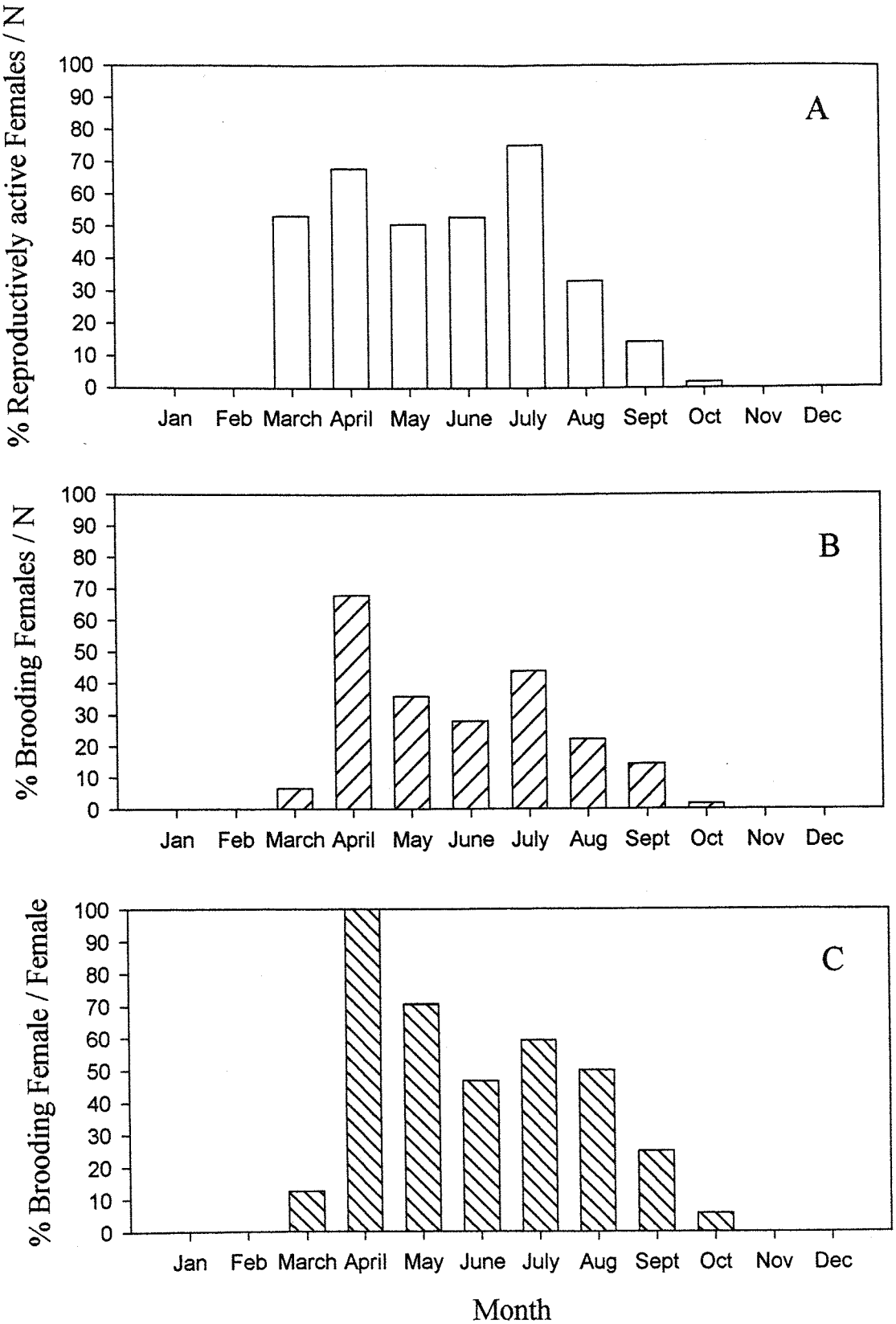


Fig. 8.1. ***Praunus flexuosus*** **females percentages on the reproduction**
A: % Reproductively active females of the total population
B: % Brooding females of the total population
C: % Brooding females of the female population

The number of embryos in the marsupium was not significantly different (T=406, p=0.9) from the number of juveniles released per female. The number of embryos brooded or juveniles released per female was maximum in June (Fig. 8.2), when both the overwinter generation and the spring generation were breeding.

The biggest broods (36 newborn juveniles) were produced in June (Fig. 8.2) by the females of the overwinter generation, which were the largest individuals in the population (22-20mm length). Spring generation females (15mm length) released an average of 12 newborn juveniles in June. Brood size decreased progressively from July to October when there were only four newborn juveniles per female (Fig. 8.2).

Different breeding generations are clearly visible from the analysis of brooding female sizes (Fig 8.3). From April until June overwinter females carried broods. The spring generation bred in June, July and August, and the summer generation bred in September and October.

Newborn juvenile size (Fig. 8.4) ranged from 2.3 mm to 4 mm (length from eyestalk to telson). From April to August the size of newborn juveniles varied between 3.2 and 3.5 mm. The maximum size was in April and after September the mean size decreased to the minimum in October.

Reproductive effort (ratio of number of embryos brooded to female size) (Fig.8.5), was maximal in May/June, when the overwinter generation bred.

Both the overwinter and spring generations, bred during May and June and the spring generation bred with maximum reproductive effort in June. Reproductive effort decreased significantly from spring (1.2) to summer (0.5) ($F=24$, $p<0.0001$).

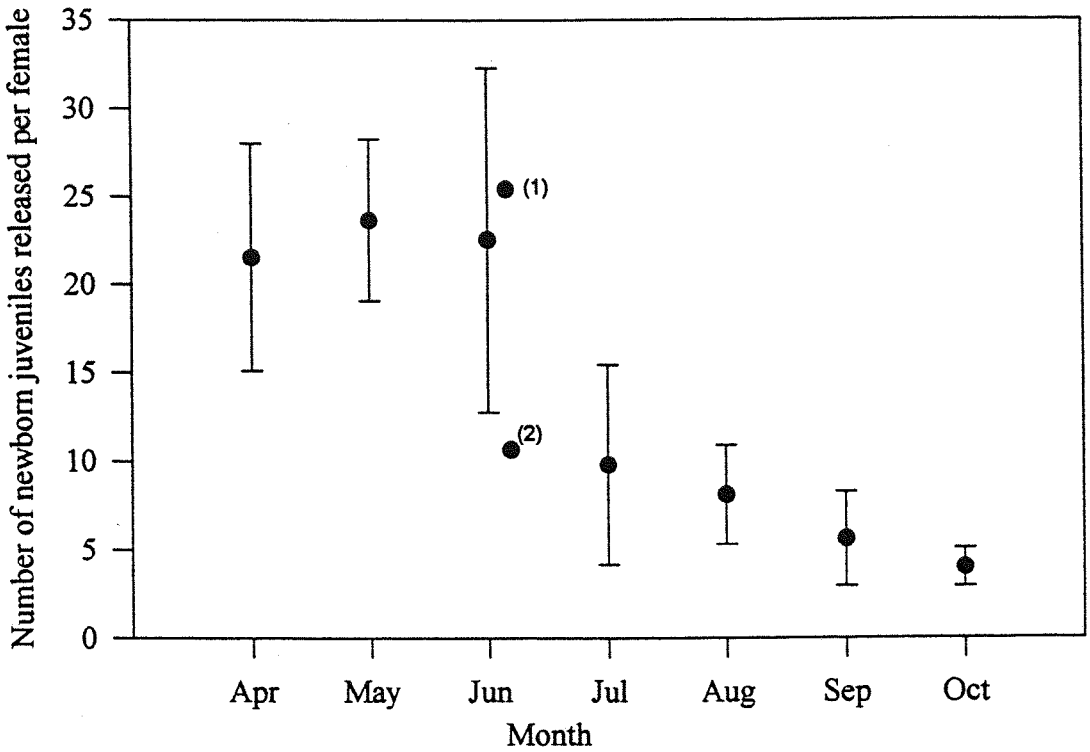


Fig. 8.2. Number of newborn juveniles (Mean, SD) released per female (brood size) of *Praunus flexuosus*. (1) - by overwinter females, (2) - by spring females.

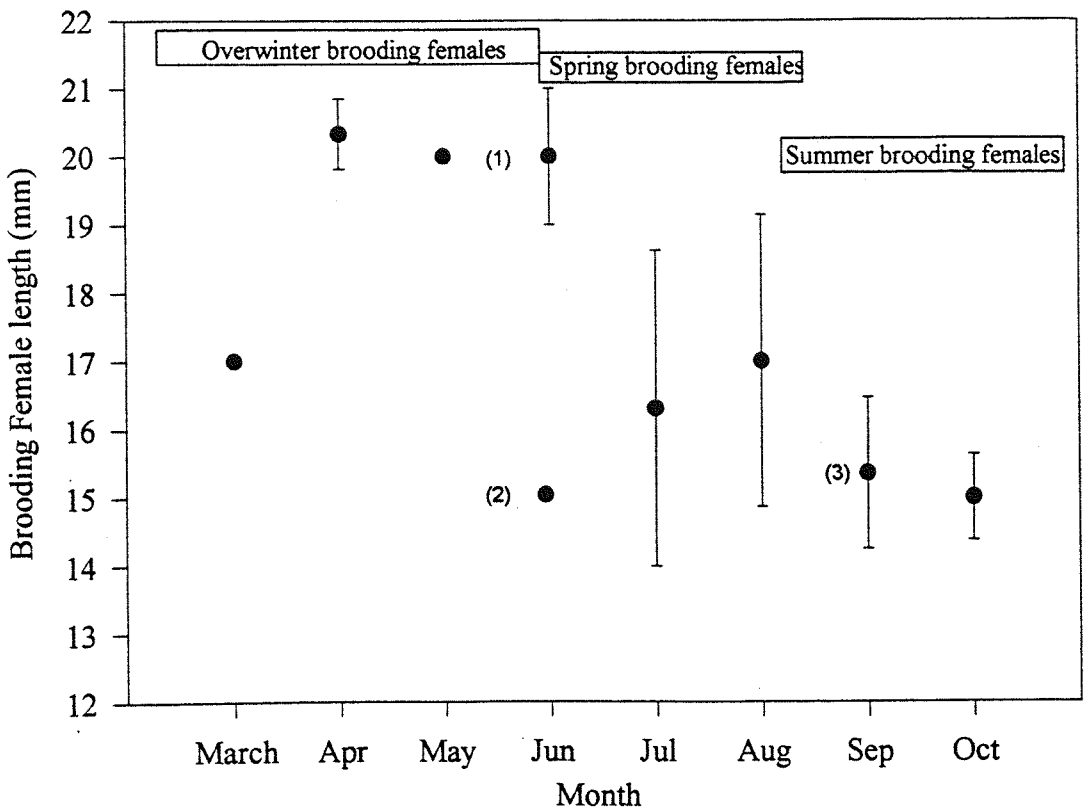


Fig. 8.3. Brooding Female length (mm) of *Praunus flexuosus* (Mean, SD). (1) overwinter generation, (2) spring generation, (3) summer generation.

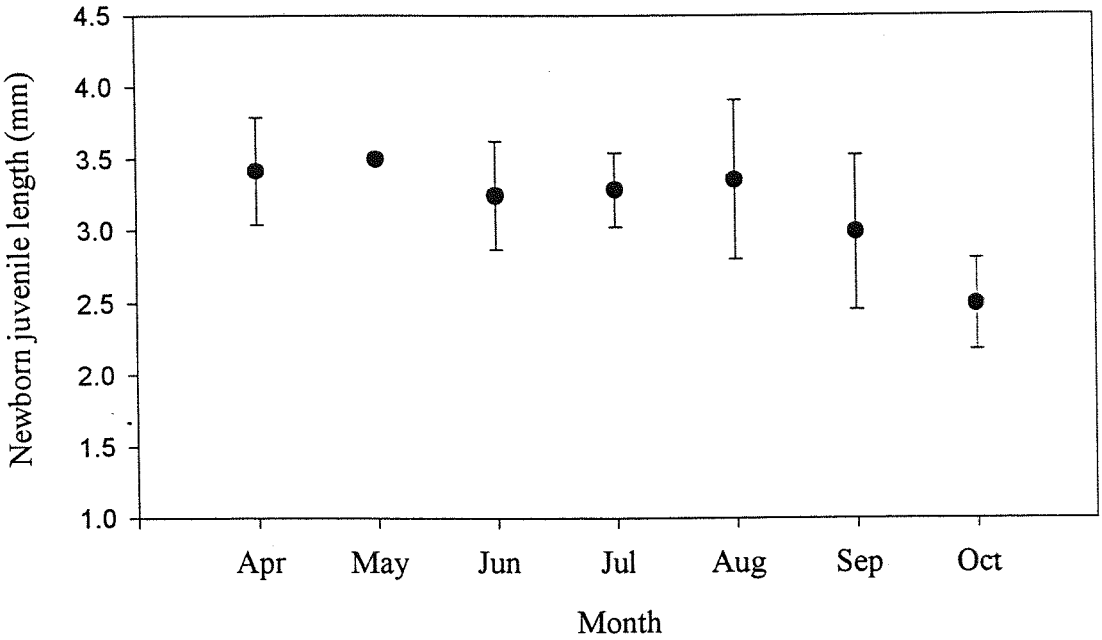


Fig. 8.4. Length (mm) of newborn juvenile (Mean, SD) released by *Praunus flexuosus* brooding females.

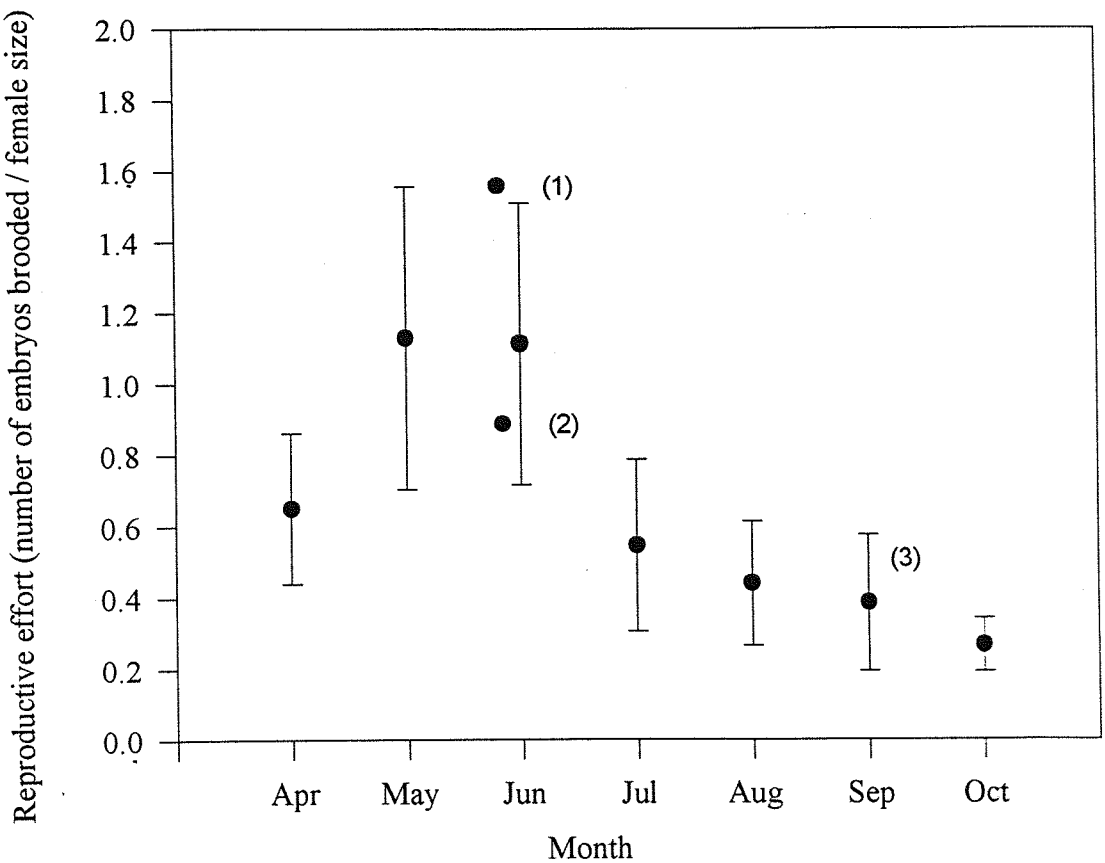


Fig. 8.5. Reproductive Effort of *Praunus flexuosus* (Mean, SD)
(ratio of number of embryos brooded to female size)
(1) overwinter generation, (2) spring generation, (3) summer generation

8.3.2 Effects of temperature

Experiment 2

Incubation periods were shorter at higher temperatures. Incubation time estimated from the survey of brooding females in the aquarium through the reproductive period under natural conditions ranged from 16 to 28 days. Because temperature and food availability are known to affect the incubation time, animals were maintained at constant food availability, and temperature was varied, following the seasonal variation of field temperature (see Chapter 3, Fig.3.3). The longest incubation time (28 days) was in spring and late summer/early autumn, coinciding with the lowest temperature of the breeding season, whereas the shortest incubation time (16 days) was recorded at the highest temperature of the breeding season (July/August). Growth was very intense in summer, and newborn juveniles reached sexual maturity in one month in June/July and early August.

Brood production was delayed in the population kept at constant temperature (10 °C) from March to June. Females began incubating embryos in early May and did not release juveniles until the end of May. The group of mysids kept at field temperatures released juveniles one month before, in April at 15 °C, which coincided with first juveniles released in the field. Those females that released juveniles in April, carried the next brood within 5 days and released a second brood in May (16-18 °C), before the 10 °C population had completed incubation of the first brood.

The incubation period of the mysids maintained at 10 °C was at least 15 days longer and the brood size was smaller than mysids at environmental temperatures. In May brooding female size was 17 mm at 10 °C, and brood size was 12-20 juveniles. On the other hand, brooding female size was (20-22 mm) and the brood size (22-29 juveniles), at field temperatures.

Brooding female size ranged from 16 to 21 mm long in June at 10 °C, whereas under field conditions (20 °C) the range was 12 to 21 mm. The size range of the population maintained at field temperature shows the presence of two different breeding generations (overwinter and spring). At 10 °C there was only one breeding generation (overwinter).

Temperature is the main driving factor controlling growth and reproduction processes. Although similar reproductive parameters occurred at two different temperatures (Fig. 8.6), important changes were apparent. A 10 °C decrease of temperature resulted in a reduction in number of brooding generations, numbers of fertile females, and growth rate. The reduction

The reduction of temperature increased incubation time and lowered the number of juveniles produced by the overwinter generation. Juveniles exhibited a very slow growth rate at 10 °C (2 mm in 20 days) whereas at 20 °C the same growth occurred in 4 days. There was only one breeding generation (overwinter) in the population at 10 °C and very few juveniles reached maturity by the end of the summer. At 20 °C there were two breeding generations developed in June and individuals of a third generation produced at the end of the summer. Regarding the 10 °C population, the overwinter generation did not die after breeding in spring and continued breeding during the summer months. However in the 20 °C population the overwinter generation died after breeding in June, and the spring and summer generation bred throughout the summer.

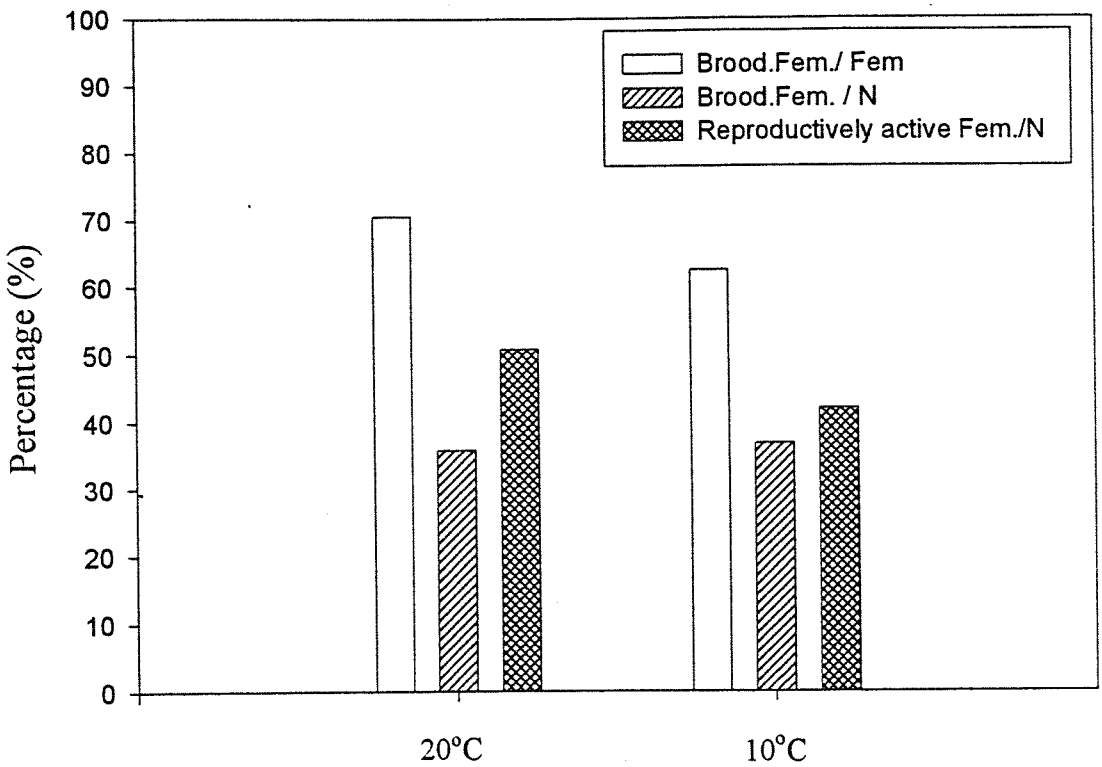


Fig. 8.6. Effects of temperature in the reproductive strategy of *Praunus flexuosus* population maintained at 20°C and 10°C. Bars represent the percentages of brooding females of the female population, brooding females of the total population and reproductively active females of the total population

Experiment 3

Temperature increase affected incubation time for brooding females in the May population. Comparison of the incubation time of females maintained at 16 °C (field temperature) and at 21 °C (experimental temperature) showed a decrease of 6 days at the higher temperature.

8.3.3 Ammonia, nitrate and nitrite effects on reproduction

Experiment 4

Nitrogenous compounds in seawater can affect reproductive processes. Acute sublethal toxicity effects on reproduction were produced by enhanced levels of ammonia, nitrate and nitrite. Reproduction of *Praunus flexuosus* was suppressed completely at the following levels: ammonia 0.3 mg l^{-1}, nitrate 20 mg l^{-1}, nitrite 2 mg l^{-1}, in 96h at pH 8. When individuals were transferred to control seawater, reproduction occurred normally below those levels.

8.3.4 Copper effects on reproduction

Experiment 5

Copper had a dramatic effect on the ability of mysids to retain broods. Toxicity tests conducted in June 1998 on brooding females at 17 °C (field temperature) showed a significant reduction ($F=5.6$, $p=0.001$) in the percentage of brooding females with increasing copper concentration and exposure time (Fig. 8.7). In the controls brooding females ranged from 100% to 80% over the 8 days of test. At 5µg l^{-1} Cu_a the proportion of brooding females decreased to 70% at 96h and to 40% on 8 days of exposure. At 25µg l^{-1} Cu_a brooding female proportions were similar to levels at 5µg l^{-1} Cu_a . At 75 µg l^{-1} Cu_a brooding females decreased to 50% in 96h and 40% in 8 days. At 200 µg l^{-1} Cu_a the brooding females decreased to 40% in 3 days and to 20% by day 6.

The percentage of abortions (total loss of the brood) recorded also during the experiment mentioned above increased significantly with copper concentration and time of exposure ($H=18.2$, $p=0.001$), being significantly different from control ($p<0.05$) at any copper treatment (Fig. 8.8). After 8 days abortions occurred in 20% of females exposed to 5 µg l^{-1} Cu_a, and in 30% of females exposed to 25, 75 and 200 µg l^{-1} Cu_a.

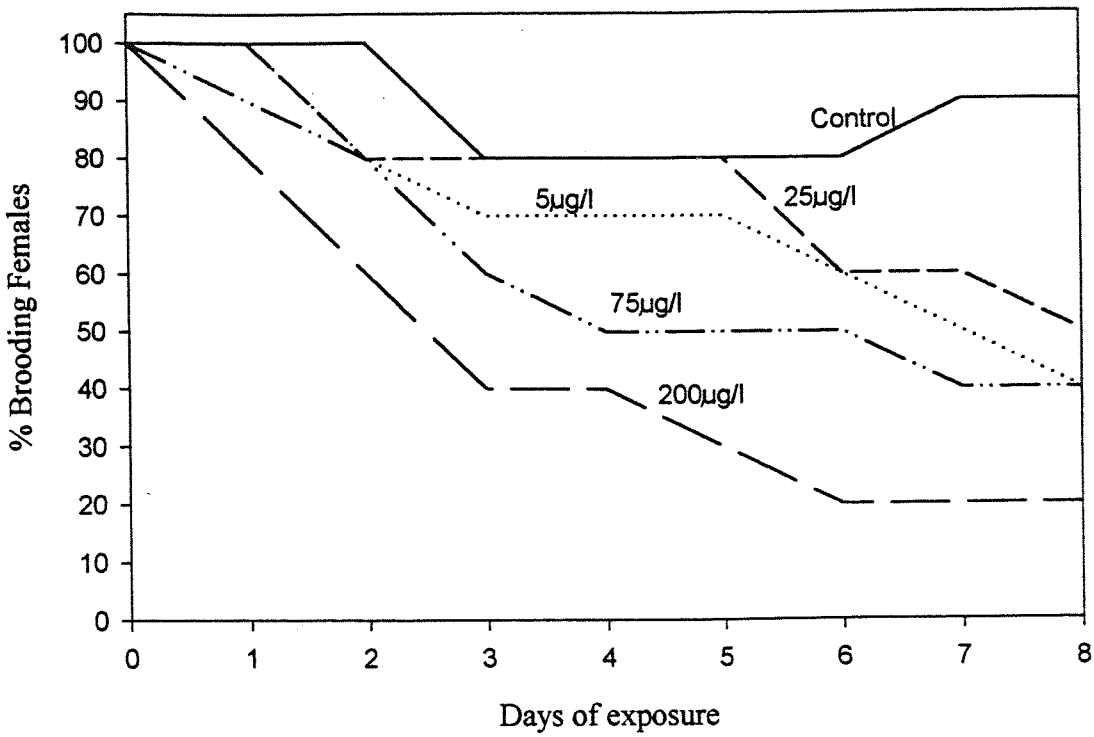


Fig. 8.7. ***Praunus flexuosus*** **brooding females (%) exposed to different copper additions for 8 days at 17°C in June 1998**

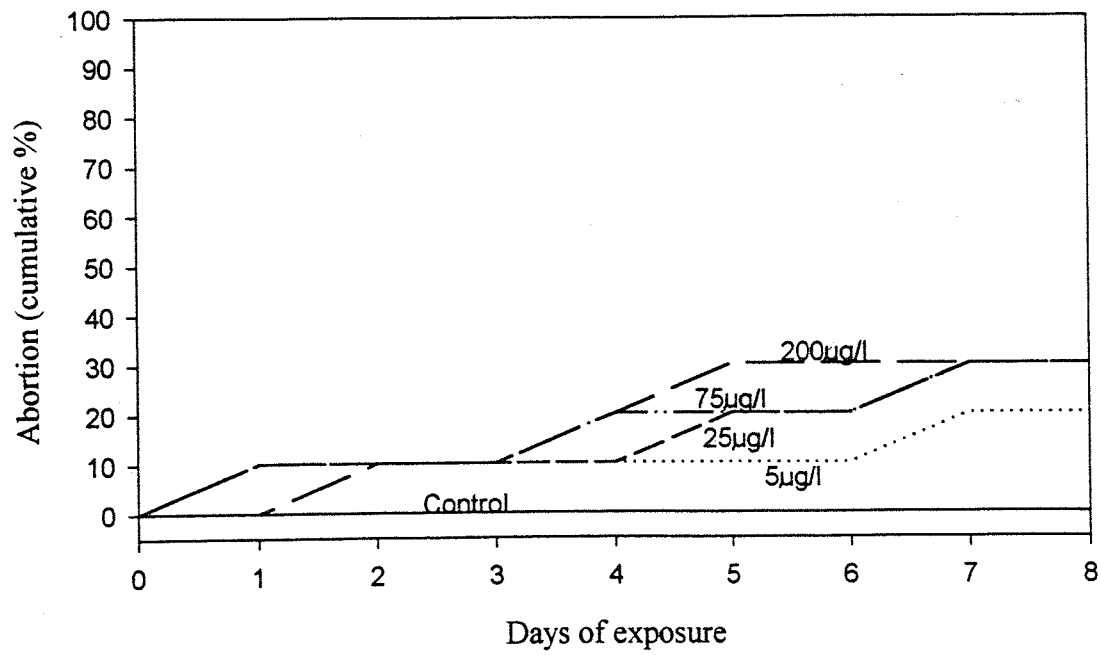


Fig. 8.8. ***Praunus flexuosus:*** **Abortions (cumulative %) or total loss of the brood, occurring in brooding females exposed to copper at 17°C for 8 days in June 1998.**

Partial abortions (loss of some embryos of the brood) were observed at any copper exposure level. Controls released an average of 14 juveniles per female. Brood size (number of juveniles/female) decreased to 8 juveniles per female at the lowest copper concentrations, and 3 juveniles per female at the highest concentrations.

The effects of copper in tests performed in August showed higher toxicity than in June. Proportions of brooding females in controls were the same in both tests, but the decrease in number of brooding females and increase of abortions with increasing copper exposure was more pronounced in August than in June.

The percentage of brooding females after 96 h of exposure decreased from 100% in controls to 60%, 50%, 40% and 10% with increasing copper concentration in August. The decrease continued to 0% at 5, 75 and 200 $\mu g\ l^{-1}$ Cu_a and to 10% at 25 $\mu g\ l^{-1}$ Cu_a after 7 days of exposure (Fig. 8.9).

Levels of abortions increased significantly with copper and time of exposure (H=16.4, p=0.002), reaching at 6 days of exposure, high values of 50% at 5$\mu g\ l^{-1}$ Cu_a , 60% at 25 and 75 $\mu g\ l^{-1}$ Cu_a and 80% at 200 $\mu g\ l^{-1}$ Cu_a (Fig. 8.10).

The reduction in brooding females and increase of abortions observed at any copper treatment. The reduction observed in August 1997 at 20 °C was not significantly different from those observed in August 1998 at 20 °C (T>90, p>0.05), except for the abortions at 5$\mu g\ l^{-1}$ Cu_a in August 1997 which were significantly higher (T=57.5, p=0.003). The percentage of brooding females decreased to 0 % at 5$\mu g\ l^{-1}$ Cu_a (Fig. 8.11) because abortions increased to 70% (Fig. 8.12) after 4 days of exposure. The high rate of abortions in August 1997 could be related to differences in embryo development. Although all embryos were at the nauplioid stage, the embryos at 5 $\mu g\ l^{-1}$ Cu_a were 4 days earlier in the nauplioid stage development.

Effects of copper measured in June and August did not differ significantly (H=12.8, p=0.114). The abortion rate was lower in June 1998 than in August, but not significantly at 5, 75 and 200 $\mu g\ l^{-1}$ Cu_a. The decrease in broods with copper exposure did not only reflected the mortality of brooding females but included abortions (total loss of brood) that some females suffered.

At all levels of copper exposure brood size (number of juveniles released per female) was reduced, from an average of 11 juveniles released per female in controls in August to 2 - 3 juveniles.

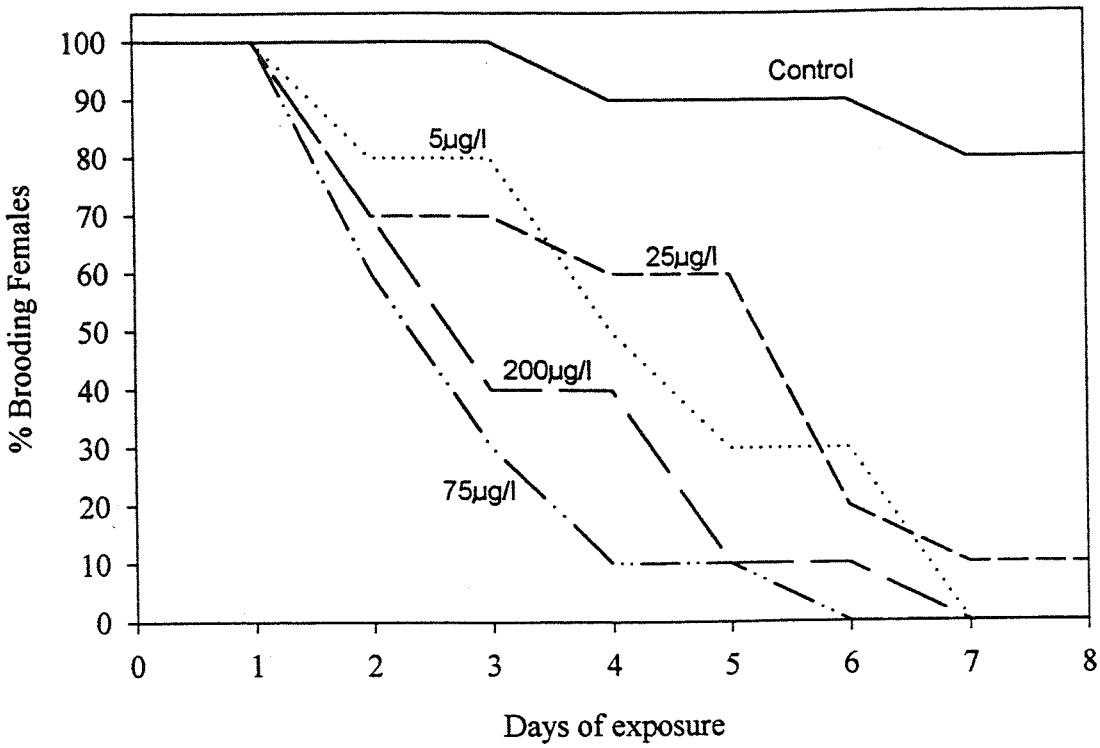


Fig. 8.9 ***Praunus flexuosus*** **brooding females (%) exposed to different copper additions for 8 days at 20°C in August 1998**

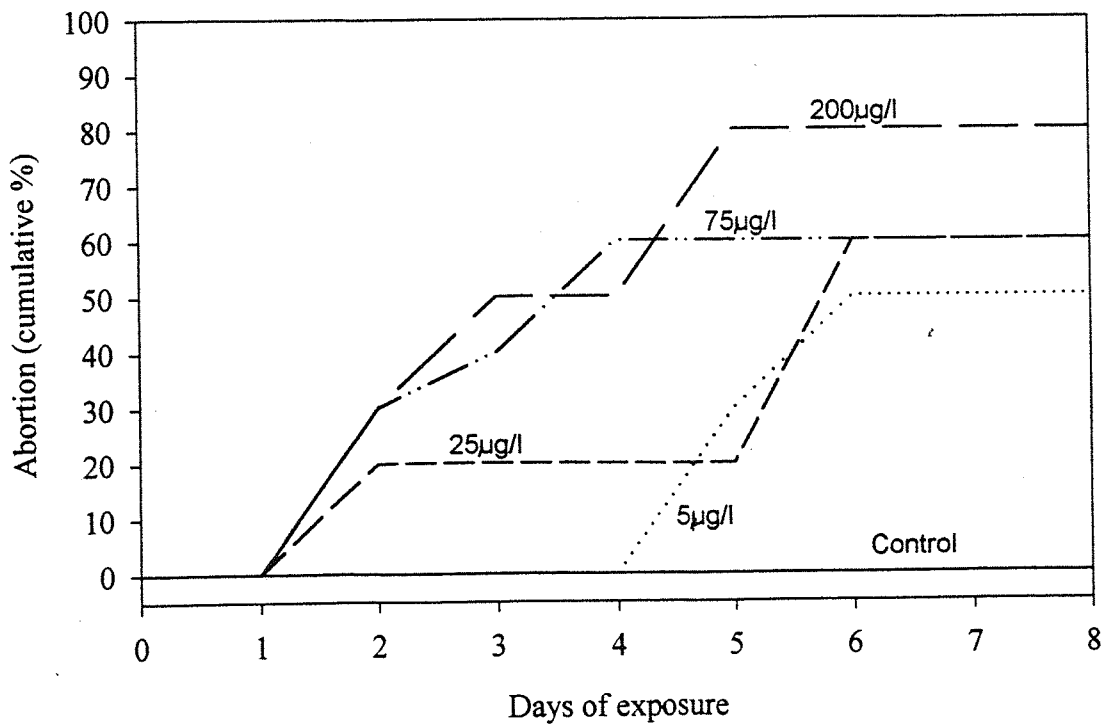


Fig. 8.10. ***Praunus flexuosus*****: Abortion (cumulative %) or total loss of brood, occurring in the brooding females of** ***Praunus flexuosus*** **exposed to copper at 20°C in August 1998**

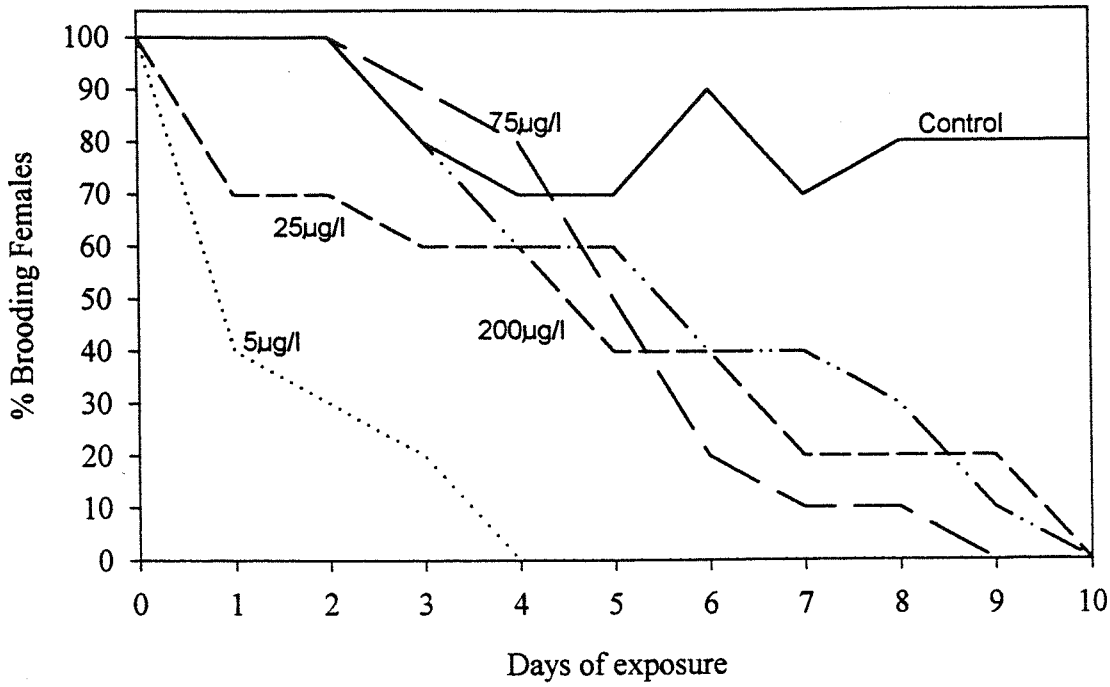


Fig. 8.11. ***Praunus flexuosus*** **brooding females (%) exposed to different copper additions for 10 days at 20°C in August 1997.**

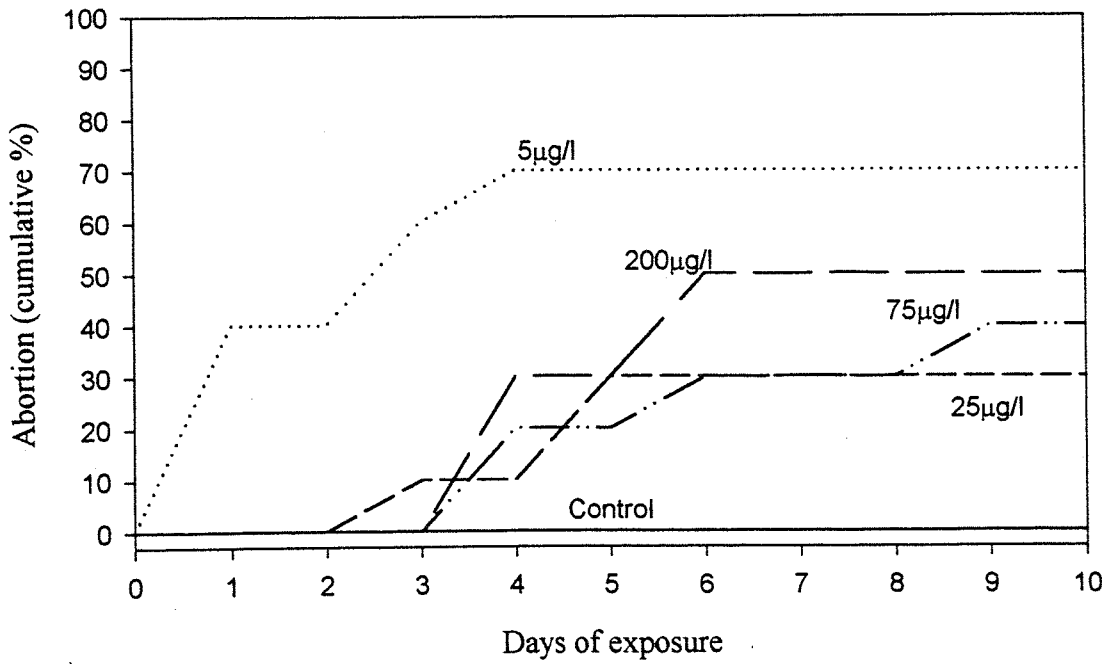


Fig. 8.12. ***Praunus flexuosus*****: Abortions (cumulative %) or loss of the total brood, occurring in brooding females exposed to copper at 20°C in August 1997.**

Females that survived the toxicity test were observed for additional 10 days under the same copper exposure conditions. Controls were releasing juveniles and successfully producing the next broods (eggs were fertilised and developed to embryos). Females exposed to copper were able to lay a few eggs, but there was no fertilisation and therefore no production of broods.

The inability to fertilise eggs and produce broods was a common effect in all copper treatments. The production of yolk in ovaries and eggs was decreased by copper. Survivors of all experiments presented disrupted behavioural patterns (as described in Chapter 5), changes in coloration, and partial necrosis. Normal ventilation of the marsupium was active only at lower copper exposure levels. At higher copper levels the marsupium was not ventilated and was kept closed. This suggests that females suppressed ventilation of the marsupium to protect the brood at high copper levels. Some empty marsupiums presented malformations (Plate 8.1), where the shape was deformed and damage of the oostegites was evident.

8.3.5 Ability to recover from the copper effects

Experiment 6

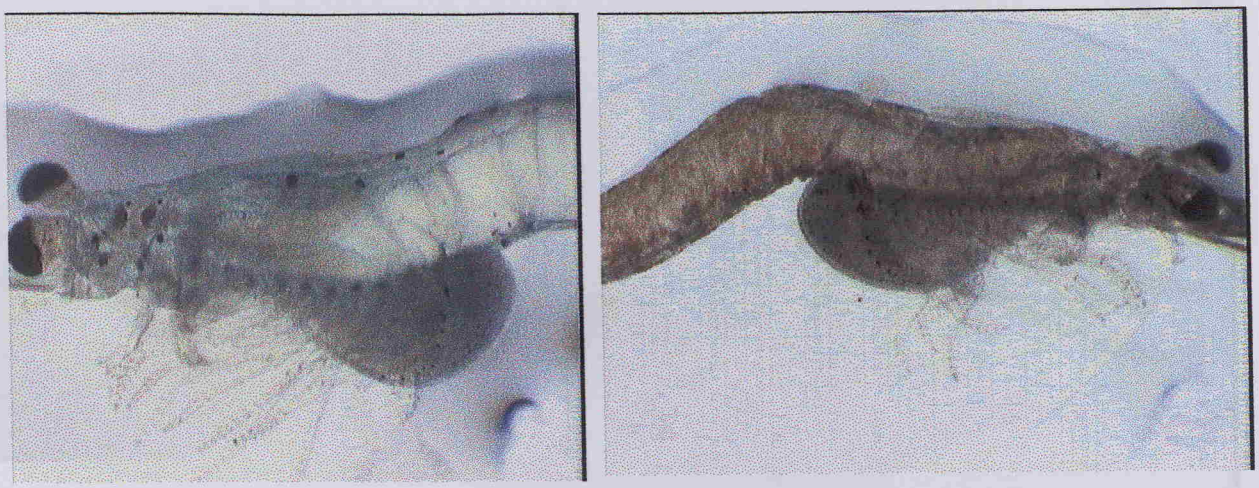
Female and male survivors from the June 1998 experiments were transferred to clean conditions for 14 days. Brooding did not occur in any mysid that had previously been exposed to copper. 40% of the females in recovery produced yolk or a few eggs in their ovaries. 60% of females had empty ovaries.

The same experiment was performed on the survivors in the August 1998 experiments for 10 days. In order to test if the observed effects were related to malfunctions of males, control males were transferred to the female tanks. In these trials there was failure of fertilisation and no broods were produced. Only one female carried fertilised eggs, by a male never exposed to copper. This female discharged or lost the eggs after 24 hours. Fertilisation of eggs and embryo development failed in control seawater in specimens previously exposed to copper.

Malformations of the marsupium observed with copper exposure and necrosis recorded in the internal tissues near the ovaries did not recover within 14 days under control seawater conditions. However, some recovery towards the normal coloration occurred at the thorax, abdomen, and antennal gland in mysids previously exposed to the lower copper concentrations (5 and 25 $\mu g\ l^{-1}$ Cu_a) (Plate.8.2). Swimming capability and daily activity returned to normal response levels in these cases.

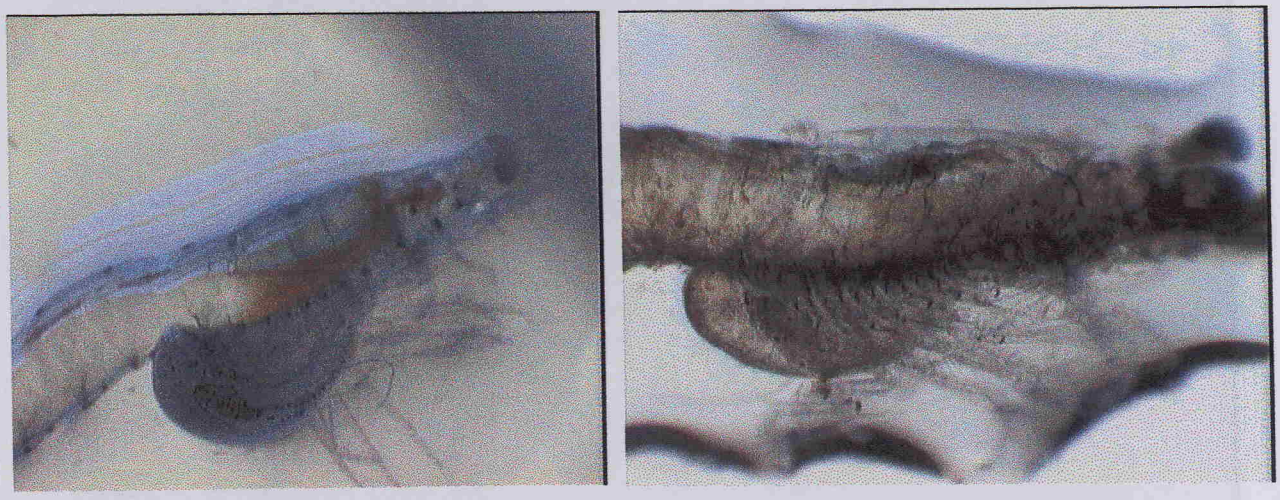
Plate 8.1. *Praunus flexuosus* brooding females exposed to copper. August 1998 experiments

Control **Copper exposed**



A **B**

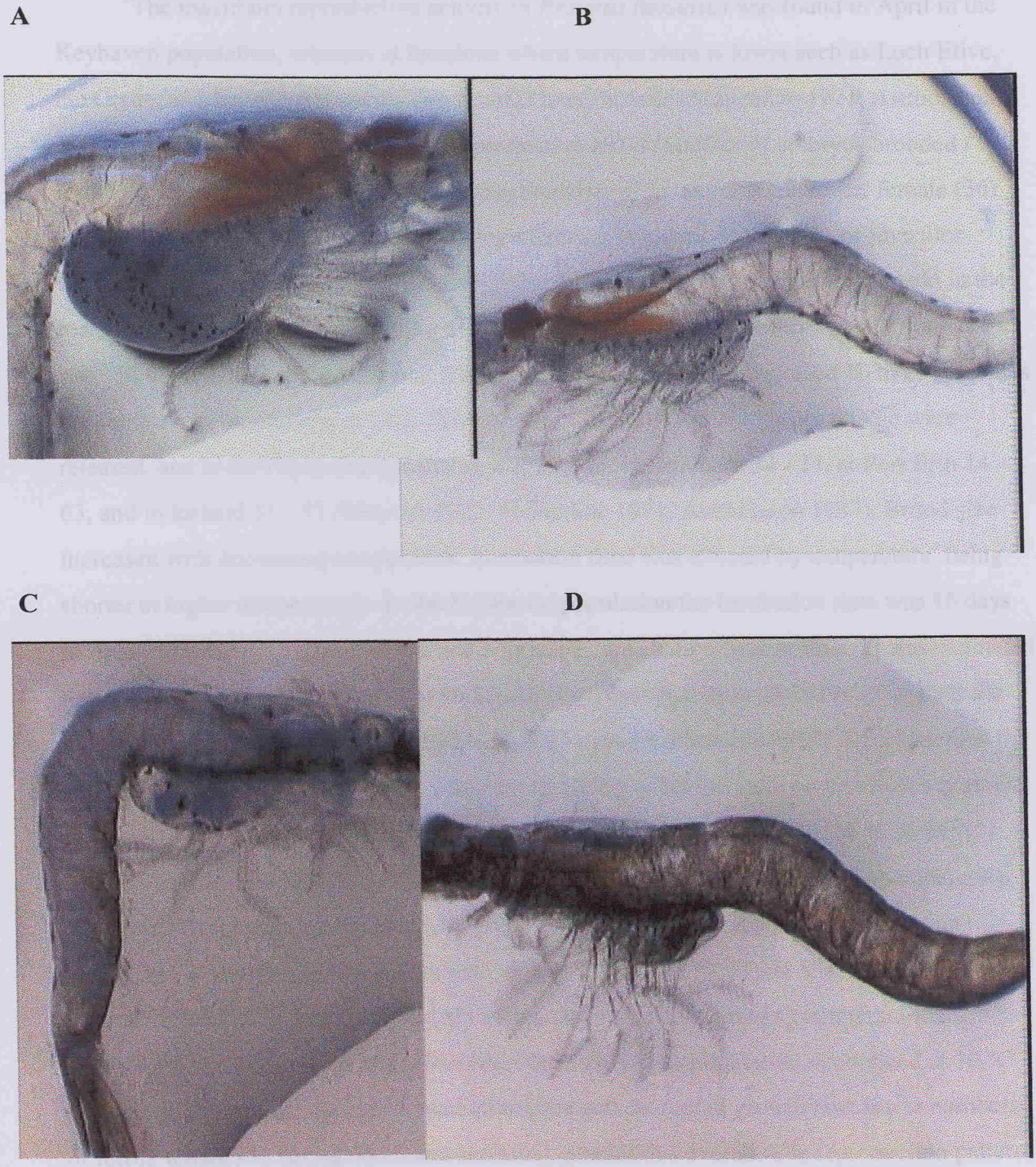
A. Specimen Control with embryos developing in the marsupium, ovaries (dorsal side) were producing yolk (globules). Formation of the next eggs in the ovaries.
B. Specimen exposed to 75 μg l^{-1} with embryos developing. Ovaries are not producing eggs. Note the colour difference from control.



C **D**

C. Control specimen with embryos well developed and eggs in the ovaries to produce the next brood.
D. Copper exposed specimen with empty marsupium and lack of eggs. Ovaries exhibited necrosis.

Plate 8.2. *Praunus flexuosus* brooding females exposed to copper for one week in June 1998 and transferred to control seawater for 2 weeks.



A. Control specimen with embryos developing in the marsupium.
B. Specimen previously exposed to 5 μg $l^{-1}Cu_a$ exhibited recovery of colour as in controls. Marsupium and ovaries are empty.
C. Specimen previously exposed to 25 μg $l^{-1}Cu_a$ laid few eggs but they were not fertilised
D. Specimen previously exposed to 75μg l^{-1} Cu_a exhibited malformation in the marsupium and necrosis in ovaries.

8.4 DISCUSSION

The maximum reproductive activity in *Praunus flexuosus* was found in April in the Keyhaven population, whereas at locations where temperature is lower such as Loch Etive, the Clyde, and Iceland this occurs two months later, in June (Mauchline 1971, Astthorsson 1980) (see Chapter 3). The maximum reproductive effort (number of embryos brooded / female size) occurs in May/June. The highest number of juveniles released per female (36) are produced in June by females of the overwintering population. Numbers of juveniles released per female was not significantly different from the numbers of embryos held in the marsupium, indicating that mortality of embryos in the marsupium of this population was low. In the main breeding season at Keyhaven, *Praunus flexuosus* released 10 to 23 juveniles per female. In comparison, at Loch Etive between 12 and 43 juveniles per female were released, and in the Clyde (Millport) this was 20 - 45, in Denmark 14 - 27, at Port Erin 14 - 63, and in Iceland 31 - 72 (Blegvad 1922, Mauchline 1971, Astthorsson 1987). Brood size increased with decreasing temperature. Incubation time was affected by temperature, being shorter at higher temperatures. In the Keyhaven population the incubation time was 16 days in July/August and 28 days in April and September. Incubation time at Roscoff was estimated to be 15 days in August and 21 days in September (Nouvel & Nouvel 1939). In Iceland the incubation time at 11°C was estimated to be ~ 25 days (Astthorsson 1987). Reproduction appears to be adapted to compensate the strong effects of temperature on incubation periods.

Brooding females subjected to an increase in temperature of 5 °C (16 °C to 21 °C) reduced the incubation time by 6 days. A decrease of 10°C (20 °C to 10 °C) in temperature, resulted in an increase in incubation time of 15 days. Similar effects of temperature on incubation time were noted for *Neomysis integer* by Kinne (1955) and Vlasbom & Elgershuizen (1977). The reduction of incubation time with increasing temperature also reduces the breeding period and affects the life history. The population maintained at 10 °C through the spring had increased incubation time and decreased growth rate, lower numbers of fertile females in spring and fewer brooding generations overall. The reproduction pattern observed in the Keyhaven population maintained at 10 °C is similar to the one recorded for *Praunus flexuosus* populations located in lower temperature environments in Scotland and Denmark (Blegvad 1922, Mauchline 1971). Temperature is therefore a major factor influencing variations in reproduction, because it affects incubation time, breeding biology, growth and the life cycle (Wittmann 1984).

Reproductive processes were rapid indicators of stress and highly sensitive to variations in the nitrogenous compounds (nitrate, nitrite and ammonia) and copper contamination. The sublethal toxicity (inhibition of reproductive processes) observed in *Praunus flexuosus* at 0.3 mg NH_3 l^{-1} after 96h at pH 8, is comparable with the acute toxicity (96h LC_{50}) at 1.7 mg NH_3 l^{-1}; and chronic toxicity (32d LC_{50}) at 0.232 mg NH_3 l^{-1} recorded on juveniles of *Mysidopsis bahia* at pH 8 (Miller *et al.* 1990). These data showed that nitrogen compounds (ammonia, nitrates and nitrites) can be toxic and can significantly influence the results of toxicity tests. In order to avoid the additional stress and toxicity created by nitrogen compounds on the assessment of toxic effects of a contaminant, experiments should be conducted at pH and nitrogen compound concentration within the naturally experienced range. In the present study copper addition to the seawater in toxicity tests did not change the pH or nitrogen compound levels. Results of a toxicity test, where pH and nitrogen compounds levels have not been controlled, or monitored, must be questionable.

Copper effects on reproduction were very disruptive. Brood survival was reduced to zero within 10 days in August. In June brood survival decreased within 8 days from 90% in controls to a range between 50% at lower copper concentrations and 20 % at the higher copper concentrations. Metals such as cadmium, mercury and zinc affect reproduction in mysids and other peracarids (amphipods), resulting in reduced numbers of juveniles released, reduced fertility and fecundity, and delay in sexual maturation (Nimmo *et al.* 1977, 1979; Gentile *et al.* 1982, 1983; Maltby & Naylor 1990, Khan *et al.* 1992, Conradi & Depledge 1999). Pesticides produced similar effects on reproduction in a range of mysids (Nimmo *et al.* 1980, McKenney 1982, McKenney 1986). The effects produced by copper in this study were more rapid and sharper than in the studies reported previously. No brood of the population in summer survived to the 10th day of copper exposure at a range of 5 to 200µg l^{-1} Cu_a.

Survival of broods was higher in June than in August because the abortion rate was lower in June than in August. Nevertheless the differences were not significant. The sizes of females in both experiments were similar (females in June were 14-16 mm long and in August 15-16 mm long). However, the metabolic rates of the spring and summer generation, with a 3 °C temperature difference between the seasons, were different (see Chapter 7). Females exposed to copper in June (17 °C) were from the spring generation and females exposed in August (20 °C) from the summer generation. Such differences may be responsible for the lower abortion rate observed in June. Comparison of the abortions observed at 5 µg l^{-1}

with the other treatments in August 1997 suggest that the first days of nauplioid development stage are more susceptible to abortion than a later nauplioid development stages.

Newborn juveniles released in copper contaminated seawater did not survive more than 24h, but some embryos incubated in the marsupium by the female survived for more than 8 days. SEM images of the marsupium in control females showed the oostegites perfectly closed. This suggests that the female can isolate the embryos from the external environment. The possible protection of the brood in the marsupium has been studied in mysids and other peracarids (Janssen & Hoese 1993, Charmartier & Charmartier-Daures 1994, Morrit & Spicer 1999, Johnston pers. comm). Experimental work with embryos of mysids suggest that female brood care is restricted to aeration of the embryos and physical protection (Johnston pers.comm.). Protection from copper contamination may occur when the female holds the marsupium closed, as observed in our experiments. Such isolation from the external environment can not be maintained for long periods because the marsupium fluid needs to be oxygenated and cleaned of embryo wastes, as control females do through ventilation. Marsupium morphology in the isopod *Glyptonotus antarcticus* suggested that the marsupium provided a mechanical protection and additional nutrition was provided by maternal secretion. In *G. antarcticus* constant oxygenation of the marsupium environment is made available through a special opening at the posterior of the marsupium (Janssen & Hoese 1993). The ability of mysid females to regulate the composition of marsupial fluid and protect embryos from external fluctuations such as salinity has been reported (McLusky & Heard 1971, Vlasbom & Elgershuiezen 1977). The euryhaline isopod (*Sphaeroma serratun*) female has also been reported to protect embryos in the marsupium from osmotic stress (Charmantier & Charmantier-Daures 1994). In the amphipod *Orchestia gammarellus* brood development in the marsupium is protected from strong osmotic fluctuations (Morrit & Spicer 1999). It may be possible that female mysids are able to provide some protection for embryos in the marsupium from the copper contamination present in the seawater. Therefore, malformations or malfunctions of the marsupium structure caused by copper may affect the protection of the embryos and survival of the brood.

The common reproductive response to copper in the June and August tests was the inability to breed. In both cases, after the release of broods (which were reduced in size) or abortion (total loss of brood), no more broods were produced. Fertilisation of eggs was similarly affected in all experiments. Fertilisation does not occur in the presence of copper.

Recovery experiments conducted in control seawater with individuals previously exposed to copper did not produce a recovery of the ability to reproduce. Individuals maintained in clean conditions for 14 days after any copper exposure were unable to produce broods. Less than 40% of females produced eggs, which, even when they were produced, were low in number, and in the majority of cases the eggs were malformed. Although individuals exposed to the lowest concentrations showed recovery of behavioural responses and reduction of change in colour, reproduction did not occur. Effects of pollution on the reproduction of mysids (Nimmo *et al.* 1977, 1979; Gentile *et al.* 1982, 1983; Khan *et al.* 1992) were recorded as a delay in the formation of broods and sexual maturity, and a decrease in brood size and fecundity. However, the inability to reproduce and the failure to fertilise eggs seen in this study has not been previously recorded.

The inhibition of reproduction observed in this study was caused by copper with the first effect being disruption of fertilisation. Fertilisation of the eggs only occurred in a non copper-contaminated environment by a male never exposed to copper. Results suggest that copper produced a long term disruption to males and females and that sperm is also damaged by copper. Necrosis of tissues occurred in sexually mature individuals. Toxic effects on sperm and male gonads are poorly studied. Sperm damage (DNA alteration) in plaice was produced by the organic contaminants PAH, PCB, PCDC (Nagler & Cyr 1996). Chitons exposed to copper failed to spawn. No abnormalities in oogenesis were observed but resorption of sperm in the male gonads occurred (Pashechenko & Tyurin 1997). In this study, marsupiums of *Praunus flexuosus* were frequently deformed. If eggs were fertilised (in a previously copper exposed female on recovery by a non-copper exposed male), incubation of the eggs to embryos failed. Copper decreased the production of yolk affecting egg quality and caused malformation of the marsupium affecting the ability to protect the development of the eggs to embryos, reducing viability.

Aberrant gonad development and carapace malformation was recorded for females and males of *Mysidopsis bahia* and *Mysidopsis bigelowi* exposed to cadmium during a 51 day test (Gentile *et al.* 1982). As the percentage of abnormalities increased, reproduction was inhibited and mortality occurred. The only cadmium concentration (5μg l^{-1} Cd) where no effect on reproduction was observed did not show significant mortality. In the present study the inhibition of reproduction was not a reflection of mortality. The 96h LC_{50} was estimated to be 30.8μg l^{-1} added copper (see chapter 5). Inhibition of reproduction occurred to a similar degree from the lowest to the highest levels of copper exposure.

Newborn juveniles did not survive at any copper exposure, and juveniles who were 1-3 weeks old, showed a very high mortality with copper (see Chapter 5). Juveniles did not achieve sexual maturity when exposed to copper and the only possibility for a population to continue is for juveniles to be released into a non-copper contaminated environment.

Brooding females exposed to copper may discharge the brood to minimise the energetic cost incurred from the incubation of the brood. The transfer of energy used in reproduction to survival of females has been suggested for amphipods (Maltby & Naylor 1990, Conradi & Depledge 1999). *Praunus flexuosus* is iteroparous and can delay breeding until better conditions occur. *P. flexuosus* showed a higher abortion rate at low copper exposure (5µg $l^{-1}Cu_a$) in August experiments when embryos were at an early development stage. Broods exposed at 5 µg $l^{-1}Cu_a$ were aborted (total loss of the brood) when embryos were at an early nauplioid stage, but when embryos were at post-nauplioid stage only part of the brood was lost. Broods exposed at high copper levels (75 µg $l^{-1}Cu_a$) did not show any difference in abortion rate with embryo development stage.

Disruption of metabolic processes was considered to be the cause of reduced growth and reproduction in *Mysidopsis bahia* exposed to insecticides (McKenney 1986). The decrease in number of juveniles released, sexual maturity and delay of breeding recorded, were related to the effect of insecticide on lipid metabolism. Interference in lipid metabolism created by contaminants has been measured in mysids and other crustaceans (McKenney 1982, 1985, Carr *et al.* 1980, Lee *et al.* 1981, Capuzzo *et al.* 1984). *Mysidopsis bahia* showed a shift to lipid metabolism with metal and pesticide exposure, suggesting that the increase in lipid metabolism created a lack of lipids available to produce gametes normally (McKenney & Matthews 1990). In the present study metabolism was significantly affected at all levels of copper exposure (see Chapter 7), but the O:N ratio showed that it shifted more to protein metabolism. Copper exposure in *Praunus flexuosus* caused a greater reliance on protein catabolism for energy production. *Praunus flexuosus* in natural conditions showed a higher lipid metabolism in winter when reproduction activity is dormant, and a lower lipid metabolism in summer when reproduction was active (see Chapter 7) although protein was the main metabolic substrate throughout the year. In the present study, lipid catabolism was decreased by copper, suggesting that lipids should be available for reproduction, but yolk production was reduced. There is no evidence of lipid restriction, although previous studies have referred to lack of lipids. The extreme catabolism of protein may cause an unbalance or lack of structural proteins that affects synthesis of yolk. The alteration of metabolism with

copper observed in this study (see Chapter 7) may require the transfer of energy from reproduction. Endocrine disruption in marine invertebrates occur when exposed to anthropenic chemicals (Depledge & Billinghurst 1999). The hormonal system involved in the reproductive processes was disrupted by metals (Cd, Zn), causing the impairs in gonadal follicule in mussels (Kluytmans *et al.* 1988), oogenesis in sea urchins (Khristoforova *et al.* 1984, Gnezdilova *et al.* 1985). In this study copper exposure decreased the production of yolk, gonads presented necrosis, and fertilisation failed. Such effects may be explained if the hormonal system was disrupted by copper.

8.5 SUMMARY

The reproduction pattern for *Praunus flexuosus* at Keyhaven, has a maximum reproductive activity in April and maximum reproductive effort in May/June. Temperature is the main factor underlying variations in reproduction. Increasing temperature decreases incubation time and the reproductive pattern appears to be adapted to compensate for temperature effects.

Reproduction processes were very good and rapid indicators of stress, and sublethal toxicity of nitrogenous compounds and copper. Reproduction was inhibited at the following levels: ammonia 0.3 mg l^{-1}, nitrate 20 mg l^{-1}, nitrite 2 mg l^{-1}, in 96h at pH 8. Fertilisation of the eggs did not occur at any copper treatment. Brooding females exposed to copper suffered a high abortion rate. The few juveniles that were released did not survived more than 24h in the presence of copper.

Copper effects on *Praunus flexuosus* were very fast and more disruptive that in other peracarids, where toxicant (copper, cadmium, oil, pesticides) effects were observed as a decrease on brooding females and brood size. Survival of the embryos carried by the females exposed to copper was minimal. Necrosis and morphological damage occurred with copper exposure, would decrease marsupium protection of the embryos that a female in normal conditions could provide. Alterations in metabolism occurred with copper exposure, which may affect normal reproductive functions.

The inability to produce broods observed in this study appears to be a long term effect, as specimens previously exposed to copper did not produce any brood after 2 weeks in seawater controls. Results suggest that copper damages male and female reproductive processes. Fertilisation failed suggesting that copper damaged sperm. Disruption on the hormonal system involved in the reproductive processes may explain the disruption observed in the female gonads, where yolk production decreased impairing the production of eggs, and necrosis occurred.

CHAPTER 9

GENERAL CONCLUSIONS AND DISCUSSION

CHAPTER 9: GENERAL CONCLUSIONS AND DISCUSSION

The toxicity of dissolved copper in seawater was examined in a common coastal population of mysids. The life cycle and ecophysiology of *Praunus flexuosus* in the Solent (Southampton) were examined under natural conditions throughout the year, and responses to dissolved copper were determined in the laboratory.

The results of the toxicity tests in this study showed pronounced seasonal variations in copper toxicity. Lethal and sublethal characters (survival, behaviour, metabolism, reproduction, and bioaccumulation) in response to copper toxicity were significantly different between winter and summer. Responses measured indicated highest copper toxicity in summer.

Considering toxicity testing as tool for an assessment of pollution, this thesis provides evidence that, for this type of organism, seasonal variation was a very important factor to include in toxicity testing design. For example, while the mortality of *Praunus flexuosus* population after 10 days of copper exposure (5, 25, 75 and 200 $\mu g\ l^{-1}$ Cu_a) was insignificant in winter, lethal effects (96h LC_{50} = 30.8$\mu g\ l^{-1}$) occurred at every copper exposure level in summer (Chapter 5).

Variations in the pollutant delivery to the aquatic environment, organism physiology (age, weight, sexual and moulting cycles) and changes of ambient water quality parameters may result in seasonal variations in the pollutant concentrations accumulated by the organisms (Phillips 1980, White & Rainbow 1987, Yamamoto *et al.*1987, Swaileh & Adelung 1995, Brown & Depledge 1998).

In the present study seasonal variations were found not only on the copper accumulated, but also in the effects of copper in survival, behaviour, metabolism and reproduction. Toxicity tests were carried out at different seasons, exposing specimens (in different seasonal physiological states) to the same pollutant concentration and water quality parameters. The variations in toxicity observed were related only to the physiological changes of the organisms with season.

The variations in toxicity were not related to variations in copper concentrations at the different tests. The reasons supporting (Chapter 4) the conclusion are a) the labile or Chelex-available copper concentrations did not vary significantly during 48h (time of seawater renewal) in toxicity tests; b) the water quality parameters were maintained constant; c) only temperature and photoperiod varied at the different tests, which do not affect significantly the

copper speciation (Byrne *et al.* 1988). Moreover, copper speciation is dominated by organic complexation (Buffle 1988, Bruland *et al.* 1991, Donat & Bruland 1995, Wells *et al.* 1998). Therefore, it is assumed that the amount of labile copper was similar at the different tests. Although organic complexation during the toxicity test was observed (as in Hall *et al.* 1997), a decrease of the total dissolved copper was only significant when the organisms were under stress, suggesting that the organisms themselves affected organic complexation. Such suggestion was also proposed by Zirino *et al.* (1998).

In order to determine whether there are seasonal variations in the biology of the mysid population in uncontaminated conditions, different aspects of the biology were examined. Every biological parameter studied showed seasonal differences in uncontaminated conditions. Field and laboratory studies of a *Praunus flexuosus* population collected at an unpolluted location allowed characterisation of the life cycle. Growth was most active in spring and summer, when reproduction processes occurred and the rest of the year individuals overwinter (Chapter 3 and 8). The variations in the life cycle for this species at different geographical locations suggested that intensity of the season plays an important role. Although metabolism (respiration and excretion) in *Praunus flexuosus* was protein based throughout the year (Chapter 7), there were significant variations with the season. The use of lipid and/or carbohydrate decreased in spring and summer. In the other hand, the metabolism in *P. flexuosus* was found to be relatively independent of temperature (Chapter 7). This was shown by the Q_{10} found in spring at the population held at 10 and 20 °C, and the metabolic rates measured at 20°C in spring and summer. Total copper content on the body mass of specimens in the field and controls of toxicity tests were significantly different between winter and summer, being higher in summer. Similar results in bioaccumulation were found in other crustaceans in non-polluted environments (Yamamoto 1987, Swaileh & Adelung 1995).

Contamination usually occurs with several chemicals and species in the real environment. In this study toxicity tests were carried out in the absence of sediment, and only one species was exposed to one chemical (copper) because of the technical limitations to study the chemical and biological parameters concerned with toxicity.

Results from the toxicity tests showed that copper toxicity in *P. flexuosus* was significantly higher in summer than in other seasons, and responses showed a low tolerance to copper when comparing with other mysid species and peracarids (Chapters 5,6,7,8).

The assessment of toxicity using the 96h LC_{50} was a more sensitive method in summer than in winter. Although no lethal effects were observed in winter at a range from 5 to 300 $\mu g\ l^{-1}$ Cu_a after 10 days of exposure, metabolism and behavioural responses in winter suggested that copper exposure longer than 10 days could result in lethal effects (Chapter 5 and 7). The behavioural and metabolic responses to copper were very sensitive parameters. Bioaccumulation analysis (Chapter 6) showed that copper was accumulated at a higher rate in summer than in winter, confirming the congruence of the results observed with the previous parameters.

Metabolism differences may increase the metal uptake affecting toxicity. The aim of this study was to test the seasonal effect, including temperature, photoperiod and natural physiological changes, avoiding temperature stress produced by non-field temperature conditions in the laboratory.

The effects of copper on reproduction observed in this study (Chapter 8) were very disruptive. Copper caused the death of the broods and long term inhibition of reproduction. Survival of the embryos was significantly higher in spring than in summer but fertilisation failed at any copper concentration at both seasons (spring and summer). Individuals failed to recover the ability to reproduce. Results (Chapter 8) suggested that copper damaged sperm, eggs and gonads resulted in the inability to fertilise. Endocrine disruption in marine invertebrates occur when exposed to anthropogenic chemicals (Depledge & Billinghurst 1999), and metals could impair in different reproductive processes. The hormonal system involved in the reproductive system of *Praunus flexuosus* could be disrupted by copper.

This study did not examine biochemical parameters and cellular responses. At the moment, there are no studies on biochemical responses to metal on mysids that could suggest a specific mechanism responsible for the seasonal variation in toxicity observed in this study.

Metabolic responses to copper indicated that copper may interfere with calcium metabolism, ionic regulation, enzymatic processes or other biochemical processes that are especially critical during moulting. Differences in metal uptake and in metal metabolism with the season may occur, as growth, reproduction and moulting is more active in spring-summer than in winter. Specimens showed the highest sensitivity to toxicity in summer, when those physiological functions are most active, and especially at moulting. In addition, the mechanisms of metal metabolism and ability to detoxify may suffer some variation when the physiological state changes, affecting consequently the sensitivity to toxicity.

For example, increase in metal uptake occurs during moulting processes (White & Rainbow 1984, Rainbow 1997). There are shared pathways and interactions between calcium regulation and trace metals (Wright 1995). During moulting copper uptake from the seawater would be greater. In this study moulting specimens did not survive in toxicity tests. Saroglia & Scarano (1979) and Cripe (1994) observed an increase in sensitivity to metals during moulting in shrimp and mysids.

On the other hand, metal metabolism changes occur during moulting. The concentration of circulating hemocyanin decreases dramatically during moulting (Hagerman 1983), and its degradation releases significant amounts of copper into the cytosolic metal pools (Engel 1987). Changes in the metals bound to metallothioneins (MT) occurred over a period of 90 min after ecdysis in the blue crab (Engel & Brouwer 1991). Metal competition with calcium and with zinc in metallo-enzyme glutamate dehydrogenase may occur, affecting enzymatic processes involved in moulting and osmoregulation of cell volume (Hochachka & Somero 1973).

The exposure to environmental contaminants in organisms induces biochemical responses involved in the regulation, excretion or detoxification systems (Stegeman *et al.* 1992). Environmental contaminants may enhance oxidative stress in aquatic organisms (Winston 1991). Antioxidants defences could be overcome by prooxidant agents resulting in many physiological disfunctions. For example, glutathione peroxidase can potentially detoxify as it acts as an antioxidant, binding a number of metals (Christie & Costa 1984, Hanna & Mason 1992).

At the same time many biochemical mechanisms such as metallothioneins synthesis, enzyme system activity (including antioxidants) vary with season, as well as physiological functions of the individual such as growth, moulting and reproductive cycle (Overnell *et al.* 1987, Engel & Brower 1991, Collier *et al.* 1995, Eggens *et al.* 1996, Hylland *et al.* 1998, Mouneyrac *et al.* 1998, Rotchell *et al.* 1999).

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APPENDICES

APPENDIX I

A. Chelex Column Extraction Method

Filtered (0.4µm) sample was passed through a column with the chelating resin Chelex-100 (Bio-Rad) 200 mesh to achieve the separation of the metal. A Teflon (FEP) separation funnel (125ml) was connected by an adapter to a Bio-Rad 13ml poly-propylene column which was filled with 200mg of Chelex resin (sodium form). All equipment was acid washed in 50% nitric acid and throughly rinsed with Milli-Q water prior to use. The Chelex resin in the column was cleaned prior to use using a micropipette to add to the resin 2 ml of 2M nitric acid to remove the metal ions and 3ml of Milli Q water to remove the excess of acid. To convert the resin into the ammonium ion form, 2ml of 2M ammomiun hydroxide was added. Finally to remove the excess of ammonia 3ml of Milli Q water was added. Solutions were allowed to pass through the column under gravity.

Samples for available copper measurements were unacidified and at a natural pH (~8). Samples for total copper measurements were acidified (1µl/ml with SBD HNO_3). Efficient copper metal extraction on the Chelex 100 resin required pH ≥ 5 and flow rate $<$ 4ml min^{-1} (Fang 1994). Acidified samples were post-neutralised, buffered with 1M ammonium acetate at pH 5.5 and passed through the Chelex column at a rate of 2.5 -3 ml/min using a peristaltic pump. Salts and unwanted cations on the resin needed to be removed before elution of the metal from the resin. The pre-elution procedure consisted of addition of 2ml of Milli Q water to remove excess seawater salts, addition of 2ml of 1M ammonium acetate to remove Ca and Mg salts and addition of 2ml of Milli Q water to remove the excess of ammonium acetate. After the resin was prepared, 2x1ml of 2M nitric acid were added for the removal of the metal ions and the eluate collected in a vial. Gravimetric measurements of the volume of the samples and eluates were made. Sample concentrations were measured with a 1100B (Perkin-Elmer) Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS). Concentrations of copper in the concentrates were transformed on the copper concentration values ($\mu g\ l^{-1}$) of the original sample using an Excel spreadsheet.

APPENDIX I

Preparation of Reagents

1M Ammonium acetate. 143ml of isothermally distilled (ITD) ammonia plus 57ml sub-boiled (SB) acetic acid, made up to 1L with sub-boiled distilled (SBD) water.

2M Ammonium hydroxide. 143ml of ITD ammonia (~7M) to 500ml with Milli Q water.

2M Nitric acid. 63ml of SBD concentrated nitric acid (16M) to 500ml Milli Q water.

The copper standard solution was prepared from a Fisons standard solution (1mg ml^{-1}) and 2M SBD nitric acid.

Blanks used were Milli Q water and Chelex stripped open ocean sea water from the Hebridean Shelf.

GFAAS standard working solutions were 0, 5, 25, 75, 200 $\mu g\ l^{-1}$.

Toxicant solution 100ppm copper (100 $\mu g\ ml^{-1}$) was prepared from the Fison standard solution (1mg ml^{-1}) and Milli Q water.

B.1. Calibration of pipette

To ensure the autopipettes were correctly adjusted and being used properly, they were calibrated gravimetrically

	Mean	SD	RSD
25µl pipette (5-40µl)	0.0258	0.000096	0.379963
125µl pipette (40-200µl)	0.1274	0.00014	0.112332

B.2. Calibration of the spectrophotometer

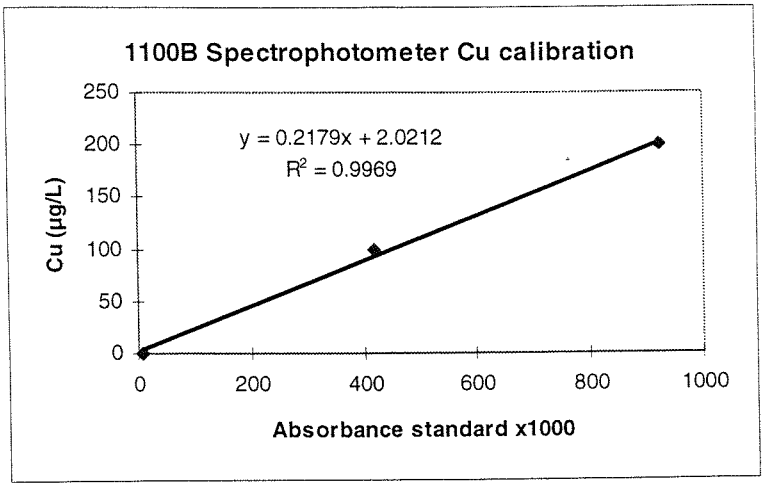

Fig. A.1. Calibration of the Spectrophotometer GFAAS (1100B).

C. Method performance experiments

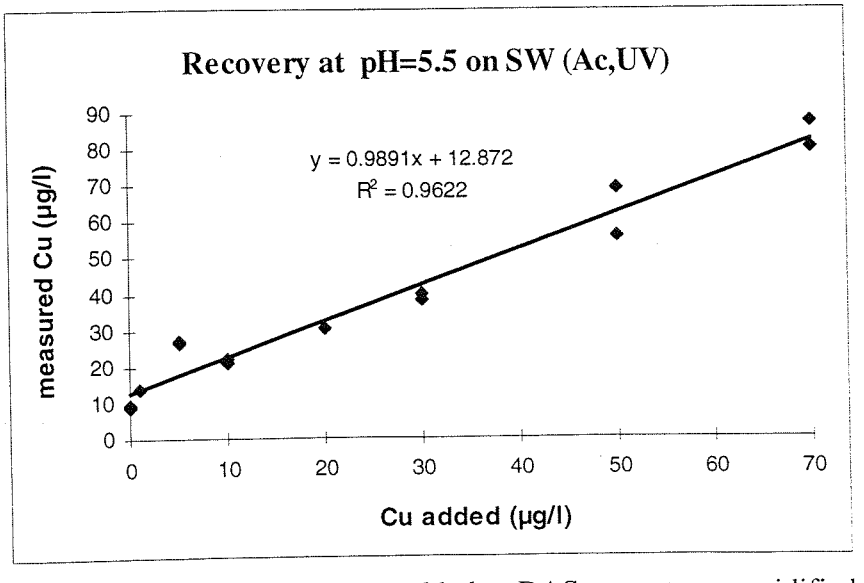

Fig. A.2. Recovery of copper added to BAS seawater on acidified and UV irradiated samples at pH 5.5. (total copper)

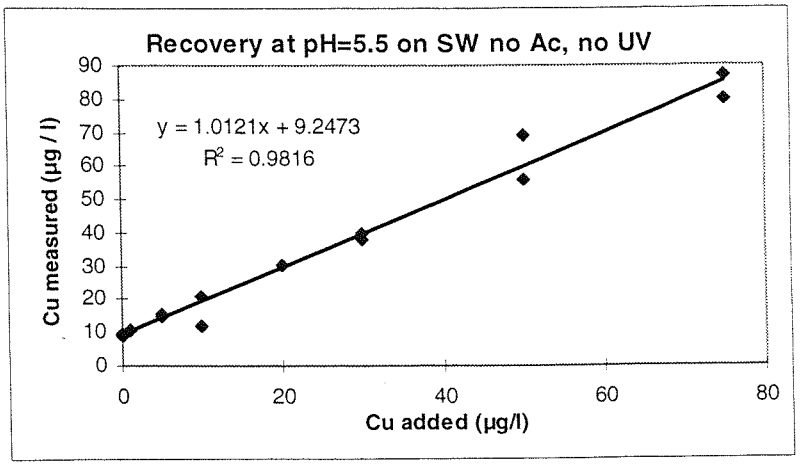

Fig. A.3. Recovery of copper added to BAS seawater on no-acidified and no UV irradiated samples at pH=5.5. (Chelex-available)

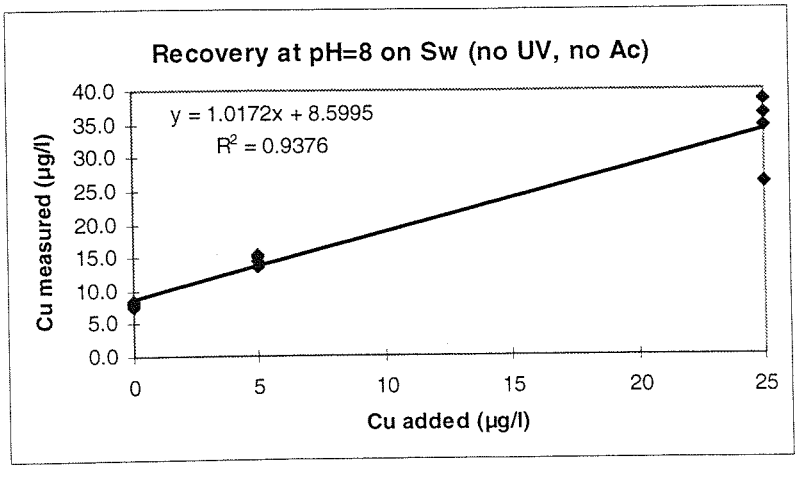

Fig. A.4. Recovery of copper added to BAS seawater in no acidified and no UV irradiated samples at pH 8. (Chelex- available copper)

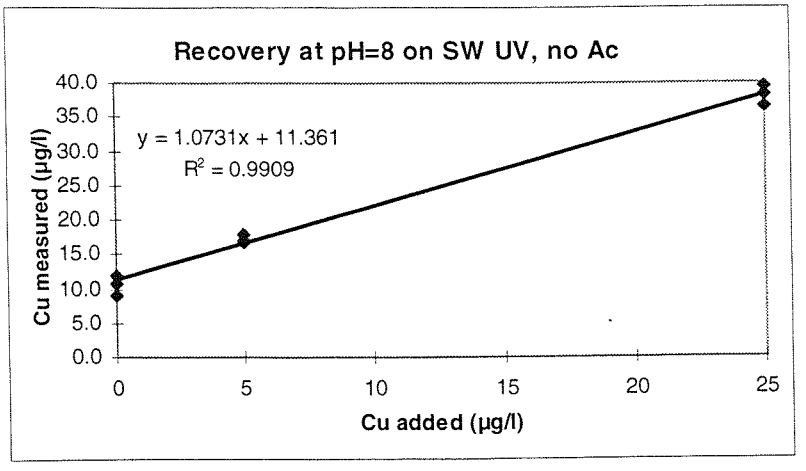

Fig.A.5. Recovery of the copper added to BAS seawater on UV irradiated and no acidified samples at pH 8. (Chelex-available)

Copper concentrations (µg l^{-1}) of the same sample with different treatments.

no Ac.UV	2.84	2.25	2.75
Ac.UV.	6.78	5.69	6.57
ratio	0.4	0.4	0.4

D. Calculations

Overall recovery was calculated from the regression analysis of copper measured against copper spikes added (y=mx+b).

Recovery values are given in % Recovery, m= Cu measured/ Cu added.

Detection limit = 2 * SD of blank value (95%),

Relative Standard Deviation RSD = (SD/Mean) * 100.

(%) Frequency of the life cycle stages (brooding female, female, male, juvenile) during the year

Month	BroodFem.	Female	Male	Juvenile	N
January	0.0	42.0	30.0	28.0	59
February	0.0	41.0	43.0	16.0	46
March	6.6	46.6	43.3	3.3	67
April	68.0	0.0	32.0	0.0	62
May	36.0	12.0	33.0	19.0	72
June	30.0	26.0	25.0	24.0	86
July	44.0	30.0	24.0	2.0	74
August	52.0	12.0	22.0	14.0	53
Sept.	14.0	57.0	25.0	4.0	48
October	2.0	29.0	34.0	35.0	56
November	0.0	34.0	28.0	39.0	42
December	0.0	36.0	22.0	41.0	47

Size - length (mm) of females, males and juveniles along the year

Month	Females		Male		Juvenile	
	Mean	SD	Mean	SD	Mean	SD
January	13.20	1.10	12.50	0.58	7.75	0.50
February	16.67	1.03	14.00	0.89	10.00	1.00
March	15.56	1.26	13.23	1.09	10.50	0.58
April	20.11	0.99	16.60	0.55	/	/
May	17.20	4.03	15.09	2.23	9.00	0.71
June	15.33	2.63	12.20	1.63	9.45	1.57
July	14.07	1.94	13.07	1.98	8.00	0.00
August	14.31	3.88	11.44	1.75	7.00	0.82
Sept.	13.73	2.67	11.29	1.11	7.67	0.58
October	13.74	2.09	12.87	1.18	6.64	1.36
November	14.67	3.21	11.40	1.52	8.00	0.82
December	14.25	2.50	11.50	0.58	7.00	1.00

APPENDIX II

Chelex method performance at pH 5.5

Samples no UV irradiated, no acidified

Cu (µg/l) added	Cu (µg/l) measured
Blank	0.06
Blank	0.04
Blank	0.05
Blank	0.09
Blank	0.12
0	8.65
0	9.66
1	10.64
1	10.59
1	10.48
5	15.79
5	14.94
10	12.14
10	20.72
20	30.32
20	30.64
30	38.36
30	39.84
50	56.01
50	69.04
75	87.20
75	80.03

Samples acidified and UV irradiated

Cu (µg/l) added	Cu (µg/l) measured
Blank	0.08
Blank	0.08
Blank	0.07
Blank	0.09
Blank	0.16
0	8.71
0	9.76
1	11.75
1	12.96
5	27.32
5	26.40
10	21.92
10	20.79
20	31.54
20	30.22
30	38.58
30	38.74
50	57.16
50	67.89
70	79.31
70	86.76

Chelex method performance at pH 8.8

Samples no UV irradiated no acidified

Cu (µg/l) added	Cu (µg/l) measured
Blank	0.09
Blank	0.04
Blank	0.08
Blank	0.01
0	7.60
0	8.38
0	8.13
0	7.68
5	13.40
5	14.90
5	14.50
5	15.20
25	36.39
25	34.48
25	38.54
25	26.05

Samples UV irradiated

Cu (µg/l) added	Cu (µg/l) measured
Blank	0.08
Blank	0.06
Blank	0.07
Blank	0.01
0	10.70
0	11.88
0	11.99
0	9.05
5	17.21
5	17.16
5	17.88
5	16.94
25	38.18
25	36.58
25	39.35

APPENDIX II

Background total dissolved copper (µg/l) in toxicity test and field samples

Tox.Test sample	Cu (µg/l)
Dec1996,1	1.89
Dec1996,2	2.01
Dec1996,3	2.08
Feb1997,1	6.14
Feb1997,2	5.43
Feb1997,3	6.38
Ju/Aug1997,1	8.59
Ju/Aug1997,2	8.40
Ju/Aug1997,3	8.84
Ju/Aug1997,4	8.24
Ju/Aug1998,1	5.97
Ju/Aug1998,2	5.49
Ju/Aug1998,3	5.41
Ju/Aug1998,4	6.32

Field sample	Cu (µg/l)
F.Dec96,1	0.80
F.Dec96,2	1.82
F.Dec96,3	1.63
F.Dec96,4	1.91
Jan1997,1	0.58
Jan1997,2	0.84
Jan1997,3	0.53
Jan1997,4	0.75

Copper concentrations on samples after storage

control (SW), copper spike 5 or 25 µg/l (sp)
a-no treatment, b-acidified in storage bottle, c- acidified outside the storage bottle

Sample	µg/l Cu
SWBlank1	0.02
SWBlank2	0.01
SWBlank3	0.06
SWBlank4	0.08
SWBlank5	0.01
SWBlank6	0.02
SWBlank7	0.02
SW1a	2.56
SW2a	3.09
SW3a	2.86
SW1b	6.55
SW2b	6.41
SW3b	6.68
SW1c	4.37
SW2c	4.44
SW3c	4.41

Sample	µg/l Cu
sp5a	3.96
sp5a	4.12
sp5a	3.74
sp5b	7.54
sp5b	7.59
sp5b	7.6
sp5c	5.68
sp5c	6.01
sp5c	5.69
sp25a	19.6
sp25a	20.3
sp25a	18.1
sp25b	35.74
sp25b	36.12
sp25b	36.41
sp25c	31.26
sp25c	32.78
sp25c	32.83

Chelex-available dissolved copper measured during toxicity test

Time 0		Time 24h		Time 48h	
Sample	Cu (µg/l)	Sample	Cu (µg/l)	Sample	Cu (µg/l)
Control SW1	6.72	Cont(no mysid)	7.92	Cont(no mysid)	9.11
Control SW2	6.52	Cont(no mysid)	7.44	Cont(no mysid)	9.17
Control SW3	6.14	Control	8.36	Cont(no mysid)	9.00
Cont(no mysid)	7.62	Control	7.07	Cont(no mysid)	8.16
Cont(no mysid)	8.49	Control	5.99	Control	7.60
Cont(no mysid)	7.82	Control	6.40	Control	8.70
Control 1	6.98	5 added	11.39	Control	7.92
Control 2	8.08	5 added	11.21	Control	7.92
Control 3	8.05	5 added	11.15	Control	7.34
Control 4	7.57	5 added	8.90	Control	7.13
Control 5	8.34	25added	19.07	Control	6.61
Control 6	7.89	25added	19.50	5added	10.44
5 added	10.57	25added	18.87	5added	9.88
5 added	10.78	25added	19.07	5added	10.76
5 added	10.75			5added	10.27
5 added	11.51			25added	18.62
25added	20.90			25added	18.70
25added	20.40			25added	19.19
25added	19.94			25added	18.21
25added	22.15				

Total dissolved copper measured in the toxicity test

Time 0	
Sample	Cu (ug/l)
Cont(SW)	16.53
Cont(SW)	13.88
Cont(SW)	14.14
Cont(SW)	12.09
Cont(no mysid)	13.67
Cont(no mysid)	15.07
Cont(no mysid)	14.94
Control1	16.06
Control2	15.54
Control3	15.48
Control4	15.53
5 added	17.76
5 added	17.90
5added	22.21
5added	19.35
25added	32.86
25added	32.10
25added	32.08
25added	32.25

Time 48h	
Sample	Cu (ug/l)
Cont(no mysid)	15.36
Cont(no mysid)	14.32
Cont(no mysid)	15.48
Control1	13.55
Control2	13.64
Control3	14.72
5 added	15.68
5 added	16.05
5 added	15.28
5 added	16.67
25added	26.65
25added	25.87
25added	25.46
25added	25.75
25added	25.44

APPENDIX II

% Mortality of the different life cycle stages when exposed to copper (C,5,25,75,200μg/l Cu_a) in December at 10°C and February at 5°C for 10days

Exposure	% Mortality	
(μg/l Cu_a)	Dec.	Feb.
C	0.0	0.0
5	0.0	1.3
25	1.3	0.0
75	0.0	0.0
200	0.0	0.0
	N=30	N=30

% Mortality of the different life cycle stages when exposed to copper (C,5,25,75,200μg/lCu_a) in August at 20°C

N=10 for every life cycle stage

		% Mortality		
	Hours of exposure	Juvenile	Female	Male
Controls	12	0	0	0
	24	0	0	0
	48	0	0	0
	72	0	0	0
	96	0	0	0
	168	0	0	0
	240	0	0	0

		% Mortality		
	Hours of exposure	Juvenile	Female	Male
5μg/l Cu_a	12	0	0	0
	24	0	10	0
	48	0	10	0
	72	0	10	0
	96	0	30	0
	168	0	30	0
	240	0	30	0

		% Mortality		
	Hours of exposure	Juvenile	Female	Male
25μg/l Cu_a	12	0	0	0
	24	40	0	0
	48	50	20	20
	72	50	40	40
	96	50	60	40
	168	50	60	40
	240	50	60	40

APPENDIX II

	Hours of exposure	% Mortality Juvenile	Female	Male
75μg/l Cu_a	12	0	20	0
	24	40	40	0
	48	40	40	0
	72	100	50	0
	96	100	60	40
	168	100	80	90
	240	100	90	90

	Hours of exposure	% Mortality Juvenile	Female	Male
200μg/l Cu_a	12	60	20	0
	24	100	30	20
	48	100	30	30
	72	100	30	30
	96	100	70	80
	168	100	80	90
	240	100	80	90

Behavioural responses to copper after 96h of exposure in summer
(C,5,25,75,200μg/l Cu_a) in August at 20°C
Behavioural responses: N-normal, Ab- abnormal, D-disruptive, SE- severe effect.

	C	5	25	75	200
Sensory capacity	N	N	N	A	D
Swimming	N	N	A	D	SE
Equilibrium	N	N	N	A	D
Feeding	N	N	N	A	D
Daily activity	N	N	A	D	SE
Morphological changes	N	N	A	D	SE
Cannibalism	N	N	N	A	D

APPENDIX II

Total copper content in body mass (μg/g.drywt) after 7 days of copper exposure (C, 5, 25, 75, 200 μg/l) in December at 10°C

N=5, samples were grouped to reach the minimum of 5mg.drywt

Copper exposure	μg/g.dry wt	Copper exposure	μg/g.dry wt
C	49.65	75	57.14
C	42.55	75	68.00
20	47.46	100	75.5
20	34.48	100	57.89
35	60.00	200	72.22
35	62.96	200	95.45
50	78.43	300	86.57
50	52.33		

Total copper content in body mass (μg/g.drywt) after 10 days of copper exposure (C, 5, 25, 75, 200 μg/l) in February at 5°C

Copper exposure	μg/g.dry wt	Copper exposure	μg/g.dry wt
C	42.47	75	70.00
C	38.10	75	60.00
C	32.35	75	68.00
C	43.08	75	55.56
C	43.33		
		200	82.76
5	34.78	200	47.89
5	37.50	200	104.17
5	36.36	200	66.13
5	48.98		
5	43.40		
25	35.59		
25	40.38		
25	50.77		
25	35.29		
25	50.60		

Total copper content in body mass (µg/g.drywt) after 10 days of copper exposure (C, 5, 25, 75, 200 µg/l) in August at 20°C

N=5, samples were grouped to reach the minimum of 5mg.drywt

Copper exposure	µg/g.dry wt	Copper exposure	µg/g.dry wt
C	50.00	25	108.93
C	49.68	25	72.09
C	50.78		
		75	95.35
5	70.77		
5	63.06	200	77.05
5	83.33	200	87.10
		200	67.21
		200	58.88

Total copper content in body mass (µg/g.drywt) of females after 7 days of copper exposure (C, 5, 25, 75, 200 µg/l) in June at 17°C

Samples were grouped to reach the minimum of 5mg.drywt

Copper exposure	µg/g.dry wt
C	31.33
C	42.86
5	49.43
5	44.74
25	46.95
25	44.52
75	74.32
75	19.63
200	70.37
200	73.62

Total copper content in body mass (µg/g.drywt) of females maintained for 2 weeks in control seawater, after the 7 days of copper exposure in June at 17°C

previous exposure	µg/g.dry wt
C	38.55
C	33.6
C	32.08
C	43.92
5	37.4
25	40.38
75	35.71
200	63.04

APPENDIX II

Metabolic rates (Oxygen consumption-Ox, Ammonia Excretion- Amm, and O:N ratio) at different seasons

W-Winter, Sp-Spring at 10 and 20°C (June) and S- Summer

Oxygen consumed (μgOx/h/mg.drywt)

Ox-W	Ox-Sp10	Ox-Sp20	Ox-S
0.974	1.640	1.800	5.600
1.162	1.440	1.630	3.200
0.610	1.380	2.340	2.470
1.570	1.130	3.130	3.080
1.820	2.030	2.220	3.700
0.700	1.280	2.650	5.170
1.656	2.120	2.860	2.590
0.944	0.890	3.170	1.940
1.380	2.080	3.980	2.950
0.950	3.440	6.090	2.550

Ammonia excreted (μmolAmm/h/mg.drywt)

Amm- W	Am.-Sp10	Am.-Sp20	Amm-S
0.002	0.038	0.057	0.064
0.012	0.039	0.064	0.029
0.008	0.048	0.072	0.038
0.014	0.034	0.040	0.023
0.006	0.016	0.049	0.026
0.008	0.007	0.069	0.060
0.009	0.071	0.055	0.034
0.007	0.066	0.124	0.020
0.005	0.068	0.074	0.039
0.006			0.027

O:N ratio

O:N- W	O:N-Sp10	O:N-Sp20	O:N-S
6.270	18.900	2.900	5.467
4.970	9.120	4.370	6.928
7.280	1.680	2.750	4.057
5.610	2.290	2.820	8.349
11.320	2.540	2.080	8.795
8.870	1.470	3.600	5.371
17.910	3.030	4.060	4.691
10.240	7.280	4.980	5.912
	4.510	5.140	4.764
			5.964

Oxygen consumption at the life cycle stages in June at 10 and 20 °C

Brooding Female- Bf, Female-F, Male - M, Juvenile - J, and Newborn juvenile -y

μgOx/h/mg.drywt

BF-10	BF-20
1.218	2.574
1.411	1.490
1.468	2.491
1.428	0.945
1.532	1.859

F-10	F-20
0.980	1.572
1.377	1.514
1.322	1.631
1.085	0.234
1.575	2.219

M-10	M-20
1.232	2.646
2.032	3.127
2.240	4.474
0.855	3.174
0.792	1.533

J-10	J-20
2.000	3.976
3.303	3.571
7.373	9.877
3.373	4.137
4.707	6.086

Y-10	Y-20
12.163	8.417
8.598	4.880
8.305	2.726
8.780	6.866
	5.538

Ammonia excretion at the life cycle stages, in June at 10 °C and 20 °C
Brooding Female- Bf, Female-F, Male - M, Juvenile - J, and Newborn juvenile -y
µmolAmm/h/mg.drywt

Bf-10	Bf-20	F-10	F-20	M-10	M-20
0.039	0.036	0.038	0.039		0.057
0.062	0.033	0.039	0.040	0.007	0.033
0.041	0.038	0.034	0.049	0.016	0.064
0.047	0.028	0.048	0.030	0.003	0.072
0.053	0.034	0.060	0.038	0.002	0.046

J-10	J-20	y-20	y-10
0.040	0.069	0.093	0.112
0.071	0.055	0.127	0.081
0.066	0.124	0.084	0.007
0.096	0.125	0.115	0.011
0.068	0.074	0.228	

O:N ration at the life cycle stages in June at 20 and 10°C
Brooding Female- Bf, Female-F, Male - M, Juvenile - J, and Newborn juvenile -y

Bf-10	Bf-20	F-10	F-20	M-10	M-20
2.03	4.49	1.68	2.81	18.90	2.90
1.48	2.85	2.29	2.82	9.12	5.92
2.33	4.09	2.54	2.08	18.56	4.37
1.98	2.35	1.47	4.88	25.80	2.75
1.88	3.37	1.70	3.65		3.88

J-10	J-20	y-10	y-20
3.26	3.60	7.04	5.48
3.03	4.06	7.18	2.40
7.28	4.98	6.89	2.03
2.29	2.28	63.48	3.72
4.51	5.14		1.52

Winter Oxygen Consumption after copper exposure
24h, 96h and 10days of copper exposure (C, 5,25,75,200 µg Cu /l)
µgOx/h/mg.drywt

	Control	5	25	75	200
24h	0.974	1.327	2.077	1.030	1.215
	1.162	1.454	1.152	0.760	0.645
	0.606	1.300	1.993	0.170	0.091
	1.574	0.903	1.073	0.390	0.521
	1.823	1.273	1.237	0.480	0.397
96h	1.212	1.110	1.307	1.130	2.556
	1.387	1.278	0.567	1.050	0.938
	0.492	1.083	2.286	0.730	0.713
	1.152	1.441	0.924	1.380	0.645
	0.784		1.293	1.200	0.373
10d	0.700	0.729	1.179	0.170	1.478
	1.656	1.238	0.348	0.580	0.496
	0.944	0.444	1.157	0.160	0.533
	1.377	1.035	0.386	1.270	0.623
	0.945	0.826	0.391	0.280	0.497

Summer Oxygen Consumption after copper exposure
24h, 96h and 10days of copper exposure (C, 5,25,75,200 ug Cu /l)
µgOx/h/mg.drywt (*) moulting or moribund cases

	C	5	25	75	200
24h	5.598	5.668	*2.033	*1.317	*0.351
	3.197	2.993	1.736	2.740	*1.906
	2.468	3.505	2.601	2.157	2.742
	3.081	4.045	1.450	1.336	2.945
	3.673	4.698	1.405	2.849	2.645
96h	3.742	3.554	2.472	1.738	1.713
	1.978	1.388	2.148	2.347	1.194
	1.285	1.351	2.022	1.491	*1.265
	2.043	1.580	1.404	*3.202	*0.975
	1.584	2.523		2.681	*3.559
10d	*5.171	1.703	0.281	*0.727	3.171
	2.589	1.797	1.912	*2.084	1.783
	1.938	1.843	1.574	1.131	2.151
	2.949	1.794	1.923	1.682	2.293
	2.545	2.404	1.681	*2.296	*2.697

APPENDIX II

Winter Ammonia Excretion after copper exposure
24h and 10d of copper exposure (C, 5, 25, 75, 200 µg.Cu/l)
µmolAmm/h/mg.drywt

C-24h	5-24h	25-24h	75-24h	200-24h
0.0025	0.0007	0.0054	0.0093	0.0151
0.0116	0.0013	0.0024	0.0168	0.0069
0.0076	0.0008	0.0082	0.0054	0.0199
0.0135	0.0008	0.0062	0.0148	0.0145
0.0103	0.0014	0.0011	0.0132	0.0103

C-10d	5-10d	25-10d	75-10d	200-10d
0.0078	0.0043	0.0118	0.0087	0.0272
0.0091	0.0072	0.0065	0.0156	0.0162
0.0067	0.0078	0.0143	0.0044	0.0224
0.0048	0.0110	0.0093	0.0097	0.0204
0.0058	0.0081	0.0088	0.0106	0.0220

Summer Ammonia Excretion after copper exposure
24h and 10days of copper exposure (C, 5,25,75,200 µg Cu /l)
µmolAmm/h/mg.drywt

C-24h	5-24h	25-24h	75-24h	200-24h
0.064	0.042	0.188	0.038	0.069
0.029	0.039	0.071	0.086	0.075
0.038	0.063	0.087	0.090	0.093
0.023	0.059	0.049	0.081	0.074
0.026	0.108	0.046	0.119	0.061

C-10d	5-10d	25-10d	75-10d	200-10d
0.060	0.106	0.111	0.101	0.134
0.034	0.044	0.101	0.156	0.093
0.020	0.074	0.100	0.094	0.091
0.039	0.072	0.079	0.097	0.081
0.027	0.088	0.078	0.121	0.117

O:N ratio in Winter after copper exposure
24h and 10days of copper exposure (C, 5,25,75,200 µg Cu /l)

C-24h	5-24h	25-24h	75-24h	200-24h
24.69	67.47	23.83	6.91	5.03
6.27	79.48	30.54	2.82	5.81
4.97	73.61	15.18	1.99	0.29
7.28	55.35	10.79	1.63	2.24
11.39	56.79	69.4	2.28	2.41

C-10d	5-10d	25-10d	75-10d	200-10d
5.61	10.48	6.25	1.2	3.4
11.32	10.82	3.34	2.31	1.91
8.87	3.56	5.05	2.23	1.48
17.91	5.89	2.61	8.16	1.91
10.24	6.34	2.76	1.65	1.41

O:N ratio in Summer after copper exposure

24h and 10days of copper exposure (C, 5,25,75,200 ug Cu /l)

C-24h	5-24h	25-24h	75-24h	200-24h
5.47	8.42	0.68	2.17	0.32
6.93	4.74	1.52	1.99	1.58
4.06	3.47	1.88	1.50	1.84
8.35	4.30	1.85	1.03	2.48
8.79	2.73	1.91	1.50	2.73

C-10d	5-10d	25-10d	75-10d	200-10d
5.38	1.00	0.16	0.45	1.48
4.69	2.57	1.19	0.84	1.20
5.91	1.57	0.99	0.75	1.48
4.76	1.55	1.57	1.08	1.77
5.96	1.72	1.34	1.19	1.44

Brooding females oxygen consumption in summer after copper exposure

24h, 96h and 10days of copper exposure (C, 5,25,75,200 µg Cu /l)

µgOx/h/mg.drywt

C-24H	5 - 24H	25-24H	75-24H	200-24H
1.859	2.646	2.304	1.518	1.733
2.491	2.514	1.789	1.079	1.152
2.567	1.742	1.729	1.846	1.761
1.490			1.777	1.713

C-96H	5-96H	25-96H	75-96H	200-96H
1.800	2.473	2.251	2.177	1.779
2.100	1.959	0.134	2.252	0.389
2.400	1.423	0.157	0.346	0.389
				1.564

C-10D	75-10D	200-10D
1.659	0.369	0.950
1.472	0.406	1.280
2.204	0.253	0.401
		0.728

APPENDIX II

Females percentages on the reproduction
% Brooding females of the female population (Brood.F/F),
% Brooding females of the total population (Brood.F/N),
% Reproductively active females of the total population (Rep.F/N)

Month	% Brood.F/F	% Brood.F/N	%Rep.F/N
March	12.50	6.60	53.33
April	100.00	68.00	68.00
May	70.58	35.82	50.75
June	46.66	28.00	53.00
July	59.26	43.83	75.34
Aug	50.00	22.20	33.30
Sept	25.00	14.28	14.28
Oct	5.58	1.69	1.69

Percentages of brooding females in the population maintained at 20°C and 10°C
% Brooding females of the female population (Brood.F/F),
% Brooding females of the total population (Brood.F/N),
% Reproductively active females of the total population (Rep.F/N)

	20oC	10oC
Brood.F/F	70.58	62.5
Brood.F/N	35.82	36.84
Rep.F/N	50.75	42.11

Reproduction parameters under field and laboratory conditions:
Brooding female size (length-mm) - F(mm), newborn juvenile size (length-mm) - y(mm), nun of newborn juveniles/embryos brooded (Brood size), and reproduction effort (Rep.effort)

March	**F(mm)**			
Mean	20			
SD	0			
N	10			
April	**F(mm)**	**y (mm)**	**Brood size**	**Rep.Effort**
Mean	20.33	3.42	21.60	0.65
SD	0.52	0.38	6.43	0.21
N	12	6	12	12
May				
Mean	20.00	3.50	23.70	1.13
SD	0.00	0.00	4.57	0.43
N	22	13	22	22
June				
Mean	20.00	3.25	22.59	1.11
SD	1.00	0.38	9.73	0.40
N	39	23	39	39

Aug-98 % Brooding Females

Day	C	5	25	75	200
0	100	100	100	100	100
1	100	100	100	100	100
2	100	80	70	60	70
3	100	80	70	30	40
4	90	50	60	10	40
5	90	30	60	10	10
6	90	30	20	0	10
7	80	0	10	0	0
8	80	0	10	0	0

% Abortion occurred on the brooding females when exposed to copper (C, 5, 25, 75, 200μg/l) for 10days in Aug.1997, eight days in June and Aug. 1998.

Aug.97 % Abortion

Day	C	5	25	75	200
0	0	0	0	0	0
1	0	40	0	0	0
2	0	40	0	0	0
3	0	60	10	0	0
4	0	70	10	20	30
5	0	70	30	20	30
6	0	70	30	30	50
7	0	70	30	30	50
8	0	70	30	30	50
9	0	70	30	40	50
10	0	70	30	40	50

Jun.98 %Abortion

Day	C	5	25	75	200
0	0	0	0	0	0
1	0	10	10	10	0
2	0	10	10	10	10
3	0	10	10	10	10
4	0	10	10	20	20
5	0	10	20	20	30
6	0	10	20	20	30
7	0	20	30	30	30
8	0	20	30	30	30

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