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Effects of copper on the acorn barnacle Elminius modestus (Darwin) (Cirripedia:Thoracica).

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EFFECTS OF COPPER ON THE ACORN BARNACLE *Elminius modestus* (Darwin) (Cirripedia:Thoracica)

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A mi abuelo que hubiese estado muy orgulloso
y a mi Titi que lo está

"In our intuitive perceptions, which may be truer than our science and less impeded by words than our philosophies, we realise the indivisibility of the earth - its soil, mountains, rivers, forests, climate, plants and animals, and respect it collectively not only as a useful servant but as a living being"

Aldo Leopold (1887-1948)

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CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

Marine pollution is nowadays a major public issue and the subject of countless scientific studies as a result of a dramatic increase in social awareness. This has been heightened mainly by the occurrence of environmental disasters, such as the spillages from the oil tankers 'Exxon Valdez' in Alaska and the 'Brear' in the Shetland Islands (UK), which have contributed to the already growing evidence regarding the extent to which pollution has caused and continues to cause severe environmental degradation.

Pollution should be understood as a potential hazard (in the form of substances or energy) introduced into the environment by man (see Holdgate, 1979), where toxicity would be the expression of that hazard in the form of a negative impact on the communities characteristic of the environment concerned and could lead to the gradual elimination of the most sensitive species.

The need to protect marine life against pollution has led to monitoring the materials entering the marine environment, as a result of domestic, agricultural and industrial activities, which involves the determination and quantification of the waste substances and their effects when they reach the sea (see Holdgate, 1979; Alloway & Ayres, 1993).

The main aim of the current study is to assess the suitability of the acorn barnacle *Elminius modestus* (Darwin) as a species with which to investigate the effects of a

common metallic pollutant, copper. An attempt is made to use the organism as a general indicator of both chronic and acute copper effects in the marine environment.

Copper is a widely distributed element in nature with a total annual turnover in the marine environment of about 7 million metric tons, originating from atmospheric fallout and terrestrial run-off, of which some 1% can be attributed to anthropogenic input mainly through industrial emissions and both urban and industrial effluents, as well as minor sources such as antifouling paints and industrial cooling systems (Nriagu, 1979). The metal can occur in 3 oxidation states, Cu(II) being the most common as for all the other elements belonging to the first transition series (see e.g. Cotton & Wilkinson, 1962). It is highly reactive exhibiting considerable biological activity.

In the sea copper can exist in several different chemical forms or species. It can be dissolved (ionic and complexed), particulate or incorporated into biota, depending on a number of physico-chemical and hydrodynamic phenomena (see reviews by Schmidt, 1978a; Lewis & Cave, 1982; also Engel *et al.*, 1981). The dia-positive state [Cu(II)] dominates the aquatic chemistry of copper and it is in this ionic form that copper is especially toxic to marine organisms (Steemann Nielsen & Wium-Andersen, 1970; Sunda & Guillard, 1976; Andrew *et al.*, 1977; Sanders *et al.*, 1983; Sunda & Ferguson, 1983; Ahsanullah & Florenz, 1984). Both inorganic and organic ligands present in seawater can complex copper (Hatfield & Whyman, 1969), reducing the amount of

Cu(II) and giving rise to other, less toxic (Gillespie & Vaccaro, 1978), soluble species (see compilation by Leckie & Davis, 1979).

Naturally occurring organic ligands are responsible for most of the copper complexation that takes place in estuarine (Mantoura *et al.*, 1978; Engel *et al.*, 1981) and marine (Zuehlke & Kester, 1981) waters. Organic copper complexes constitute the major copper species in those environments (Mantoura *et al.*, 1978; Zuehlke & Kester, 1981). The extent of such complexation is determined by the concentration and chemical composition of dissolved organic matter, salinity and pH (Sunda & Hanson, 1979; Engel *et al.*, 1981; Zuehlke & Kester, 1981).

The nature of naturally, occurring, organic copper ligands has long been a matter of discussion (see review by Schmidt, 1978a; compilation by Leckie & Davis, 1979; review by Lewis & Cave, 1982). Kemling *et al.* (1983) summarised the current view that organic ligands originate largely from primary producers either by excretion of complexing agents or from the decomposition of dead organisms. Steemann Nielsen & Wium-Andersen (1971), Swallow *et al.* (1978) and Fisher & Fabris (1982) showed that products excreted by phytoplankton, including glycolic acid, carbohydrates, nitrogenous substances and vitamins (Fogg 1966, 1977), could act as copper complexing agents (see also Fogg & Westlake, 1955; McKnight & Morel, 1979; Van den Berg *et al.*, 1979; Härdsted-Roméo & Gnassia-Barelli, 1980; Fisher & Fabris, 1982;

Slauenwhite & Wangersky, 1991). In coastal waters, organic ligands such as fulvic (Manning & Ramamoorthy, 1973) and humic acids (see Mantoura *et al.*, 1978) have likewise complexing capacity.

Complexed and particulate copper is eventually removed from solution through precipitation, co-precipitation and sedimentation (see reviews by Schmidt, 1978b; Lewis & Cave, 1982; Förstner & Salomons, 1983). Speciation is, therefore, as important a parameter as total metal concentration when considering copper toxicity in aquatic media.

Trace amounts of copper are essential to marine life (see Bowen 1966; White & Rainbow, 1985), but excess levels are extremely toxic (see reviews by Hodson *et al.*, 1979; Lewis & Cave, 1982; Amiard-Triquet, 1989) (see Alloway & Ayres, 1993). However, such toxicity varies with the metal species and the availability of those species for biological uptake (see reviews by Lewis & Cave, 1982; Bryan, 1984; Smies, 1983). Dissolved copper appears to be more biologically available than particulate copper (see review by Schmidt, 1978b) and the ionic form more so than complexed forms (Sunda & Guillard, 1976; Anderson & Morel, 1978).

Copper is essential for marine organisms as a direct constituent of many enzymes and indirectly for the synthesis of non-enzymatic proteins (see reviews by Lewis & Cave, 1982; White & Rainbow, 1985). There are 20 to 30 copper-containing enzymes which are involved in metabolical processes such as electron transport reactions and membrane permeability control (see review by Lewis & Cave, 1982).

In contrast, the toxicity of copper is well documented with several comprehensive reviews (see Eisler, 1979; Hodson *et al.*, 1979; Lewis & Cave, 1982; Jørgensen *et al.*, 1991). Generally the sensitivity of marine organisms to copper exhibits both intraspecific and interspecific variability as a result of the interaction of numerous abiotic and biotic factors such as salinity temperature, pH, oxygen content, fluctuating metal concentrations, complexation, acclimation and diet (see Sprague, 1970; Hodson *et al.*, 1979; Bascom, 1983; Mance 1987). These factors would also modify metal availability in the water column and the rate of metal uptake by the organisms so that copper toxicity is either enhanced, decreased or even prevented.

Toxicity is normally assessed through acute and chronic dose-effect tests (see Holdgate, 1979; Committee of Analysts, 1983). Time and dose interact such that short-term tests are acute using high concentrations of toxins and resulting in the often quoted median lethal dose (LD_{50}= the dose that kills 50% of the organisms) used for comparison, the derivation of Maximum Allowable Concentrations (MACs) and the establishment of Environmental Quality Standards (EQS) (see Holdgate, 1979)

The intrinsic validity of acute toxicity tests, however, requires standardisation of test conditions. Thus, tests should ideally be performed on large numbers of animals of equivalent physiological condition and life cycle stage, which should also be genetically uniform and acclimated to

test conditions (see Holdgate, 1979; Snell *et al.*, 1991). Extrapolation to natural communities is often compromised by the experimental conditions of the tests *per se*, the lack of data for more than a small number of tests species used so far and the short duration of the exposure period, which does not allow prediction of the long-term consequences of pollutants (see reviews by Sprague, 1968, 1970; Draggan & Giddings, 1978; Holdgate, 1979; Mance, 1987; Persoone, 1988; Persoone *et al.*, 1989; Alloway & Ayres, 1993).

Chronic toxicity tests generally investigate the long-term effects of low concentrations of pollutants focussing on an organism's survival, growth and reproduction. Chronic toxicity is often assessed through a median effective dose (ED_{50}= the dose that induces a response in 50% of the population). Chronic tests are more expensive that acute tests, but perhaps have more relevance to episodic contamination of the marine environment.

Copper toxicity is expressed mainly through inhibition of the activity of enzymes (e.g. amylase, trypsin, ATPase) involved in biochemical and cellular processes vital for life, such as oxidative phosphorilation, membrane permeability, protein synthesis and electron transport reactions (see reviews by Hodson *et al.*, 1979; Lewis & Cave, 1982). The results of such inhibition are often seen initially in a lowering of physiological rate processes or a change in behaviour, which have a direct influence on an organism's growth and reproduction and on the eventual success of the population in a contaminated environment

(Ferrando *et al.*, 1993).

A major consequence of chronic metal contamination is the accumulation above ambient concentrations of both essential and non-essential trace metals by marine invertebrates (see reviews by Eisler, 1981; Bryan, 1984; Amiard-Triquet, 1989). The degree of accumulation, however, varies with metal dose; size, sex and physiological state of the organism; temperature and salinity (see review by Depledge & Rainbow, 1990; see also Canli & Furness, 1993); tidal flow and phytoplankton productivity in the surrounding water (see Ireland, 1974).

The interest in the accumulation of metals by marine invertebrates stems from their potential use to monitor marine pollution. Historically, pollution was monitored through chemical analysis of seawater and marine sediments, which tends to be expensive and provides an inadequate picture of the development of a pollution problem without extensive long-term sampling (see Phillips, 1977, 1980). Alongside seawater sampling, the use of bioaccumulator organisms (as pollution indicators) has been advocated since they are easily available and their tissues show analytically feasible metal concentrations which reflect the changes in metal availability in their surrounding environment integrated over time (see Phillips, 1977, 1980).

Because of the diversity of habitats and marine species there is no universal indicator organism (Bryan, 1984). Acorn barnacles have shown potential for use as

bioindicator organisms (Walker *et al.*, 1975a; Barbaro *et al.*, 1978; Barber & Trefry, 1981; Rainbow, 1985; Phillips & Rainbow, 1988; Powell & White, 1990). They are sessile animals, abundant and widely distributed on rocky substrata within the intertidal zone (Darwin, 1854) and hence easy to sample. Being filter feeders, they could be used to monitor dissolved and suspended particulate metals (see requirements for indicator species in Phillips, 1976, 1977). Species like *E. modestus* are capable of tolerating brackish water, can be reared in the laboratory with few problems (Moyse, 1960; Thighe-Ford *et al.*, 1970), have a relatively fast larval development allowing a speedy turnover, and can be made to settle on a wide range of surfaces tailored to suit subsequent manipulation of the organisms (Lucas *et al.*, 1979). Furthermore, juvenile growth is also relatively fast, and in the case of *E. modestus* sexual maturity can be reached in 8 weeks (Crisp, 1958). Finally, acorn barnacle species such as *B. eburneus*, *B. (Semibalanus) balanoides*, *E. modestus*, *B. amphitrite* and *B. crenatus* have been shown to accumulate metals such as copper, zinc, cadmium, iron, manganese, calcium and lead (Clarke, 1947; Ireland, 1973, 1974; Walker *et al.*, 1975a; Barbaro *et al.*, 1978; Rainbow, 1985; Rainbow & White, 1989; Powell & White, 1990; Zauke *et al.*, 1992).

Accumulation of metals can result in a built up to toxic levels within the organism's tissues, but acorn barnacles are known to reduce such effects by sequestering metals in their soft tissues in the form of physiologically inert, insoluble

organic and inorganic granules (Bernard & Lane, 1961; Walker *et al.*, 1975a, 1975b; Walker, 1977; Thomas & Ritz, 1986; Pullen & Rainbow, 1991).

However, the use of bioindicator organisms to monitor pollution (biomonitoring) is a retrospective hazard assessment and biomonitor organisms reflect the physical and chemical changes experienced during a period where harm (if any) may have already been done (Persoone, 1988). As a result, increasing attention is now being given to an ecotoxicological approach, involving whole communities and a predictive evaluation of the negative impact of pollutants as a means of determining whether or not a pollutant might be released into the sea and in what quantities (see Persoone & De Pauw, 1991). The formulation of standardised, representative and validated toxicity tests (see review by Persoone, 1988), such as that developed by Snell & Persoone (1989) using rotifers, thus becomes increasingly important. Acorn barnacles, and especially acorn barnacle nauplii, could likewise fulfil the conditions laid down by these authors regarding, mainly, uniformity of the physiological, developmental and, to some extent, genetic homogeneity of the test organisms, both in an easy and low-cost manner.

Acorn barnacles appear to fulfil the necessary prerequisites for the selection of indicator species and, at the same time, are suitable organisms for use in ecotoxicological studies on both the acute and, in particular, the chronic effects of pollutants in the marine

environment.

Studies on the effects of copper on barnacles have spanned the life cycle of the organisms. They are heavily concentrated on the adult forms even though the larvae, as with any other marine invertebrate larvae and juvenile stages, are generally more sensitive to pollutants than the adults (see review by Mance, 1987; see also Connor, 1972; Ahsanullah & Arnott, 1978; Birge & Black, 1979; Lewis & Cave, 1982; Davenport & Redpath, 1984). The ability to work with equal ease on all stages of a barnacle life history must enhance its usefulness. For example in setting up water quality criteria for environmental protection when usually adult sensitivity alone has been used in the past (see Ahsanullah & Arnott, 1978).

Clarke (1947) was one of the first to test the effects of copper on barnacle larvae using *B. eburneus* nauplii and *B. improvisus* cyprids. As with most toxicity studies, Clarke (1947) focused on the lethality of the metal at high concentrations. Such acute studies provide data which are difficult to interpret and compare. Table 1 shows the results of acute toxicity tests on the nauplii of a number of barnacle species in the form of short-term LD_{50} (see Sprague, 1970) values and derived from static test procedures performed under different conditions of salinity and temperature. Not only was exposure time specific to each test, but also the criteria for establishing death of the nauplii varied.

Species	LD_{50}	Time	S	T	Reference
B. eburneus	60	29	-	-	Clarke, 1947
B. balanoides	410	6	-	-	Pyefinch & Mott, 1948
B. crenatus	260	6	-	-	Pyefinch & Mott, 1948
B. amphitrite	200-350	24	20	30	Lang *et al.*, 1979
B. improvisus	150-200	24	26	30	Lang *et al.*, 1979
B. improvisus	80	24	20	15	Lang *et al.*, 1980
B. improvisus	>200	24	20	30	Lang *et al.*, 1980
B. improvisus	80	96	15	15	Lang *et al.*, 1981
B. improvisus	>160	96	15	30	Lang *et al.*, 1981
E. modestus	235	16	-	15	Toseland, 1976 unpub.

Table 1 Acorn barnacle nauplii LD_{50} values (ppb) reported to date in the literature (with the exception of Corner & Sparrow's (1956) estimated 24-h LD_{50} at >6,000 ppb), Time in hours, S=salinity, T=temperature

Nevertheless, the objective of the studies was not simply concerned with the production of LD_{50}s for the different barnacle species or the establishment of water quality criteria, but also with the investigation of acute toxicity mechanisms and possible factors affecting them. Comparable LD_{50}s could be more easily obtained by standardising the experimental procedure as has been done for fish (see Committee of Analysts, 1983). However, the study of copper toxicity to barnacle larvae needs further experimentation over longer-term periods, using concentrations that reflect the situation in the environment.

Studies on the sublethal effects of copper on barnacle larvae started with Pyefinch & Mott (1948), who showed that settlement of *B. (Semibalanus) balanoides* cyprids was inhibited above 24 ppb. In addition, they pointed out the differences in sensitivity between the different life stages of barnacle species, showing that cyprids were more tolerant

to copper than nauplii and that the adults were the most tolerant of all (1.5 and 1.7 adult/larva LD_{50} ratio for *B. (Semibalanus) balanoides* and *B. crenatus* respectively) (see also Clarke, 1947). Bernard & Lane (1963) focused on the oxygen consumption of *B. amphitrite* cyprids, which they linked to motor activity, showing that addition of copper up to a threshold concentration increased respiration rates, but that the rate decreased progressively as more copper was added.

More recent work usually deals with stage II nauplii, where copper uptake could occur mainly through diffusion across the body cover due to their high surface/volume ratio. Such work includes that of Lang *et al.* (1979, 1980, 1981), who monitored mortality, rate of development, normality of moulting, spontaneous swimming speeds and photobehaviour during 48 or 96 h exposure to copper in *B. improvisus*. Their work showed reductions in survivorship and positive phototactic response, as well as in the rate of both development and swimming activity (speed and pattern). In addition, all the nauplii that successfully moulted from stage II to stage III exhibited morphological abnormalities, consisting of twisted short setae and deformed appendages. Morphological abnormalities in larvae (or embryos) exposed to sublethal pollutant levels, and especially metals, have also been observed in other marine invertebrates such as gastropod (see review by Ravera, 1991) and bivalve molluscs (see Martin, 1981).

Royo-Gelabert (1991, unpublished), recognising the need

to monitor larval growth over extended periods of copper exposure, studied copper effects on *B. amphitrite* nauplii over the period of their development and assessed the effects on all six naupliar stages. Her work showed reduced survivorship and morphological abnormalities in the nauplii, which occurred above a threshold concentration of 1,000 ppb and were similar to those described by Lang *et al.* (1979, 1980, 1981), including enlarged (bulbous) bases to the setae on the endopodites of all six limbs. In addition, her study revealed the existence of a different mechanism for acute and chronic toxicity, as well as the importance of phytoplanktonic exudates in reducing copper's toxic action. Reduced copper toxicity via complexation had also been observed by Toseland (1976, unpublished) on *E. modestus* nauplii, using EDTA as a chelating agent.

Clarke (1947) also pioneered the study of the acute toxicity of copper in adult barnacles, and showed that mortality of copper-exposed animals was proportional to concentration over the range tested. At the chronic level, he showed that copper retarded the development of newly metamorphosed *B. improvisus* and prevented the formation of the calcareous base. Pyefinch & Mott (1948) also observed retarded development in *B. (Semibalanus) balanoides* and *B. crenatus*. Furthermore, Clarke (1947) detected copper induced behavioural alterations in fully grown adults, in which, at copper citrate concentrations of 600 ppb for *B. (Semibalanus) balanoides* and 550 ppb for *B. eburneus*, cirral

beating slowed down after 12 h and where increasing exposure time progressively reduced activity until the animals died. However, Clarke (1947) also observed that poisoned animals, i.e. those with open valves, inactive, but reacting to touch, could recover within a day if placed in clean seawater. Pyefinch & Mott (1948) likewise observed activity changes with copper exposure in adult *B. crenatus*, where 6 h exposure to copper levels above 310 ppb reduced barnacle activity (cirral beating) to about 9%. More recently, Powell & White (1989) also studied cirral activity in copper exposed *B. (Semibalanus) balanoides* and *B. crenatus* at both lethal and sublethal levels. They showed a significant overall reduction in the level of activity with copper as well as a decrease in beat frequency at copper levels that were proved to be lethal. Such levels were also shown to induce changes in the type of cirral activity, from normal beat to pumping.

Changes in cirral activity with copper exposure might result in an altered metabolic load, given the relationship between activity and metabolism (Crisp & Southward, 1961; Newell & Northcroft, 1965). Thus, Newell (1973) suggested that environmental stress might reduce the scope for activity and the respiration rate would hence fall below the standard rate characteristic of the organism. Reductions in oxygen consumption under copper exposure have been observed in both molluscs (Brown & Newell, 1972) and crustaceans (Corner & Sparrow, 1956). Corner and Sparrow (1956) suggested that reductions of about 25% in the respiration rate of copper-exposed *Artemia salina* constituted a response to specific

inhibition of respiratory mechanisms (cellular respiration). Published literature does not seem to contain any example concerning barnacles. However, Bowles (1993, unpublished) showed that, over a range of sublethal copper concentrations, the respiration rate of *E. modestus* adults increased below 60 ppb, whereas above that level it fell progressively to below the values observed prior to copper addition (see also Burkitt, 1994 unpublished). A similar pattern of response to copper concentration had already been observed by Bernard & Lane (1963) in the case of the cypris larva of *B. amphitrite*. Thus, increased respiration in *E. modestus* could signify a rise in metabolic rate necessary to cope with added copper levels below a certain threshold concentration. Above that concentration, the respiration rate would fall as copper toxicity either reduced activity or disrupted normal metabolism.

Copper exposure also affects growth and development of adult acorn barnacles. Weiss (1948) observed that *B. amphitrite*, *B. improvisus* and *B. eburneus* grown on copper antifouling paints, copper alloys or close to copper-coated surfaces were loosely attached (hence easily removed) and showed distortion of both the shell margins and the shell plates. The author also observed that the three species exhibited different resistance to the development of malformations, which probably resulted from their different copper tolerances, *B. amphitrite* being the least sensitive (Weiss, 1947). Not only, the morphology of acorn barnacle

base and parietal plates, but also that of the opercular plates can be affected by environmental contamination. Furman's (1989, 1990) study on the geographical variation of *B. improvisus* over a wide geographical range by reference to biochemical and morphometric characteristics first suggested that pollution could be responsible for the gross distortion of the opercular plates observed in specimens from Gdynia in the Baltic sea. Royo-Gelabert & Yule (1994) demonstrated the existence of a link between the morphology of *E. modestus* opercular plates and copper concentration in the laboratory, which was parallelled by the relative shapes of field barnacles from polluted and unpolluted sites within a 200 mile range of the west coast of Britain. The plates of contaminated animals were relatively larger.

Metal pollution, including that caused by high copper levels, has also been shown to act differentially on genotypes within a population. Thus, Nevo *et al.* (1978) and Patarnello *et al.* (1991) observed that the genotype frequencies of *B. amphitrite* from contaminated sites showed consistent differences with those from clean locations. It is not known whether the differences are associated with post-settlement mortality of certain genotypes susceptible to pollution or whether the observed pattern results from differential larval settlement.

The current work aims to demonstrate that a very hardy, fast growing, acorn barnacle species can be used to investigate both acute and chronic effects of pollutants on intertidal organisms. *Elminius modestus* (Darwin) is an acorn

barnacle originating from Australia and New Zealand (Darwin, 1854). However, owing mainly to shipping movements, its distribution has widened globally and it can now be found on mid and lower shores in Britain, where it was first observed in 1945 (Bishop, 1947; Crisp & Chipperfield, 1948). *E. modestus* is found intertidally on sheltered coasts and estuaries, since it is able to tolerate salinity fluctuations by closure of the opercular plates.

The anterior three pairs of cirri are differentiated from the posterior and, as a result, *E. modestus* can carry out both macro-captorial and micro-filter feeding (Southward, 1955a; Crisp & Southward, 1961). Reproduction occurs continuously throughout the year, so larvae are present at all times in the plankton (Crisp, 1958) with a tendency to greater larval abundance in the summer (Knight-Jones & Waugh, 1949; Crisp, 1958). Larval development was described by Knight-Jones & Waugh (1949) and is typical of that seen in balanomorphs, but rapid, taking as little as 6 days from larval release to settlement stage (Yule, 1986). The juveniles also grow very rapidly, reaching maturity at a basal diameter of 6-7 mm in as little as 8 weeks (Crisp, 1958) and the maximum basal diameter is 1 cm.

The above characteristics enable effective study of the organism at all stages of the life history. A large part of the work is aimed at addressing the difficulties in drawing adequate conclusions from acute toxicity tests with larvae and investigating the major factors (e.g. temperature, diet,

complexation etc.) which influence the measurement of LD_{50}s.

In addition, the effects of chronic exposure to copper throughout the organism's life history are addressed aiming to indicate how physiological processes, such as ingestion or respiration rates, are disrupted leading to the death of individuals or to reduced fecundity thus endangering the survival of populations.

Finally the availability of a nearby population of chronically contaminated barnacles (Dulas Bay, N. Wales, UK) presented the opportunity to compare responses of larvae and adults with potentially different tolerances, and to investigate the degree to which tolerance may be conferred genetically. That population would also allow the assessment of the chronic effects of copper on the morphology of barnacle opercular plates, and the comparison with earlier studies (Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994).

CHAPTER TWO

GENERAL MATERIALS AND METHODS

CONTENTS

The acorn barnacle larvae used throughout this study were obtained by either of two methods: 'artificial' hatching, the most widely employed, or 'natural' hatching.

'Artificial' hatching method

Adult barnacle specimens with ripe egg masses were lightly crushed using a mortar and pestle and carefully placed in a dark polystyrene tray containing cartridge-filtered (0.2 μm) and UV-irradiated seawater (FUVSW), whereupon ripe egg masses began to hatch. The tray was covered with a dark lid, leaving an opening for a strong point light-source to attract the newly released, photopositive, stage I nauplii, which were pipetted off into a flask containing FUVSW. Only the nauplii released within the first half hour of setting up the tray with the crushed adults were used in any of the experiments.

If the eggs masses were not ripe, the adults were dissected, the egg masses removed and transferred to suitable flasks containing FUVSW, where they were kept at the required temperature, under constant gentle aeration (1 bubble.sec^{-1}), until they matured.

The above was the most common method used for hatching

the nauplii in the toxicity tests carried out throughout the work, and was that used in the methodology of each specific test unless otherwise stated.

'Natural' hatching method

Substrata covered with barnacles (e.g. pebbles, shells, etc.) were brought back to the laboratory where they were brushed clean and placed in tanks with FUVSW at the required temperature with constant aeration. The barnacles were cleaned regularly and fed daily on a diet of *Artemia sp.* nauplii (Artemia 90, Sanofi Aquaculture, Paris, France) and mixed unicellular algae (both routinely grown in the laboratory as food supply for marine invertebrates), to ensure maturation of the egg masses. The algae were cultured in a semi-continuous manner using methods similar to those described by Walne (1966). Upon maturation of the egg masses, stage I nauplii hatched naturally in the tanks. Subsequently, the nauplii were attracted to a light source in the corner of the tank and pipetted off into flasks with FUVSW.

Two basic conditions were used to maintain acorn barnacle larvae during tests, depending on the length of the experimental period. Occasionally, the culture medium was modified to suit the individual needs of a particular experiment. The same techniques were utilised to produce culture media in the adult experiments.

Glass vials

These were used mainly in long term culture experiments, ranging from 12 to 20 days, where the nauplii were fed throughout the experimental period. Thus, routine use was made of 22 ml glass vials containing 20 ml of a medium made of by FUVSW (11.9 ml), the flagellate *Rhinomonas reticulata* [Lucas] (Novarino, 1991) to give a concentration of 100 cells.μl^{-1} (8 ml) and the requisite amount of copper dissolved in deionised, distilled water (0.1 ml), with a final salinity of approximately 32 ppt.

Stock copper solution was made up to 10^6 ppb using hydrated copper sulphate ($CuSO_4.5H_2O$, AnalaR, BDH Chemicals Ltd., Poole, UK) and deionised, distilled water; it was then kept in a refrigerator and used throughout the study. The copper was supplied to each medium in a volume of 100 µl

originating from a 10 ml 'intermediate' copper solution, constituting a dilution of the 10^6 ppb stock solution in deionised, distilled water, which belonged to a series as large as the number of copper concentrations to be used in any individual experiment.

'Control' cultures were made up in an identical manner to those containing copper, but the 100 µl of added copper was substituted by 100 µl of deionised, distilled water.

The concentrations of added (nominal) copper in each naupliar culture were assumed to be confident estimates of the actual concentrations in those media, since an earlier study using *Balanus amphitrite* (Darwin) nauplii grown in culture media prepared as described above showed that there was a very good correspondence between the target copper concentrations in the cultures and those measured by atomic absorption spectrophotometry (see Royo-Gelabert, 1991, unpublished; Royo-Gelabert & Yule, 1994). Therefore, all copper concentrations given refer to that nominal amount at the outset of the experiments.

The present study made no attempt to determine either the metal speciation process within the naupliar cultures or the possible reduction in the total copper concentrations due to physical, chemical and biological processes such as precipitation, adsorption and physiological detoxification.

The added copper would have speciated in accordance to the pH and the organic matter content of the cultures (see Zuehlke & Kester, 1983). Generally the buffering capacity of seawater maintains pH at around 8. The pH of the naupliar

cultures was measured initially at 7.7 and 7.55 after 48 h with no food algae present. A similar culture containing food algae gave an initial pH of 7.7, but showed a rise to 8.4 after 48 h. Most of the toxicity tests carried out in the present study were conducted over 48 h hence the time is pertinent to the results.

A decrease in pH of 0.15 units would result in a slight (less than 2%) increase in the percentage of free ionic copper (Zuehlke & Kester, 1983), which might enhance copper toxicity to the nauplii in the acute toxicity tests. However, such enhancement would not be as dramatic as that caused by lack of organic complexation, resulting from the absence of food algae in those tests (see Chapter One: General Introduction).

An increase in pH of 0.7 would result in a 2% decrease in the percentage of free ionic copper (see Zuehlke & Kester, 1983), which would have been negligible compared to copper complexation to algal food products (see Chapter One).

The algae *R. reticulata* was supplied to the naupliar cultures either straight from the jars in which it was grown and was thus suspended in the medium used for its culture (see Walne, 1966) ('unwashed', 40% Algal Culture Medium, ACM), or centrifuged at 2,000 r.p.m. for 10 min and resuspended in FUVSW ('washed', 0% ACM). Consequently, 40% was the maximum ACM to be achieved in a 20 ml naupliar culture, since the volume of added algal suspension was 8 ml.

The culture vials were placed on a rotating wheel at 6

r.p.m. to maintain the homogeneity of the media and prevent sedimentation of the algae (see Crisp & Southward, 1961; Crisp, 1984) (see Plate 1). The wheel was kept in either the laboratory or in a refrigerated chamber depending on the temperature needed to grow the nauplii. Light conditions varied, these being natural if the wheel was in the laboratory, but artificial (12 h light/12 h darkness cycle) if it was kept in the refrigerated chamber.

The number and origin of the nauplii, temperature, salinity, light conditions, copper concentrations, etc. were parameters that varied with each test and have been specified accordingly.

Polystyrene trays

These were used in short-term experiments, ranging from 24 to 48 h, where the nauplii were not fed throughout the experimental period. The trays were transparent, square and divided into 5x5 cells, each of 5 ml volume. In most cases the distribution of the copper concentrations in these cells followed a 5x5 Latin square design. Initially, each cell contained 4 ml of a medium made up of FUVSW (3.9 ml) and the requisite amount of copper dissolved in deionised, distilled water (0.1 ml).

The stock copper solution was the same as that used in the long-term experiments, where copper was initially supplied to each cell in volumes of 100 µl that originated

from 'intermediate' copper solutions, as described above. To reduce preparation time and variability, however, it was later decided to make up the various copper concentrations by serial dilution of the highest concentration (in the range chosen for a particular experiment) using 250 ml volumes as a minimum. Each cell of the tray was then filled with the appropriate copper-containing culture medium, which was pipetted off from a larger volume.

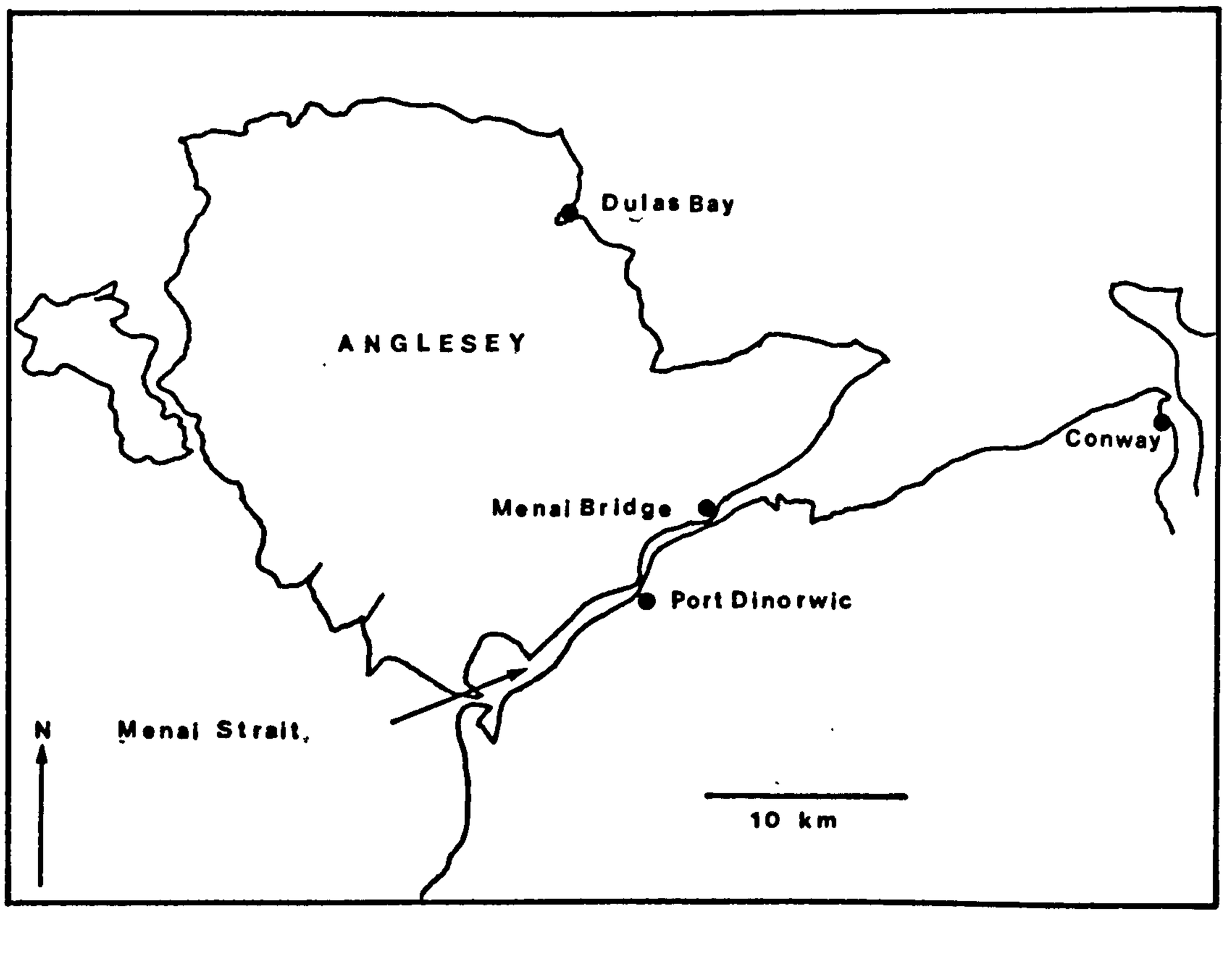
The trays were covered with transparent lids and sometimes placed in recirculating water baths to keep the media at the desired temperature. The water baths were maintained in the laboratory under natural light conditions. Specific experimental protocols are given in the relevant sections.

Adult barnacles were collected from sites that were chosen according to the presence of the species studied, viz. *Elminius modestus* (Darwin), rather than the conditions at those locations *per se*. In addition, some experiments required the specimens to have settled on a particular substratum. Dulas Bay, however, was chosen as a sampling location because of its very special physical, chemical and biological characteristics as an estuary with considerable copper loading.

The barnacles were collected at low water from sampling sites which were located on the North Wales coastline, UK, and on the island of Anglesey (see Figure 1). The Menai Strait provided loose barnacles from Menai Bridge pier and individuals on small slates from Port Dinorwic. Dulas Bay supplied *E. modestus* both loose and attached to small rocks, whereas Conway provided specimens fixed to mussel shells.

As a general rule, newly collected barnacles were allowed to recover from handling before they were used in any of the adult tests.

Figure 1
Elminius modestus collection sites on the North Wales coastline, UK, and on the island of Anglesey.



Dissolved copper concentrations in water samples used throughout the study were determined following a slight modification of the diethyldithiocarbamate method of Strickland & Parsons (1965, 1972), whereby copper was extracted from the seawater samples in an organic phase and the extinction of the extracts measured spectrophotometrically.

The method determines only the non-complexed or weakly complexed forms of the metal ions, which are considered to be the more biologically active (see Sunda & Guillard, 1976, Anderson & Morel, 1978). A significant fraction of the 'soluble' copper in the sea may be strongly bound to organic matter and not react with diethyldithiocarbamate (Strickland & Parsons, 1972). The magnitude of this bound fraction depends largely on the organic matter load of the seawater. Thus, Foster & Morris (1971) estimated that 3% copper was not directly extractable, using this method, in Afon Goch water samples with a very low organic matter content (Foster *et al.*, 1978). In contrast, the high organic content of seawater in the Menai Strait, which shows large annual fluctuations, would result in 13% to 43% of the copper content not being extracted by the method (Foster & Morris, 1971).

Nevertheless, water samples taken from the Menai Strait

during the present study consistently showed dissolved copper concentrations below 2 ppb, which are substantiated by existing literature (see Foster & Morris, 1971; Davenport & Redpath, 1984).

All the glassware used in the analysis was acid washed with 50:50 hydrochloric acid. One litre water samples were collected from the required locations using polythene bottles, which were then left in the laboratory, both for the water to acquire room temperature and for the sedimentation of any particulates.

A minimum of 2 x750 ml water volumes were processed within 8 h of collection (see Foster & Morris, 1971). If dilution of the samples was necessary, it was normally effected with FUVSW prior to the extraction. Occasionally, however, the copper content of a sample would be higher than had been expected, where the sample had not been diluted prior to extraction. In those cases, the extract would then be diluted using carbon tetrachloride, which undoubtedly led to a systematic error in copper estimation, since the sodium diethyldithiocarbamate might not have been able to complex all the copper in the sample. Otherwise, the percentage of copper extracted is around 95% and the calibration procedure takes into account the slight error (Strickland & Parsons, 1972).

The basic Strickland & Parsons (1965, 1972) method was modified in terms of the calibration procedure. Thus, in the determination of the blank, any copper in the 250 ml of deionised, distilled water was extracted by adding 2 ml of

sodium diethyl-dithiocarbamate and 3 x5 ml of carbon tetrachloride, as was done for any of the seawater samples. The water was not pre-conditioned prior to extraction as outlined by the above authors. Yule (pers. comm.) developed this modification, which gives a performance comparable to that of the original method, since deionised, distilled water was used for both the reagent blanks and a zero-copper blank not assumed in the calculations of the sample copper concentrations.

LD_{50} TESTS AND PROBIT ANALYSIS

LD_{50} (see Trevan, 1927) stands for Median Lethal Dose, the dose of any given toxic substance which kills 50% of a population of any given species that has been exposed to it for a certain period of time (see Fry, 1947; Sprague, 1969) (also known as LC_{50}, or Lethal Concentration 50, see review by Sprague, 1969).

The LD_{50} of a substance is normally derived from the results of an acute toxicity test, a test determining the relationship between the dose of the substance and the response of the tested species over a chosen exposure time (normally 48 h), where the response is usually the death of the test organisms (see Sprague, 1969) (see Chapter One). The LD_{50} value represents the outcome of the toxicity test allowing interspecific and intraspecific comparisons. Such comparisons could, however, be complicated by each author's particular interpretation of the meaning of the LD_{50} (see Sprague, 1969) and the conditions under which each toxicity test is carried out, since many parameters, such as temperature, salinity, exposure time, pH, etc. affect the outcome (Sprague, 1970) (see Chapter One).

The LD_{50} is calculated from the curve of standardised mortality against dose (see review by Sprague, 1969). The statistical method used in the present study is known as Probit Analysis and was developed by Finney (1952, 1964,

1971). The analysis takes the doses of toxin tested, the total number of test organisms and the number of 'responding' organisms for each dose, transforming the mortality percentages for each concentration into Probits. The Probits (Empirical), which express mortality in terms of standard deviations above and below the mean response, are then plotted against the logarithm of the doses tested and the equation of the resulting line is used to produce new Probits. Iteration proceeds, fitting the curve by successive approximations based on the maximum likelihood method, until the change in the logarithm of the LD_{50} is insignificant (Finney, 1952, 1964, 1971).

Comparison of LD_{50}s was performed by the method of Relative Potencies (Bliss, 1935), which measures differences between two or more comparable series of dosage mortality records (see Finney, 1971). This method calculates the Relative Dose Metameter and, from it, the Relative Potency (and 95% fiducial limits) of a set of LD_{50}s. One of the LD_{50}s to be compared is selected as the 'Reference' LD_{50} and, once the relative potencies and their fiducial limits are calculated, significant differences between the former are determined by whether or not their respective confidence intervals include the number 1 ('Reference' relative potency) (see Finney, 1971).

Barnacle ingestion rates were measured throughout this study for both stage II nauplii and adults. Larvae and adult barnacles are both filter feeders and their ingestion rate will hence be a function of their filtration rate and the food particle concentration. Assuming that the animals clear a constant volume of water with time, provided that the initial food particle concentration is not too high, the filtration rate (F) can be calculated as (see Crisp, 1984):

$$F = \frac{V}{n\ (t-t_o)} \cdot \log_e \left(\frac{C^e{}_o}{C^c{}_o} \cdot \frac{C^e{}_t}{C^c{}_t}\right)$$

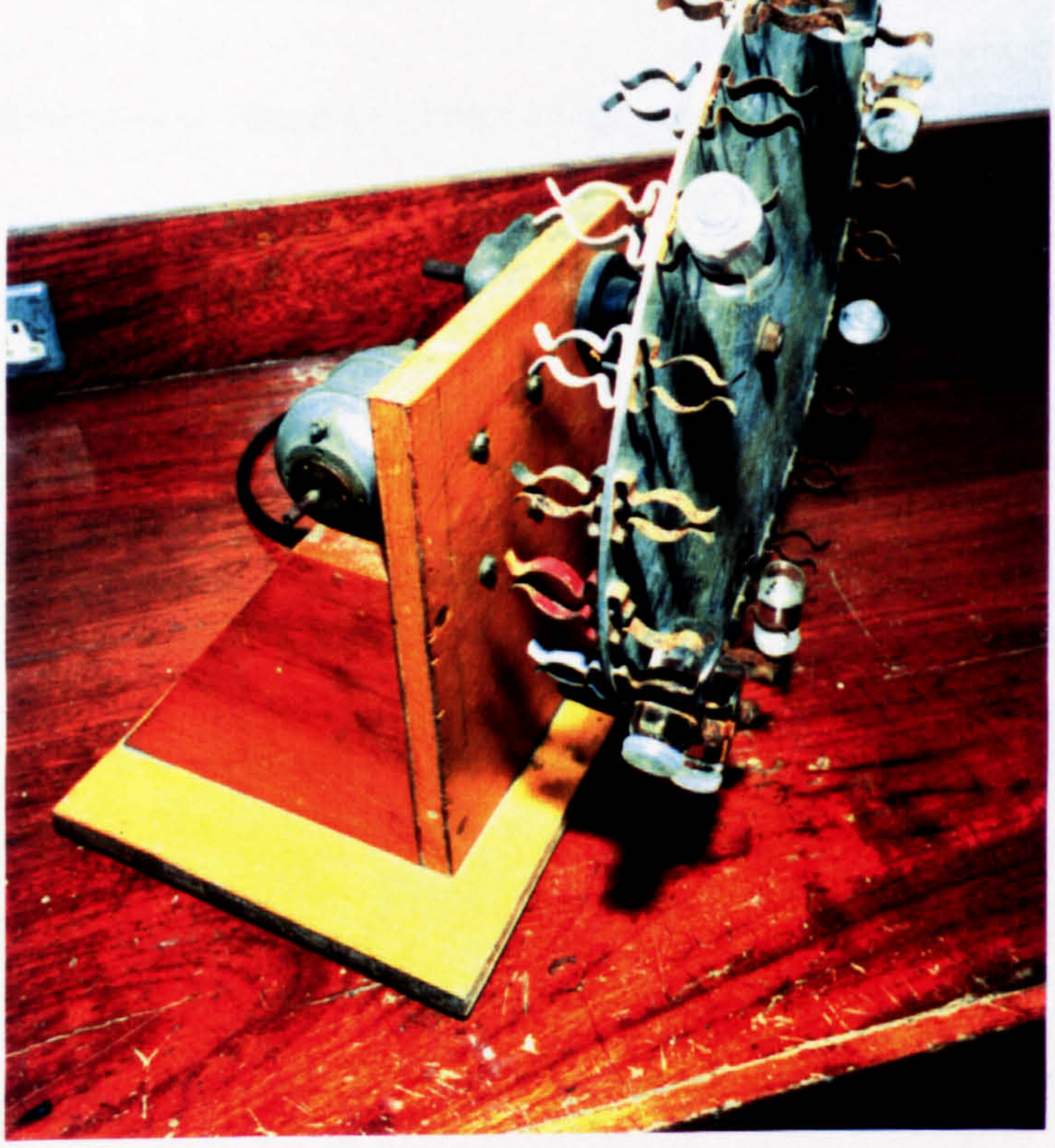
where C^e and C^c are the food particle concentrations in the experimental and 'control' cultures respectively, V is the culture volume in millilitres containing n individuals and t is the time in h.

The ingestion rate (I), can be estimated as the product of the filtration rate and the average food concentration over the time of the observations:

$$I = F. \left(\frac{C^e{}_o + C^e{}_t}{2}\right)$$

PLATES

Plate 1
Rotating wheel used in the culture of *Elminius modestus* nauplii



CHAPTER THREE

FACTORS AFFECTING COPPER TOXICITY TO *Elminius modestus* NAUPLII

CONTENTS

Copper toxicity to marine organisms exhibits great variability which results from the effects of several biotic and abiotic factors, including species, life stage, acclimation, diet, physiological state, salinity, temperature, pH, oxygen content and metal complexation (see reviews by Hodson *et al.*, 1979; Engel *et al.*, 1981; Mance, 1987) (see also Chapter One: General Introduction).

In the case of acorn barnacle nauplii, the literature contains specific examples of situations where the toxic activity of copper has been either increased, reduced or even prevented by its interaction with the above factors. Thus, the work by Lang *et al.* (1979) on the effects of copper on *Balanus amphitrite* and *B. improvisus* nauplii showed that increased temperature (within the range tolerated by the species) from 20° to 26°C increased post-exposure naupliar mortality. Similarly, Lang *et al.* (1980, 1981) found that salinity modified copper toxicity to *B. improvisus* nauplii, where a salinity increase from 15 ppt to 30 ppt resulted in a reduction in naupliar mortality. Lang *et al.* (1981) further showed that, within a 48-hour copper exposure period, mortality of unfed stage II *B. improvisus* nauplii did not differ significantly from that of well-fed larvae, although their spontaneous swimming speeds were reduced.

The first half on the present Chapter deals with long-

term toxicity tests, which are used to investigate the effects of algal diet on copper toxicity to *E. modestus* nauplii. These effects are a direct result of the interaction between copper complexation through algal products present in the naupliar culture media and the potential 'nutritional stress' caused by either inadequate quality or quantity of food, which would add to the copper stress (see Winner *et al.*, 1977).

Earlier work on the effects of copper on the larval development of *Balanus amphitrite* had shown that nauplii fed on the flagellate *R. reticulata*, which had been supplied in suspension in the medium used for its culture (Algal Culture Medium, ACM) or in an 'unwashed' form (see Chapter Two: General Materials and Methods), tolerated copper concentrations up to 1,200 ppb throughout their development (see Royo-Gelabert, 1991, unpublished).

Here, the effects of similarly high copper levels are assessed throughout the development of *E. modestus* nauplii using both 'unwashed' and 'washed' algae, as well as different ACM percentages (see Chapter Two). The algae are supplied in these different forms to try and reduce copper complexation to both algal metabolites and nutrients present in the ACM on one hand, and determine which percentage of ACM shows copper effects on both naupliar survivorship and rate of development more adequately, on the other. This part of the study also involves assessing the survival of unfed nauplii and of the algal species used to feed them, as well as naupliar ingestion rates.

Long-term tests are also used to examine whether a reduction in the experimental temperature results in a reduction of the copper toxicity to the nauplii as reported in the literature (see reviews by Hodson *et al.*, 1979; Mance, 1987).

The second half of the Chapter deals with short-term copper toxicity tests using *E. modestus* nauplii which are not fed throughout the duration of the exposure period. These tests are used to enumerate and quantify sources of variability in the methodology, such as temperature, naupliar stage and preparation of test media. This allows standardisation of the test method in order to achieve repeatability and reduce variability in determining 48-h copper-LD_{50}s. The standardised tests are then used to examine the effects of parent provenance, hatching method and of variations in the organic content of seawater on acute copper toxicity to the nauplii.

Nauplii used in any of the toxicity tests are produced by either 'artificial' release from a natural adult population living at Menai Bridge or 'natural' release from a population that had been maintained in the laboratory for 2-3 months, often producing several broods during that time, indiscriminately (see Chapter Two). Life conditions between both populations are very different, which might result in differences in the copper tolerance of the nauplii. The LD_{50}s of nauplii produced by adults of each population are then calculated in order to evaluate such hypothesis. In addition,

the 'artificial' hatching method could force the nauplii to hatch before they are ready, which may affect their fitness and could induce a reduction in their survivorship. As a result, it could lead to overestimating the mortality of nauplii used in toxicity tests. It is, therefore, tested whether artificially hatched nauplii are generally less fit that those hatched naturally.

Finally, the seawater used in the toxicity tests comes from the Menai Strait, North Wales, UK, which can carry considerable loads of both particulate and dissolved matter depending on the time of the year (see Foster & Morris, 1971). The water is cartridge-filtered (0.2 μm pore diameter) and UV irradiated prior to use to eliminate, amongst other things, organic matter (see Chapter Two). Such removal should normally be achieved with the exception, perhaps, of periods of high productivity (algal 'blooms').

Any dissolved or particulate matter reaching the naupliar cultures would, as in the case of algal metabolites, complex the cupric ions dissolved in the culture media and, subsequently, reduced copper toxicity to the nauplii. This potential complexation is investigated by carrying out different copper toxicity tests using a range of waters with different organic matter loads, having untreated water from the Menai Strait on one extreme, and artificial seawater made with 'Instant Ocean' salts on the other. The actual copper and organic matter content of the different waters is determined by chemical analysis.

Long-term copper toxicity to barnacle nauplii

The long-term toxicity test with 'unwashed' algae was carried out using nauplii, newly hatched from Menai Bridge adults, which were exposed to 25, 50, 100, 250, 500 and 1,000 ppb copper. Test concentrations were made up from 100 µl of a dilution of a 10^{-6} ppb copper stock, 11.9 ml of FUVSW and 8 ml of algal suspension to make up a 20 ml volume of culture medium (see Chapter Two). The media had a final salinity of 32 ppt, a final algal concentration of 100 cells.μl^{-1} and were contained in 22 ml glass vials (see Chapter Two). The algae had been supplied straight from the jars where they were routinely cultured in the laboratory for feeding invertebrates and so, suspended in Algal Culture Medium (ACM) or 'unwashed'. Since 8 ml of the total volume of the naupliar culture medium was made up by the algal suspension, this type of culture will be referred to as 40% ACM (see Chapter Two). The vials were placed in a wheel rotating at 6 r.p.m., which was kept in the laboratory at ambient temperature 20.5-26°C (average of approximately 24°C) under natural light conditions (see Chapter Two). The culture media were changed every 48 h when numbers of active, inactive and dead nauplii (see Bhatnagar & Crisp, 1965) as well as number of moults and stage of development (see Knight-Jones & Waugh, 1949) were recorded (with the aid of a stereoscope). Death was established through complete lack of

movement within a one-minute period (see Snell *et al.*, 1991). The length of the experimental period was 13 days, dictated by the time when >70% of the nauplii had metamorphosed to cyprid.

Copper effects on naupliar development were monitored not only through the percentage of cyprids obtained at the end of the experiment, but also through the time taken for the first cyprids to appear in the cultures, in an attempt to detect alterations or suppression of the moulting process as reported by Lang *et al* (1979, 1981) in *B. improvisus.*

The long-term test with 'washed' algae was almost identical to the one for 'unwashed' algae, but with the following exceptions: the experimental temperature was slightly lower, ranging from 21°C to 23.5°C and the length of the exposure period (13 days) was dictated by the time when >70% of the nauplii had died. The main difference, however, was that the algae *R. reticulata* were 'washed', by centrifugation at 2,000 r.p.m. for 10 min and resuspension in FUVSW, before they were added to the naupliar culture media. Thus, since all the ACM had been eliminated, these naupliar cultures will be referred to as 0% ACM (see Chapter Two).

The methodology followed in the long-term test using different ACM percentages is also very similar to the one described above; although in this case the nauplii were only exposed to 1,000 ppb copper. Test ACM percentages were 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35% and 40%, obtained by centrifugation of the algae and resuspension in FUVSW, plus

the appropriate quantity of culture medium (see Chapter Two). The test lasted 19 days, dictated by the time when >70% of the nauplii had metamorphosed to the cypris stage. The ambient laboratory temperature was 21.5°-26°C, averaging 24°C.

The experimental set up of the long-term test determining the survival of unfed nauplii using different ACM percentages was again very similar to that used in previous tests; although in this case the culture medium was made up by either 20 ml of FUVSW (0% ACM) or 20 ml of ACM (100% ACM), where each medium was replicated 4 times. The naupliar culture vials were kept in the laboratory at an ambient temperature (20.5°-26°C) and the culture media were changed every 24 h.

E. modestus nauplii were subsequently grown in uncontaminated cultures using different ACM percentages and algae. The culture media were made up by FUVSW, *R. reticulata* (to give a concentration of 100 cell.μl^{-1}) and different amounts of ACM to give final percentages of either 0% ACM, 20% ACM or 40% ACM, where each medium was duplicated. The culture vials were kept in the laboratory at ambient temperature (19.5-23.5°C) under standard culture conditions. The cultures were changed every 48 h, until the end of the experimental period after 17 days, dictated by the time when >70% of the nauplii had metamorphosed to the cypris stage.

The survival of *R. reticulata* kept for about 60 h in 0% ACM using 40% ACM as a 'control' was also tested. Each medium was replicated 3 times, where the initial algal concentration

was approximately 100 cells.μl^{-1}. The culture vials were kept at ambient laboratory temperature, 22-24°C.

The number of immobile cells and the total number of cells were calculated with the aid of a haemocytometer twice with a 6 h interval on the first day, and only once both on the second and the third day. The count was performed as follows: only the algae on the three main squares on the diagonal of each half of a haemocytometer were counted (i.e. 6 squares). The concentration of algal cells was then obtained by dividing the total number of cells on the six squares by the total volume of those squares (1.2 µl). *R. reticulata* is a flagellate hence, motile and loss of mobility is considered a good indicator of stress (see Anderson & Morel, 1978).

The test was carried out twice under identical experimental conditions, although algal growth was only monitored twice in the second test, after the first 24 h and after 48 h, at the end of the experimental period. Initial algal concentrations were 114.17 cells.μl^{-1} for 0% ACM and 105.18 cells.μl^{-1} for 40% ACM.

Naupliar ingestion rates were estimated by feeding approximately 40 stage II *E. modestus* nauplii in a culture of *R. reticulata* at ca. 100 cell.μl^{-1} suspended in 20 ml of either 0% ACM or 40% ACM inside 22 ml glass vials. The cultures were replicated 5 times with a 'control' culture vial without nauplii for each ACM percentage. The vials were placed on a wheel rotating at 6 r.p.m which was kept in the

laboratory under natural light conditions at an ambient temperature (average 26°C) for 20 h (see Chapter Two).

The initial algal concentration in the naupliar cultures was verified using a 'coulter counter' (Coulter Counter ZM, Coulter Electronics Ltd., Luton, UK). After 20 h, the cultures were fixed with 300 µl of concentrated formalin. The number of nauplii in each replicate was counted and the algal concentrations assessed with a haemocytometer. The ingestion rates of the nauplii were then calculated using the formula described in Chapter Two.

The effects of copper on *E. modestus* cultured at the lower temperature of 18°C were assessed using copper concentrations of 25, 50, 100, 250, 500 and 1,000 ppb. The nauplii, produced from adults living at Menai Bridge pier, were grown in culture media made up by copper, FUVSW and the algae *R. reticulata* in the usual manner, but using 20% ACM (see Chapter Two). The cultures were maintained in glass vials, held on a rotating wheel. The wheel was placed in a refrigerated room, which kept the cultures at 18°C, under an artificial light regime with a 12:12-h light:darkness cycle (see Chapter Two).

The culture media were changed every 48 h for 17 days, the time when >70% of the nauplii had metamorphosed into cyprid.

Short-term copper toxicity to barnacle nauplii

Initial copper LD_{50} estimations were carried out in transparent polystyrene square trays divided in (5x5) 5 ml cells (see Chapter Two). Test nauplii were hatched from an adult population maintained in the laboratory over 2-3 months, during which time they had released several larval broods. Nauplii, which were not fed during the duration of the test period, were exposed to 4 ml of a medium made up by 3.9 ml FUVSW and 100 µl copper, from 'intermediate' solutions originating from a 10^6 ppb stock (see Chapter Two) with a final salinity of 32 ppt.

Copper concentrations in the first test were 20, 40, 60, 80, 100, 120, 140, 150, 160, 170, 180 and 200 ppb, each of them duplicated. The second test used 40, 80, 120, 140, 180, 200, 220, 230, 240, 250, 260 and 270 ppb copper, which were also duplicated. The tests were separated by 96 h and some of the 'intermediate' copper solutions prepared for the first test were also used for the second one.

The trays were kept in the laboratory under natural light conditions for 48 h, after which the number of dead larvae was recorded.

The methodology to be followed in subsequent tests was standardised in order to reduce variability and allow direct comparison of results.

Firstly, it was decided to standardise the stage of the

nauplii used in the tests. Initial collection of larvae from a hatch always leads to a mixture of stage I and stage II nauplii, which could have different copper sensitivities resulting from differences in size and number of pathways for copper uptake (stage I do not feed, Darwin, 1854). Thus, the nauplii would be kept for 2 h and 30 min in FUVSW to guarantee the metamorphosis to stage II (see Harms, 1987) before they were used.

Secondly, it was decided to test copper effects on naupliar survivorship over a range of increasing temperatures in order to establish which temperature ensured adequate characterisation of such effects within the shortest time interval. Test copper concentrations were 70, 80, 90, 100, 120, 130, 140, 150, 170 and 1,000 ppb; and test temperatures were 12.5°C, 17.5°C and 22.5°C, within the range of tolerance of the species (see Crisp, 1958). Test nauplii were produced from adults living at Menai Bridge pier and the tests were carried out using polystyrene trays (see above). The trays were kept in the laboratory under natural light conditions and the experimental temperatures were achieved by placing the trays in thermostatic recirculating water baths. The nauplii were not fed during the length of the experimental period, 48 h, after which the number of dead larvae was recorded.

Thirdly, the production of test culture media was also standardised, where big volumes of culture would be freshly prepared when required. Thus, avoiding problems derived from the use of 'intermediate' copper solutions of different ages

and the preparation of the media in small volumes, as seen in previous tests. The culture media were then produced by serial dilution of a 500 ppb 1 litre initial solution, which was made up by diluting 0.5 ml of the stock copper solution (10^6 ppb) in FUVSW, with a final salinity of 32 ppt. Therefore obtaining a minimum of 200 ml of each of the required copper concentrations, 90 ml of which were used to resuspend the nauplii giving a concentration of approximately 13 nauplii.ml^{-1}. The necessary volume of both naupliar culture media and nauplii were achieved by pipetting 4 ml of each of the 90 ml solutions.

Finally, it was decided to try a new criterion to establish naupliar death. So far naupliar death had been assessed through total absence of movement, which made counting very long and tedious, since copper poisoning is a gradual process. A copper-poisoned nauplius stops swimming, lies with its dorsal area in contact with the bottom of the dish, moves internal organs and appendages spasmodically, with long periods of inactivity, and lacks the whitish colouration of larvae that have been dead for a while. A nauplius in these conditions has to be observed very carefully under the stereoscope to ascertain that it does not show any kind of movement which would place it in the 'alive' category. Here, a different criterion was used according to which a nauplius was considered to be 'alive' only when it was swimming actively in the culture medium. Larvae on the bottom of the culture dish that did not swim, but were not

necessarily without movement, were classified as 'dead', which significantly reduced the time needed for counting.

This criterion was assessed by comparing the mortality of *E. modestus* nauplii exposed to copper with that resulting from their subsequent transfer to an unpolluted medium. Approximately 30 nauplii produced by adults from Menai Bridge pier were exposed to 50, 100 and 150 ppb copper in glass vial cultures of 32 ppt salinity. The vials were placed on a rotating wheel and maintained in the laboratory at 22.5°C under natural lighting. The nauplii were not fed during the first 48 h of the experiment, after which the larvae were categorised as 'swimming' (alive) or 'non-swimming' (dead). Subsequently, all the larvae were transferred to 22 ml glass vials containing 20 ml of a copper-free culture medium, made up of FUVSW and the flagellate *Rhinomonas reticulata* (100 cell.μl^{-1}) in 20% ACM (see Chapter Two). The nauplii were maintained in these new media, under identical culture conditions to those described above, for 24 h after which the number of dead larvae was assessed again.

The 48-h copper LD_{50} of nauplii from a laboratory-maintained population was estimated using 100, 200, 300, 400 and 500 ppb copper concentrations, each of which was replicated 5 times, where the replicates were distributed within a polystyrene culture tray which was kept in the laboratory under natural illumination inside a thermostatic recirculating water bath to maintain the temperature at 22.5°C. The test lasted 48 h (larvae were not fed), when the number of dead (not swimming) larvae was assessed.

The 48-h copper LD_{50} of nauplii from a natural population was estimated in an identical manner to the previous one, with the exception that the nauplii were produced from adults living on Menai Bridge pier. In addition, the test was replicated 3 times (A, B and C) in 3 consecutive weeks in order to assess any variability that could result from the use of the newly standardised method for the preparation of naupliar culture media.

The effects of 'artificial' hatching on the survivorship of *E. modestus* nauplii, for larvae from both natural and laboratory-maintained adult populations, were assessed by testing the ability of the nauplii to develop to the cypris stage within the time period determined by the experimental temperature. The natural *E. modestus* adult population lived on rocks in Port Dinorwic which, once collected, were maintained in the laboratory for 3 days. The laboratory-maintained adult *E. modestus* population had been kept in the laboratory for several months. There were 4 treatments, each of them testing about 70 nauplii, as follows: nauplii from crushed laboratory parents, nauplii released naturally from laboratory parents, nauplii from crushed Port Dinorwic parents, and nauplii released naturally from Port Dinorwic parents; which were replicated 4 times. Nauplii were grown in 22 ml glass vial cultures consisting of 12 ml of FUVSW and 8 ml of the algae *R. reticulata*, straight from the jars where they were routinely grown in the laboratory ('unwashed'), to give a final concentration of 100

cell.μl^{-1}, at 32 ppt of salinity. The vials were placed in a wheel rotating at 6 r.p.m. kept in the laboratory at ambient temperature (20°C) under natural light conditions (see Chapter Two). The cultures were changed every 48 h, when the numbers of live and dead nauplii as well as their stage were recorded (with the aid of a stereoscope).

The method used in the tests with seawater containing different organic loads (4 carried out in 4 consecutive weeks) was the standardised one where stage II *E. modestus* nauplii 'artificially' hatched from a natural population and exposed to 100-500 ppb copper for 48 h, at 22.5°C and 32 ppt under natural illumination. The seawater used for hatching the larvae, making up the naupliar culture media, etc. was, however, different with each test. In the first one, the water came from the Menai Strait, held for three days in special 'settling' tanks and then used without filtration. In the second test, the water was obtained from laboratory supply, where water from the Strait had passed through a biological filter and then a coarse mechanical filter. The third test used artificial seawater, made up by dissolving the salts 'Instant Ocean' (Aquarium Systems, Sarrebourg, France) in deionised, distilled water. Finally, the water used in the fourth test was a mixture (50:50) of unfiltered water from the Menai Strait and standard FUVSW.

The organic matter content of the 4 waters plus that of the FUVSW, was determined, by total carbon/nitrogen assay (Roboprep CN Analyser, Europa Scientific Ltd., UK), in the first 12 h of each experimental period following the method

described by Mackereth *et al.* (1978). The method involved micro-filtration of a variable volume of water (normally two replicates per sample) onto pre-combusted glass fibre filters. The volume of sample to be filtered was determined by the change in colouration of the filters after a period of filtration. After filtering, the inorganic matter present in the samples was removed through vaporisation with concentrated hydrochloric acid. The filters were then dried at 60°C and subsequently the coloured area of each one was cut out and folded inside a pre-combusted circle of silver foil. The foil was then made into a ball which was kept in a dessicator until the moment of the analysis.

The organic matter content (carbon and nitrogen) of the samples was calculated from the traces produced by the apparatus used in the analysis, which showed the value for the area under the peak of each element as well as the calibration lines. The figures for carbon and nitrogen were obtained by using those areas, the calibration lines and the volume of sample filtered.

The copper concentration in the samples was also assessed in the first 12 h of each experimental period using 3 x750 ml replicates per water sample and following a modification of the method described in Strickland & Parsons (1972) (see Chapter Two).

The potential organic matter content of the deionised, distilled water, used to dissolve 'Instant Ocean' salts in making artificial seawater, was assessed by comparing the

colouration of the filters after micro-filtration of 2x 1 litre replicate samples and another 2 l of artificial seawater. Thus, the method employed to measure the organic load of the water used in the above tests had shown that the filters with a darker colouration corresponded to those waters with the highest organic content, and such qualitative approach was considered to be sufficient for what was required in this case.

The potential organic matter content of the 'Instant Ocean' salts used in making artificial seawater was also assessed. Thus, 3x 1-g replicates of salt (approximately) were placed in 3 crucibles of known weight. The weight of the crucibles and their content was recorded and they were then left to dry in an oven at 60°C for 12 h. Afterwards, they were weighed again and transferred to a muffle furnace where they were kept for 5 h at 550°, after which they were weighed again.

The mortality of nauplii exposed to copper using artificial seawater was compared to that of copper-exposed nauplii using FUVSW. The nauplii hatched from a natural adult population living at Menai Bridge pier. Test media were replicated (x5) and the replicates were distributed within a polystyrene culture tray in groups of 5 (see Chapter Two). Thus, the first 2 groups were a 'control' and a 180 ppb copper treatment that had been made up in FUVSW; whereas the remaining 2 groups were the equivalent, but made up in artificial seawater. The test was carried out in the standard manner at 22.5°C and 32 ppt under natural illumination and

lasted 48 h, when the number of dead larvae was assessed following the 'swimming' criterion.

Long-term copper toxicity to barnacle nauplii

i. Effects of algal diet on long-term copper toxicity: Mortality at the end of the long-term test using 'unwashed' algae, expressed as the percentage naupliar mortality and calculated from the total number of nauplii in each culture, was very low in all the cultures. The percentage of dead nauplii increased a little from the 1.16% of the 'control' culture to the 5.79% at 1,000 ppb (see Table 2).

Added [Cu] (ppb)	cont.	25	50	100	250	500	1000	Total
No. dead nauplii	4	5	7	8	9	7	7	47
No. live cyprids	80	80	85	93	82	81	61	569
Total	84	85	92	101	98	88	68	616

Table 2 Chi-square test on the survivorship and development of fed *E. modestus* nauplii, from a natural population, grown for 13 days (at approx. 24°C and 32 ppt) in different copper concentrations at 40% ACM, [Cu]=copper, cont.='control' culture

The percentage of cyprids obtained after the 13-day exposure period was very high (93-98%). In addition, cyprids were first obtained after 7 days in all treatments, a time period considered 'normal' for this species at the experimental temperature (20.5-26°C, mean of about 24°C) (see Moyse, 1960; Tighe-Ford *et al*, 1970).

There were no significant differences in the distribution of dead nauplii and living cyprids between cultures (X^2=2.395, p=0.879, df=6).

The results of the tests lead to conclude that copper toxicity to the nauplii was masked due, probably, to the components of the ACM (i.e. algal exudates and culture nutrients).

In contrast, the percentage naupliar mortality in the test using 'washed' algae was of 100%. Not only the copper-exposed nauplii died, but also the 'controls'. Above 100 ppb copper, death occurred in the first 6 h of experiment and the nauplii did not develop beyond stage II. Below 100 ppb copper, the nauplii were alive until the 8th day and reached stage IV; whereas all the 'control' larvae had died by the 13th day, several developing to stage VI (see Table 3).

Added copper (ppb)	N	100% mortality after	Stage
control	42	13 days	V-VI
25	54	9 days	IV-III
50	78	8 days	IV-III
100	70	7 days	II-III
250	74	6 h	II
500	90	6 h	II
1000	91	6 h	II

Table 3 Survivorship and development of fed *E. modestus* nauplii, from a natural population, grown for 13 days (at approx. 22°C and 32 ppt) in different copper concentrations at 0% ACM

These results showed that lack of ACM within the naupliar culture medium had enhanced copper toxicity to the

nauplii.

The percentage naupliar mortality in the test using different ACM percentages ranged from 26.41% at 1,000 ppb and 40% ACM, to 100% at 1,000 ppb and 0% ACM; where intermediate percentages of ACM resulted in intermediate values for mortality (see Table 4).

Below 20% ACM, however, naupliar mortality was very high, reaching 100% at 0%, 5% and 10% ACM in the first 96 h of experiment and where no nauplii metamorphosed further than stage II. In contrast, above 20% ACM increased percentages of ACM resulted in increased naupliar survival (see Table 4).

%ACM	Added copper (ppb)	N	Percentage mortality	Percentage cyprids	1st day cyprids	100% mortality after
0	cont.	99	100	0.00	-	11 days
0	1000	125	100	0.00	-	96 h
5	1000	126	100	0.00	-	96 h
10	1000	126	100	0.00	-	96 h
15	1000	112	91.96	5.35	9th	-
20	1000	113	58.40	32.65	9th	-
25	1000	97	51.54	45.05	9th	-
30	1000	87	35.63	50.57	7th	-
35	1000	110	30.18	68.43	7th	-
40	1000	97	26.41	78.20	7th	-

Table 4 Survivorship and development of fed *E. modestus* nauplii, from a natural population, grown for 19 days (at approx. 24°C and 32 ppt) in media made up by different percentages of Algal Culture Media (ACM) and 1,000 ppb copper

Therefore, 20% ACM was the ideal percentage to show copper effects on the nauplii without masking or enhancing them.

It follows that ACM might be providing some essential

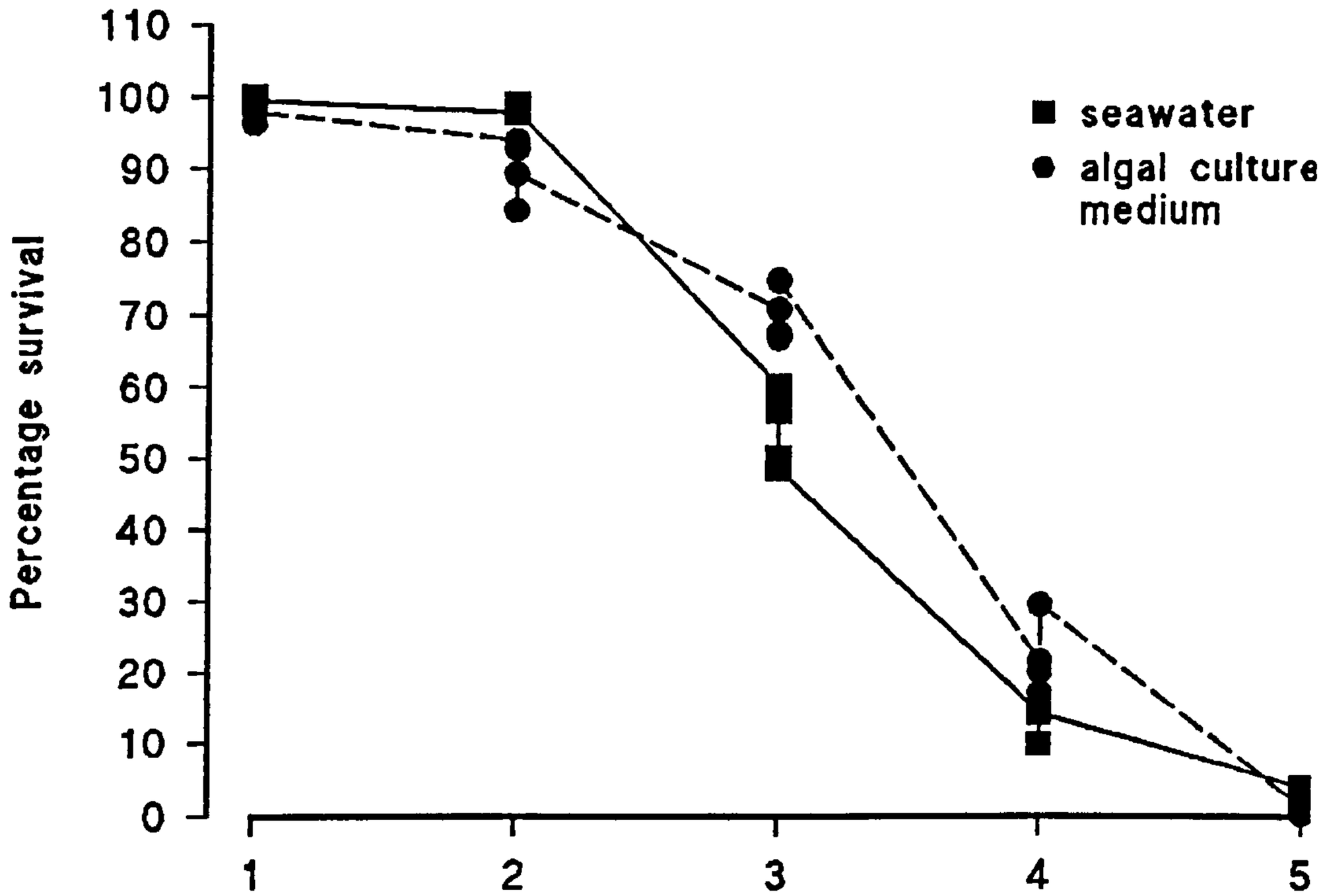
element for naupliar survival. This hypothesis was assessed by removing the algae from the naupliar culture media and testing the survival of the nauplii in different percentages of ACM. All the nauplii had died after 5 days without food, none of them reaching stage III. There were no significant differences in the mortality distributions between treatments at day 5 (X^2=2.927, p=0.513, df=3), where the mean mortality percentage at 0% ACM was 96.95±0.70 and 98.53±0.94 for 40% ACM. An Analysis of Variance (homogeneous variance at Bartlett's statistic, X^2=0.068, p=0.793, df=1) comparing the linear portion of the relationship between the percentage of surviving nauplii and time showed that there was no significant difference in slope (F= 0.18, p=0.677) (see Figure 2). These results show that ACM had not provided significant extra nutrients to the nauplii.

E. modestus nauplii were then grown in uncontaminated cultures, made up by using different percentages of ACM, and fed on *R. reticulata* to investigate the reason for the unexpectedly high mortality of 'control' nauplii observed in previous tests, and determine if variations in ACM percentages would result in variations in the survival and rate of development of the nauplii.

The percentage naupliar mortality decreased with increased ACM from 100% at 0% ACM, to 6.9% at 40% ACM. All the nauplii died at 0% ACM as had been observed in previous tests. In contrast, mortality values for 20% ACM and 40% ACM were lower than 10% and did not differ significantly (t=0.45, p=0.73, df=1) (see Table 5).

Figure 2

Percentage survival of *Elminius modestus* nauplii, from a natural population, grown for 5 days (at approx. 23°C and 32 ppt) without food in media made up of either seawater (0% ACM) or algal culture (100% ACM)



% ACM	N	Percentage mortality	Mean	Percentage cyprids	Mean
20 a	81	7.4	8.35	91.30	90.95
20 b	43	9.3		90.60	
40 a	86	6.9	7.90	91.70	90.85
40 b	56	8.9		90.00	

Table 5 Survivorship and development of fed *E. modestus* nauplii from a natural population grown for 17 days (at approx. 21.5°C and 32 ppt) in media made up of different percentages of ACM, 0% ACM (x2), 20% ACM (x2) and 40% ACM (x2)

At 20% ACM and 40% ACM most of the nauplii reached the cypris stage on the 9th day. However, the nauplii grown at 0% ACM had died in the first 10 days of the experiment period in which time they only reached stage III/IV. It is clear that lack of ACM had a significant detrimental effect on the development of *E. modestus* nauplii.

The survival of *R. reticulata* in different ACM percentages was then tested to assess if the exclusion of ACM, maybe through lack of nutrients, led to the death of the algae, which would, in turn, cause the death of the nauplii.

After 3 days, the final algal concentrations were around 100 cells. μl^{-1} for both treatments. That had been the algal concentration at the beginning of the test hence no population growth took place. Thus, the mean value for 0% ACM was 95.55±20.45 cells.μl^{-1} and 106.94±10.04 cells.μl^{-1} for 40% ACM (see Table 6).

A Two-way Analysis of Variance (homogeneous variance at Bartlett's statistic X^2=1.6721, p=0.975, df=7) determined

that there were no significant differences in the mean total cell values with either percentage of ACM (F=0.23, p=0.637) or time (F=2.86, p=0.070), and that the interaction term was not significant (F=1.18, p=0.347).

The percentage of immobile after the 3 days, which was very difficult to estimate, was again rather similar for both treatments at 42.73±9.23% for 0% ACM and 32.98±6.81% for 40% ACM (see Table 6).

		t=5 h	t=11 h	t=33 h	t=57 h
0% ACM	[Total algae]	90.27 ±11.71	90.83 ±13.77	99.44 ±15.01	95.60 ±20.50
	%Immobile algae	92.00 ±13.45	75.83 ±11.97	25.97 ± 6.04	42.73 ± 9.23
40% ACM	[Total algae]	72.28 ±12.92	97.22 ± 8.91	107.22 ±11.38	106.94 ±10.05
	%Immobile algae	90.40 ± 8.38	88.28 ±24.04	24.09 ± 9.33	32.98 ± 6.81

Table 6 Survivorship of *R. reticulata* cells grown for 3 days (at approx. 23°C and 32 ppt) in media made up of either 0% ACM (x3) or 40% ACM (x3), [algae]=cells.μl^{-1}; cell concentrations are the mean value for 3 replicate counts of the total number of cells in the haemocytometer, h=hours

A Two-way Analysis of Variance (homogeneous variance at Bartlett's statistic, X^2=5.301 p=0.623, df=7) determined that there were no significant differences in the mean percentage of immobile cells between the different ACM percentages (F=≤0.001, p=0.970) and no significant interaction (F=0.83, p=0.495). In contrast, the percentage of immobile cells decreased significantly with time (F=41.30, p≤0.001).

The results suggest that the cells were initially under

stress, perhaps due to the centrifugation, but that the presence of ACM had no significant effect on the growth (which was essentially zero within the error of counting) or health of the culture. The observation were then repeated with a fresh algal culture, in case the one used initially had reach a 'static-growth phase' in culture (see Massuti & Margalef, 1950).

After 24 h, the mean total algal concentration in 0% ACM had decreased to 73.23±1.41 cells.μl^{-1}; whereas for 40% ACM the value remained very close to the initial concentration 106.94±3.05 cells.μl^{-1} (see Table 7).

		t=0 h	t=24 h	t=48 h
0% ACM	[Total algae]	114.17	73.23±1.41	66.65±7.55
	% Immobile algae	-	16.04±2.85	20.41±6.85
40% ACM	[Total algae]	105.18	106.94±3.05	135.36±6.00
	% Immobile algae	-	21.55±5.05	21.68±2.38

Table 7 Survivorship of *R. reticulata* grown for 48 h (at 23°C and 32 ppt) in media made up of either 0% ACM (x3) or 40% ACM (x3); [algae]=cells.μl^{-1}, cell concentrations are the mean value for 3 replicate counts of the total number of cells in the haemocytometer, h=hours

At the end of the experimental period, the total algal concentration at 0% ACM had decreased even further to 66.65±7.55 cells.μl^{-1}; whereas the value at 40% ACM had increased to 135.36±6.00 cells.μl^{-1}. Thus, after 48 h the total algal concentration in 40% was double that in 0% ACM (see Table 7).

A Two-way Analysis of Variance (homogeneous variance at

Bartlett's statistic, X^2=5.48, p=0.139, df=3) determined that the differences in the mean total algal concentrations were significant both between ACM (F=118.34, p≤0.001) and time (F=5.39, p=0.049) and that the interaction term was also significant (F=13.82, p=0.006).

The individual means were, therefore, compared by Tukey's method for multiple comparisons, which showed that initial and final total algal concentration differ significantly for both percentages of ACM. Cell concentrations at 0% ACM had decreased significantly relative to those at the beginning of the experimental period; whereas the those at 40% ACM had increased significantly (see Table 8).

	Time (h) comparisons	Mean difference	
0% ACM	0-24	41.0	s
	0-48	47.5	s
	24-48	6.5	ns
40% ACM	0-24	-2.0	ns
	0-48	-30.0	s
	24-48	-28.0	s
0% ACM versus 40% ACM	0-24	9.2	ns
	0-48	-33.8	s
	24-48	-68.3	s

Table 8 Tukey's test for multiple comparisons on the mean total cell concentrations of *R. reticulata* (cells.μl^{-1}) grown for 48 h (at 23°C and 32 ppt) in media made up of either 0% ACM (x3) or 40% ACM (x3), average S.E.=4.70, minimum difference for 0.05=19.27, ns/ s=(non) significant differences

The percentage of non-mobile cells increased a little from 16.04±2.85% after 24 h to 20.41±6.85% after 48 h at 0% ACM; whereas it remained unaltered at 40% ACM (21.55±5.05 versus 21.68±2.38) (see Table 7).

A Two-way Analysis of Variance (homogeneous variance at Bartlett's statistic, X^2=2.258, p=0.520, df=3) determined that the differences in the mean percentages of immobile algae with time (F=1.60, p=0.242) were not significant, and that there were no significant differences between ACM (F=0.70, p=0.426), where the interaction term was not significant either (F=0.62, p=0.453).

Lack of ACM, therefore, led to a reduction in cell numbers, probably representing a loss of condition in the algae owing to lack of nutrients. Such reduction in growth could have, in turn, reduced the amount of algae available to the nauplii hence, affected their ability to acquire sufficient energy for development. In addition the ingestion rate in the larvae could have also been reduced if the lower quality algae were not as palatable.

It was, therefore, important to assess the feeding behaviour of *E. modestus* nauplii grown in 0% ACM using 40% ACM as a reference, in order to establish whether that percentage of ACM could induce variations in the ingestion rate.

The ingestion rates between treatments were different, where the mean value for the 5 replicate cultures at 0% ACM (277.01±93.01 cells.h^{-1}.nauplius^{-1}) was about 2 and a half times smaller than that for the replicates at 40% ACM

(684.90±234.28 cells.h^{-1}.nauplius^{-1}) (see Table 9).

% ACM		No. nauplii	F	Mean F	I	Mean I
0	a	97	4.85		361.85	
0	b	76	2.20	3.526	187.36	277.88
0	c	88	4.67	±1.35	356.85	±93.35
0	d	84	1.98		168.34	
0	e	76	3.93		315.04	
40	a	89	6.95		554.19	
40	b	78	7.04	8.422	578.96	688.10
40	c	97	6.01	±2.73	486.74	±235.32
40	d	64	9.29		748.49	
40	e	40	12.82		1072.13	

Table 9 Ingestion rates in *E. modestus* nauplii (stage II) from a natural population grown for 20 h (at 26°C and 32 ppt) in media made up of either 0% ACM (x5) or 40% ACM (x5), F= filtration rate (cells.h^{-1}.nauplius^{-1}), I=ingestion rate (cells.h^{-1}.nauplius^{-1})

A Mann-Whitney test determined that those differences were significant (p=0.0122).

Over 30 h, the total algal concentration in the 'control' cultures had changed. There had been a slight increase in the case of 0% ACM (6%) and a substantial increase (40%) in the case of 40% ACM (see Appendix). The final concentrations in the experimental 0% and 40% ACM vials were roughly equivalent hence, both groups of nauplii had enough algae to feed on. Therefore, nauplii at 0% ACM ate less due to either a direct effect of the lack of ACM on them, or an indirect effect resulting from the negative influence of 0% ACM on the algae. It could be that the presence of algal exudates in the naupliar culture media, as would be the case of 40% ACM, increased the volume of culture

swept free by the nauplii (see Yule, 1986) hence, the ingestion rate. The lack of algal exudates in the 'washed' cultures (0% ACM) would cause a lower overall ingestion rate if naupliar feeding behaviour was not stimulated.

ii. Effects of a temperature reduction on long-term copper toxicity: All the above tests had been carried out at ambient temperature in the laboratory, which at no time was lower that 20° (it was the beginning of September). It was, therefore, decided to assess copper effects on *E. modestus* nauplii cultured at the lower temperature of 18°C.

The percentage of naupliar mortality after 17 days, was very low for all the treatments, increasing from 6.41% in the 'control' culture to 18.66% at 1,000 ppb. This last value was lower than the expected minimum 50% as seen in the test that combined different ACM percentages (see Table 10).

Added copper (ppb)	N	Percentage mortality	Percentage cyprids	1st day cyprids
cont.	78	6.41	93.05	8th
25	77	10.38	88.57	8th
50	89	12.35	86.41	8th
100	85	10.58	89.41	8th
250	83	14.45	84.00	8th
500	89	8.98	90.80	8th
1000	75	18.66	80.00	8th

Table 10 Survivorship and development of *E. modestus* nauplii from a natural population grown for 17 days (at 18°C and 32 ppt) in different copper concentrations

The percentage of cyprids obtained at the end of the experiment was very high (≥80%) and similar for all the

cultures. Differences in both naupliar mortality and the total number of cyprids between cultures were not significant (X^2=7.830, p=0.250, df=6).

The reduction in temperature from the usual 20°C-26°C to 18°C, therefore, lengthened the time to cyprid by one day relative to previous cultures. In addition, it must have reduced copper toxicity, since copper appeared to have no effect on naupliar mortality. Thus, the percentage naupliar mortality at any of the copper concentrations tested did not differ significantly from that of the 'control' nauplii. Furthermore, the value at 1,000 ppb was lower at 18.66% than the one at 20% ACM of the test using different ACM percentages (58.40%) carried out at approximately 24°C.

Short-term copper toxicity to barnacle nauplii

i. Initial LD_{50} estimations: The percentage naupliar mortality increased in general with the increase in copper concentration from 3.72±2.43% in the 'control' cultures to 49.78±2.92% at 200 ppb (see Table 11).

Added copper (ppb)	N A	N B	% Mortality A	% Mortality B	% Mortality mean
control	50	55	2.00	5.45	3.72 ± 2.43
20	42	65	2.38	12.30	7.34 ± 7.01
40	33	59	9.09	8.69	8.89 ± 0.28
60	41	53	7.31	9.43	8.37 ± 1.49
80	56	69	10.71	15.94	13.32 ± 3.69
100	55	84	9.09	16.66	12.87 ± 5.35
120	45	54	15.55	16.66	16.10 ± 0.78
140	45	63	13.33	15.69	14.51 ± 1.66
150	61	54	22.95	29.62	26.28 ± 4.71
160	43	74	28.60	28.91	28.75 ± 0.21
170	50	56	44.00	35.71	39.85 ± 5.86
180	51	77	56.86	42.05	49.45 ±10.47
200	35	54	47.71	51.85	49.78 ± 2.92

Table 11 Survivorship of *E. modestus* nauplii from a laboratory maintained adult population exposed for 48 h (at approx. 22.5°C and 32 ppt) to different copper concentrations, A & B=replicate cultures

The acute toxicity test had been carried out as a means to establish the LD_{50}, the copper dose that kills half of the naupliar population, which was necessary in order to compare mortalities between toxicity tests (see Chapter Two). Here, however, the percentage naupliar mortality never reached 50% making it impossible to calculate the LD_{50} (see Sprague, 1970; Finney, 1971). Therefore, the test had to be

repeated using higher copper concentrations.

The percentage naupliar mortality increased in general with the increase in copper concentration from 1.32±1.87% in the cultures at 40 ppb to 70.07±14.03% at 270 ppb (see Table 12).

Added copper (ppb)	N		% Mortality		% Mortality mean
	A	B	A	B	
control	42	51	4.76	0.00	1.38 ± 1.95
40	24	43	0.00	4.65	1.32 ± 1.87
80	23	41	4.34	2.43	4.88 ± 0.77
120	29	41	3.44	0.00	5.22 ± 2.51
140	20	33	20.00	18.18	19.09 ± 1.28
180	30	27	13.33	22.22	21.27 ± 1.33
200	24	39	16.66	25.64	23.65 ± 2.81
220	27	31	47.05	26.00	36.52 ±14.88
230	31	31	29.03	35.80	37.41 ± 2.28
240	32	38	21.87	34.21	38.04 ± 5.41
250	32	33	59.25	33.33	46.29 ±18.32
260	28	35	42.85	42.85	42.85 ± 0.00
270	35	39	80.00	60.15	70.07 ±14.03

Table 12 Survivorship of *E. modestus* nauplii from a laboratory-maintained adult population exposed for 48 h (at approx. 22.5°C and 32 ppt) to different copper concentrations, A & B=replicate cultures

The upper range of naupliar mortalities was over 50% and, subsequently, the results were adequate to calculate the LD_{50} (see Finney, 1971). However, the LD_{50} was not calculated since the data exhibited numerous inconsistencies. Thus, mortalities obtained at copper concentrations which had also been used in the previous test were not equivalent and tended to be comparatively lower. For example, the mortality percentage at 120 ppb was 5.22±2.51%; while previously it had been 16.10±0.78% (see Table 12).

The above results could be a consequence of having used 'intermediate' copper solutions of different ages. Various attempts to measure LD_{50}s repeatedly led to extremely variable results hence the following sections describe efforts to standardise the method in order to reduce variability.

ii. Effects of increasing temperatures on short-term copper toxicity: The percentage naupliar mortality reflected changes in copper concentration and temperature, increasing proportionally with the increase in both parameters. At 1,000 ppb, however, mortality reached the expected 100% regardless of the temperature (see Table 13).

Added copper (ppb)	N			% Mortality		
	12.5°	17.5°	22.5°	12.5°	17.5°	22.5°
control	43	38	67	0.00	0.00	1.49
70	50	59	50	0.00	4.00	10.00
80	50	57	44	0.00	4.00	11.36
90	67	67	41	0.00	2.98	19.51
100	67	51	18	2.98	4.47	16.66
120	54	53	30	3.70	5.55	20.33
130	68	35	40	4.41	8.57	32.50
140	23	20	37	8.69	10.00	32.43
150	68	64	57	10.29	10.93	54.86
160	60	41	47	11.16	12.19	57.44
170	43	52	21	13.95	17.30	66.66
1000	55	19	29	100	100	100

Table 13 Survivorship of *E. modestus* nauplii from a natural population grown (at 32 ppt) for 48 h in different copper concentrations at different temperatures (degrees centigrade)

The temperature that allowed a better characterisation of copper effects on the nauplii over the duration of the

exposure period was 22.5°C, and that would be the temperature used in future tests.

iii. New criterion for establishing the death of the nauplii: The percentage naupliar mortality was affected by both the copper concentration and the time at which the dead larvae were counted, from 6.62±3.48% at Day 1 (after a lapse of 48 h) and 10.79±2.41% at Day 2 (after a total of 72 h) in the 'control' cultures, to 62.35±4.61% at Day 1 and 78.42±0.42% at Day 2 at 150 ppb (see Table 14).

Added copper (ppb)	N	% Mortality Day 1	% Mortality Day 2	% Mortality mean Day 1	% Mortality mean Day 2
control a	28	4.16	12.50	6.62	10.79
control b	22	9.09	9.09	±3.48	±2.41
50 a	26	17.39	21.73	15.83	20.92
50 b	35	14.28	20.12	±2.19	±1.13
100 a	35	29.03	61.90	44.86	69.18
100 b	18	60.70	76.47	±22.39	±10.44
150 a	25	59.09	78.72	62.35	78.42
150 b	35	65.62	78.12	±4.61	±0.42

Table 14 Survivorship of *E. modestus* nauplii from a natural population, exposed for 48 h (at 22.5°C and 32 ppt) to different copper concentrations and subsequently transferred to unpolluted media where they remained for 24 h. Day 1= values after a lapse of 48 h and Day 2= after a further 24 h

A Two-way Analysis of Variance (homogeneous variance at Bartlett's statistic, X^2=12.333, p=0.090, df=7) on the data determined that the differences in mortality between copper concentrations were significant (F=38.91, p≤0.001) and that there was also a significant effect of time (F=5.93,

p=0.041). The interaction term was not significant (F=0.71, p=0.571). Hence, the increase in mortality between Day 1 and Day 2 was consistent between treatments.

These results indicate that after 48 h of copper exposure, larvae on the bottom of the culture dish (non-swimming) had been irreversibly damaged by the metal, since after being placed in clean media with abundant and adequate food, they could not recover and died. Therefore, it would have been more appropriate to have placed them in the 'dead' category initially.

In the case of the 'control' nauplii, the increase in mortality from Day 1 (mortality inherent to the larval stock) to Day 2 could have been caused by lack of food during the first 48 h. Thus, the nauplii would have suffered 'nutritional stress' (see Lang *et al.*, 1979; Lang & Marcy, 1982). Such stress does not have a direct effect on the survival of the nauplii if imposed for less than 24 h, but it affects naupliar physiology which, in turn, could reduce survivorship (see Lang *et al.*, 1979; Lang & Marcy, 1982). Where copper was used, the 'nutritional stress' compounded that caused by the presence of the metal, thus reducing the survival of the nauplii even further (see Winner *et al.*, 1977).

In view of these results, it was concluded that the criterion for estimating naupliar mortality proposed here was adequate and would be used in future experiments.

iv. Effects of parent provenance on short-term copper toxicity to the first generation: The LD_{50}s of nauplii from both a natural and a laboratory-maintained population, living under very different environmental conditions, were calculated and compared to assess the effects of parent provenance of naupliar sensitivity to copper.

The range of copper concentrations tested in both cases was very wide (100-500 ppb) to make sure that the resultant mortalities would span 50%, as needed to calculate the LD_{50} (see Finney, 1971).

The percentage naupliar mortality in the case of nauplii from the laboratory-maintained population increased with copper concentration ranging from 0% and 3.22% at 100 ppb, and reaching 100% at 500 ppb (see Table 15).

A Latin Square Analysis of Variance of the mortality percentage of the 5 replicates of each of the 5 copper concentrations tested, was performed in order to check for systematic errors due to the position of the chambers in the culture tray. The test showed that for each concentration, there were no significant differences between replicates (column, $p=0.289$; row, $p=0.310$) hence, the position of the different cells in the culture tray did not affect the final result.

The LD_{50} was then calculated following the method developed by Finney (1971) (see Chapter Two). Thus, the lethal dose for 50% of a population of *E. modestus* stage II nauplii, from a laboratory-maintained population, exposed for

48 h at 22.5°C and approximately 32 ppt, was 252 ppb (see Table 17).

Added copper (ppb)	N	% Mortality	% Mortality mean
100 a	70	0.00	
100 b	40	2.50	
100 c	62	3.22	1.77 ± 1.64
100 d	63	3.17	
100 e	61	0.00	
200 a	52	9.61	
200 b	52	17.30	
200 c	38	21.05	14.36 ± 4.71
200 d	47	12.76	
200 e	45	11.11	
300 a	23	69.23	
300 b	29	73.68	
300 c	22	72.72	66.96 ± 19.04
300 d	28	61.11	
300 e	31	58.06	
400 a	35	91.42	
400 b	26	96.15	
400 c	39	89.74	94.31 ± 3.56
400 d	51	96.07	
400 e	56	98.21	

Table 15 Survivorship of *E. modestus* nauplii (stage II) from a laboratory-maintained population exposed to different copper concentrations for 48 h (at 22.5°C and 32 ppt), a-e=positions in culture tray

In the case of nauplii from a natural population, for all three replicates (A, B and C) and each one of the copper concentrations, the percentage naupliar mortality increased with concentration ranging from 6.59±2.39% (B) to 13.65±4.65% (C) at 100 ppb, and reaching 100% at 400 ppb. The percentages in replicates A and C appeared very similar; whereas those of replicate B were generally lower up to 300 ppb, the

maximum copper concentration survived by the nauplii. Mortalities were, however, compared in terms of the LD_{50}s (see Table 16).

Added copper (ppb)		N			% Mortality			% Mortality mean		
		A	B	C	A	B	C	A	B	C
100	a	-	55	65	-	7.27	10.76			
100	b	80	56	77	7.50	3.50	7.79			
100	c	82	55	71	13.41	7.27	11.26	13.65	6.59	10.43
100	d	44	39	71	18.18	5.12	11.26	±4.65	±2.39	±1.49
100	e	58	51	54	15.51	9.80	11.11			
200	a	76	46	65	67.10	34.78	49.23			
200	b	60	60	65	46.66	28.33	53.34			
200	c	78	46	59	41.02	36.95	40.67	56.14	30.80	52.07
200	d	51	-	57	56.86	-	63.15	±12.31	±6.12	±8.15
200	e	55	34	63	69.09	23.52	53.96			
300	a	46	62	35	97.82	98.38	94.28			
300	b	50	56	47	78.00	92.85	85.10			
300	c	52	52	27	84.61	96.15	88.88	87.85	96.21	89.85
300	d	38	50	43	89.47	96.00	93.02	±7.27	±2.13	±3.76
300	e	47	43	25	89.36	97.67	88.00			

Table 16 Survivorship of *E. modestus* nauplii (stage II) from a natural adult population exposed to different copper concentrations for 48 h (at 22.5°C and 32 ppt) A, B and C= replicates carried out in consecutive weeks, a-e=positions in the culture tray

A Latin Square Analysis of Variance of the mortality percentage of each of the 5 replicates of the 5 copper concentrations tested, was carried out prior to its calculation in order to check for significant systematic errors resulting from the position of the chambers in the culture tray. The test showed that, for each concentration, there were no significant differences between cells (A, column, p=0.829; row, p=0.059), (B, column, p=0.249; row, p=0.437), (C, column, p=0.108; row, p=0.217).

The LD_{50}s were then calculated as described before, where the lethal doses for 50% of a population of *E. modestus* stage II nauplii, from a natural population, exposed for 48 h at 22.5°C and approximately 32 ppt, were 172 ppb (A), 195 ppb (B) and 185 ppb (C) (see Table 17).

	Replicate	LD_{50} (ppb)	C.I.(95%)
Laboratory nauplii	-	252	242-261
Menai Bridge nauplii	A	172	165-181
	B	195	187-202
	C	185	177-192

Table 17 LD_{50}s (lethal doses for 50% of the population) and 95% confidence intervals, determined by the naupliar mortality percentages of *E. modestus* nauplii (stage II) from a laboratory-maintained or a natural adult population (Menai Bridge), exposed for 48 h to 100-500 ppb copper (at 32 ppt and 22.5°C)

Subsequently, the LD_{50}s were compared between themselves and, at the same time, with the one obtained in the previous test. The comparison was performed following the method of the Relative Potencies described in Finney (1971) and selecting the LD_{50} for replicate A of this test (172 ppb) as the 'reference' LD_{50} (see Finney, 1971). The method also involved the calculation of the 95% confidence intervals for each relative potency; where significant differences between potencies were determined by whether their respective confidence intervals included 1 or not ('reference' relative potency).

In this case there were no significant differences

between the relative potencies of the LD_{50}s obtained in this test; whereas the one for the previous test (nauplii from a laboratory-maintained population) was significantly higher (see Table 17 and Figure 3).

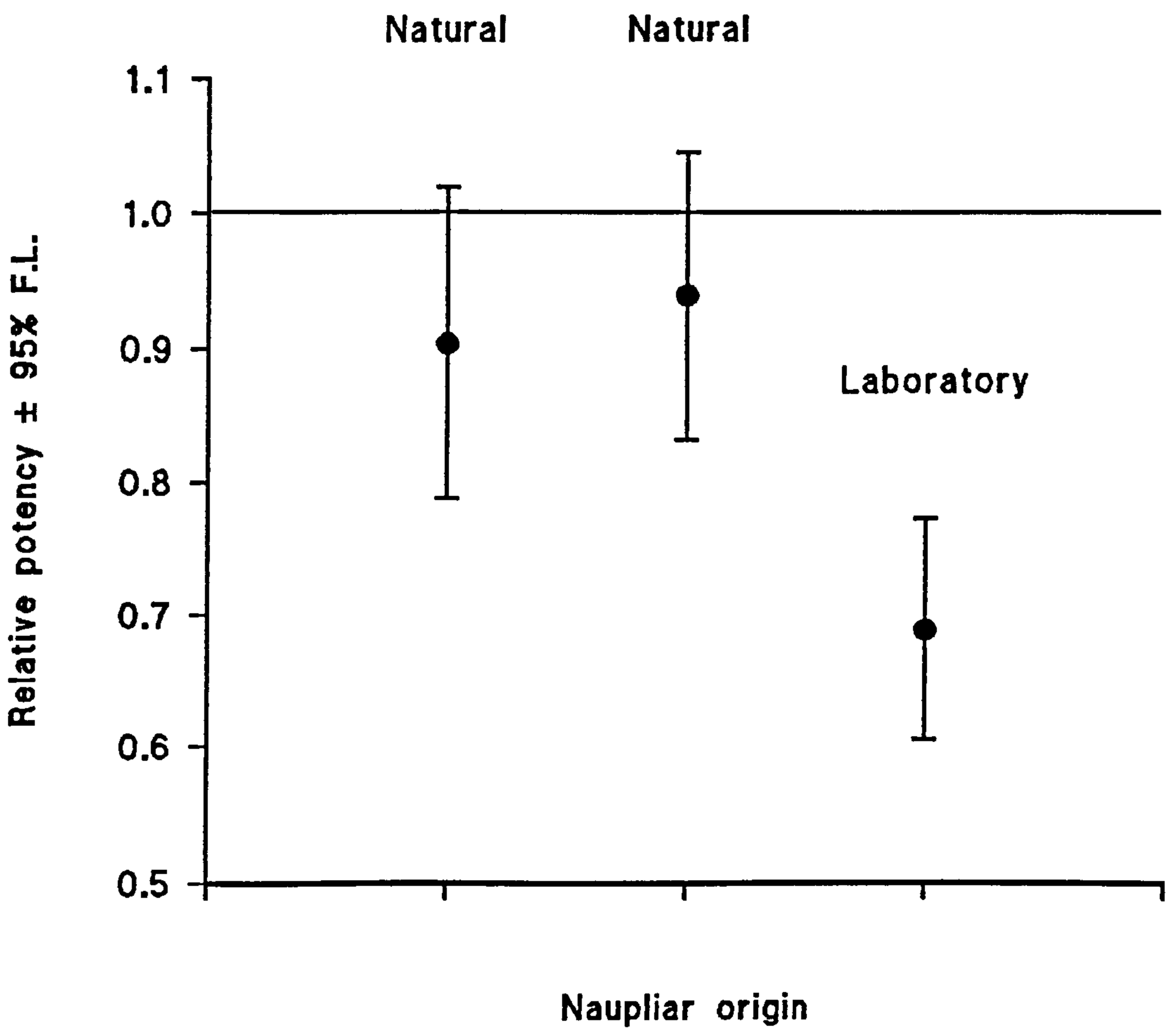
It can be concluded that either the larvae from Menai Bridge pier were less fit that those from the laboratory population or that the method used to release the larvae (artificial versus natural) altered their susceptibility to copper.

The combined effect of naupliar origin and hatching methods was assessed in order to validate the above hypotheses. Cyprids started to appear in all the cultures after 4 days determining the end of the test and showing that there had been no differences in the length of the development period between treatments. Furthermore, the percentage naupliar mortality obtained after the 4th day were again very similar (see Table 18).

A Two-way Analysis of Variance (homogeneous variance at Bartlett's statistic X^2=1.344, p=0.720, df=3) confirmed the lack of significant difference in the mean mortality percentage between nauplii from a laboratory-maintained and a natural population (F=3.79, p=0.075).

The test also showed that there were no significant differences in mortality between hatching methods (F=0.07, p=0.791) and that the interaction was not significant (F=4.67, p=0.052). Nauplii hatched naturally from the laboratory-maintained population showed the lowest mortality, although not significantly so. Presumably the

Figure 3
Relative potencies and 95% Fiducial Limits of the LD_{50}s of *Elminius modestus* nauplii (stage II) from either a natural or a laboratory-maintained adult population, exposed to 100–500 ppb copper for 48 hours (at 22.5°C and 32 ppt)



added stress of copper contamination would highlight the differences in fitness between laboratory and field larvae, which are not statistically evident under less stressful conditions.

Adult population	Hatching method	N	% Mortality	% Mortality mean
PortD	a	45	6.52	
	b 'artificial'	67	4.34	
	c	61	8.82	5.96 ± 2.47
	d	49	4.16	
PortD	a	63	6.38	
	b 'natural'	69	7.69	
	c	57	11.86	8.53 ± 2.34
	d	84	8.19	
Lab.	a	52	4.76	
	b	43	4.34	
	c 'artificial'	43	5.90	6.18 ± 2.18
	d	48	9.75	
Lab.	a	45	2.38	
	b 'natural'	36	4.65	
	c	49	4.65	4.19 ± 1.22
	d	52	5.08	

Table 18 Survivorship of *E. modestus* nauplii (stage II) from either natural (PortD) or laboratory-maintained (Lab.) adult populations, hatched by either an 'artificial' or a 'natural' method and grown in the laboratory for 4 days (at 20°C and 32 ppt) until cyprids started to appear, a-d=replicate cultures

v. Effects of the organic content of seawater on short-term copper toxicity: In each test, and for the range of copper levels tested, the percentage naupliar mortality increased with concentration. Values at 100 ppb ranged from 5.23±2.13% for the filtered Menai Strait water, to 55.8±9.93% for the artificial seawater. Naupliar mortality percentages

of 100% were reached at 500 ppb for both the unfiltered and the filtered Menai Strait waters and the 50:50 water mixture, and at 400 ppb for the artificial seawater. None of the nauplii survived copper levels above 400 ppb (see Table 19).

Added copper (ppb)	% Mortality ± stdev			
	U	F	A	M
100	8.71±2.80	5.23±2.13	55.75±9.93	5.28±1.60
200	24.56±8.75	20.55±8.33	79.45±5.30	25.91±11.30
300	58.09±5.91	72.68±7.13	98.59±0.90	77.14±7.68
400	94.75±5.10	98.93±1.48	100.00	98.26±3.89

Table 19 Survivorship of *E. modestus* nauplii (stage II), from a natural adult population, exposed to different copper concentrations for 48 h (at 22.5°C and 32 ppt) using different waters, U=unfiltered water from the Menai Strait, F=filtered water from the Menai Strait, A=artificial seawater, M=mixture of unfiltered water from the Menai Strait (50%) and FUVSW (50%)

The percentages of naupliar mortality for both the unfiltered and the filtered Menai Strait waters and the 50:50 water mixture appeared to be similar; whereas the values for the artificial seawater were considerably higher.

The tests were further compared through the LD_{50}s, after a Latin Square Analysis of Variance was performed on the percentage mortalities of each of the 5 replicates of the 5 copper concentrations tested in order to check for systematic errors due to the different position of the chambers. The test showed that, for each copper concentration, there were no significant differences between cells. The LD_{50}s were then

calculated as described earlier and compared with those obtained in the previous test, which determined the LD_{50} of *E. modestus* nauplii from a natural population using FUVSW (three replicates). The comparison followed the method of the Relative Potencies (Finney, 1971) already described; where the LD_{50} from replicate A of the previous test was again chosen as 'reference' (see Table 20).

	Replicate	LD_{50} (ppb)	C.I.(95%)
FUVSW	A	172	165-181
	B	195	187-202
	C	185	177-192
Unfiltered Menai Strait water		250	239-269
Filtered Menai Strait water		241	231-251
Artificial seawater		139	129-149
Mixture (50:50) of unfiltered and FUVSW		236	226-246

Table 20 LD_{50}s (lethal doses for 50% of the population) and 95% confidence intervals, determined by the naupliar mortality percentages of *E. modestus* nauplii (stage II) from a natural adult population exposed to different copper concentrations for 48 h (at 22.5°C and 32 ppt) using different waters

The method arranged the LD_{50}s in three groups according to similarities between them. The first group contained the relative potencies of LD_{50}s higher than 200 ppb which correspond with both unfiltered and filtered Menai Strait waters and the 50;50 water mixture; all waters that, supposedly, presented the highest organic matter loads. The

second group contained the relative potencies of LD_{50}s between 150 ppb and 200 ppb corresponding to the previous test which used FUVSW. The third group contained just one relative potency, which corresponded to the LD_{50} of nauplii exposed to copper in artificial seawater, the lowest of them all (<150 ppb), and also the one with, supposedly, the lowest organic load (see Table 20 and Figure 4).

Copper concentrations in the different waters were very similar and lower than 2 ppb, except in the case of the artificial seawater which showed copper levels that were, at least, 20 times higher (see Table 21).

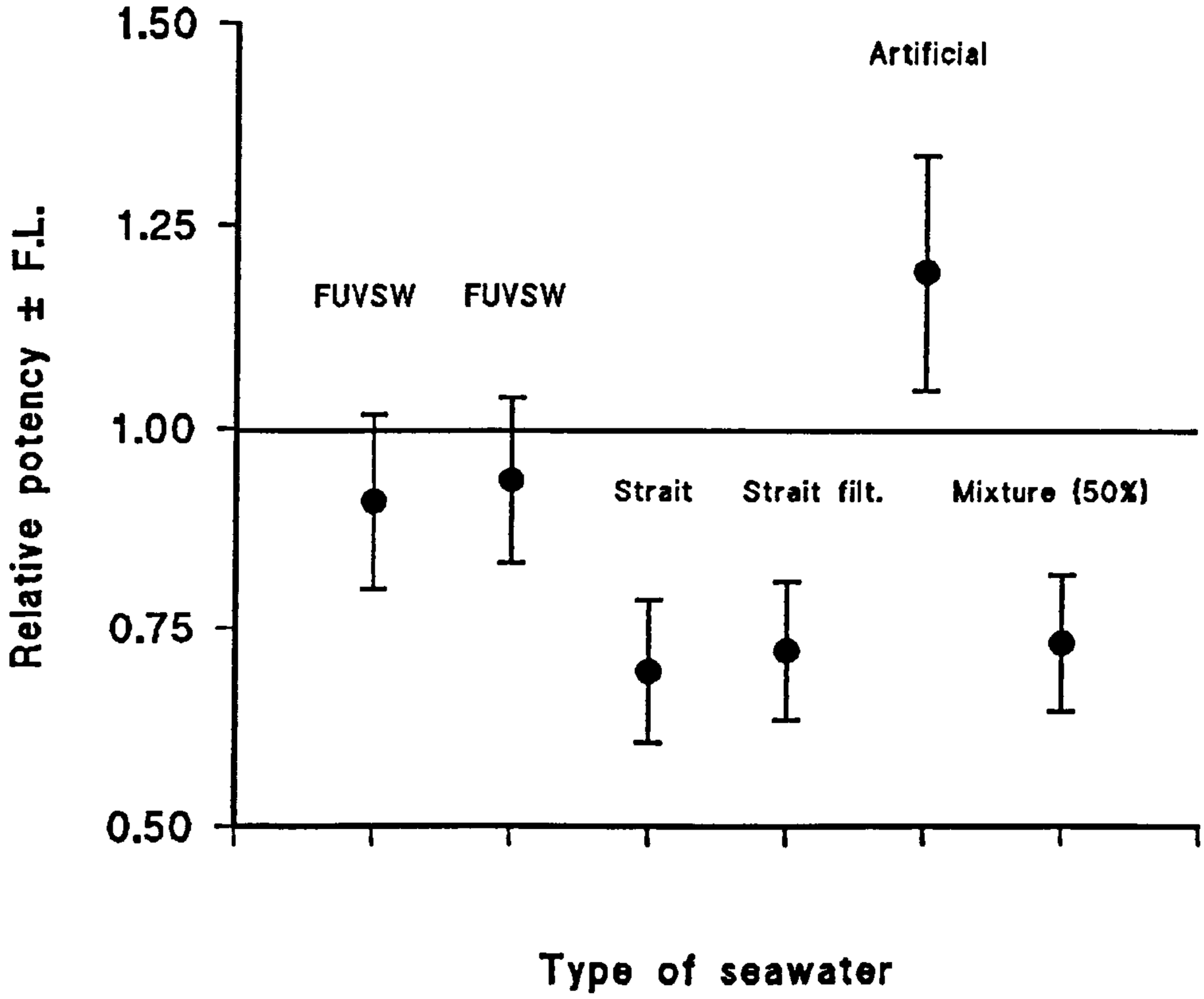
	Menai Strait unfiltered	Menai Strait filtered	Artificial	50:50 mixture	FUVSW
a	1.027	0.911	39.11	1.825	1.130
b	0.744	0.987	42.60	1.013	0.687
c	1.072	0.554	42.30	1.395	1.676
d	-	-	-	-	0.256
Mean	0.947 ±0.177	0.817 ±0.231	41.336 ±1.934	1.411 ±0.406	0.937 ±0.608

Table 21 Copper concentrations (ppb) of the waters used in the different toxicity tests, a-d=replicate measurements

The organic matter analysis showed that the water samples followed a pattern increasing in value from FUVSW (0.011 µg C.ml^{-1} and 0.002 µg N.ml^{-1}) to the unfiltered water from the Menai Strait (0.434 µg C.ml^{-1} and 0.053 µg N.ml^{-1}) (see Table 22).

The artificial seawater, however did not take the expected position in the range, showing a substantial organic

Figure 4
Relative potencies and 95% Fiducial Limits of the LD_{50}s of *Elminius modestus* nauplii (stage II) from a natural adult population exposed to 100–500 ppb copper for 48 hours (at 22.5°C and 32 ppt) using different waters, FUVSW, Strait=unfiltered water from the Menai Strait, Strait filt.=idem filtered, Artificial=artificial seawater, Mixture (50%)=50:50 mixture of unfiltered Menai Strait water and FUVSW



content (0.161 µg C.ml^{-1} and 0.053 µg N.ml^{-1}) which had been expected to be negligible.

Water	µg C.ml^{-1}	µg N.ml^{-1}	Volume filtered
FUVSW	0.011 ±4.433.10^{-3}	0.002 ±1.932.10^{-4}	5000
Menai Strait filtered	0.022	0.002	4000
Artificial	0.161	0.018	640
50:50 mixture	0.216 ±0.087	0.0293 ±2.097.10^{-3}	425
Menai Strait unfiltered	0.434 ±0.132	0.053 ±2.195.10^{-3}	260

Table 22 Mean organic matter content: carbon and nitrogen of the waters used in the toxicity tests

It was, therefore, decided to analyse its components (deionised, distilled water and 'Instant Ocean' salts) to the determine the origin of the organic matter detected.

The whitish colour of the filter papers used to filter the deionised, distilled water remained unaltered after filtration. In contrast, the ones used to filter the artificial seawater became dark brown, as seen earlier, indicating that the organic matter detected in the artificial seawater did not originated in the water.

The organic matter would, therefore, have originated in the 'Instant Ocean' salts. The pack containing the salts did not, however, disclose the specific ingredients hence, the organic content was determined by the differences between the

dry and the ash weights.

The differences between dry and ash weight of the salts indicated that they contained organic matter amounting an average 18.33±1.16% (see Table 23).

	Wet weight	Dry weight	Ash weight	Differences dry-ash weight	% Organic matter
a	1.662	1.639	1.359	0.28	17.08
b	1.415	1.392	1.122	0.27	19.39
c	1.413	1.392	1.134	0.26	18.53
					Mean 18.33±1.16

Table 23 Percentage organic matter in the 'Instant Ocean' salts used to make up artificial seawater, weights are in grams, a-c=replicate measurements

The organic content of the salts, and in turn of the artificial seawater, however, did not appeared to have complexed the added copper, since the LD_{50} of the artificial seawater was as low as expected for a water with negligible organic content. It could, therefore, be suggested that the organic matter was present in the salts in order to complex their high copper content, an average of 41.336±1.934 ppb when dissolved in deionised, distilled water, to avoid damage to aquarium organisms. This hypothesis was evaluated by comparing the survivorship of copper-exposed *E. modestus* in FUVSW versus that of nauplii in seawater, and it would be validated if there were no differences between them.

The percentages naupliar mortality obtained after the 48 h appeared to be very similar for both treatments and a Two-way Analysis of Variance (homogeneous variance after arcsin

transformation at Bartlett's statistic X^2=2.429, p=0.512, df=3) confirmed that there were no significant differences in naupliar mortality between waters (F=2.00, p=0.176). The test also showed that there were significant differences between the mortality of 'control' and 180 ppb exposed nauplii for both waters as expected (F=560.29, p≤0.001) and that the interaction term was not significant (F=1.50, p=0.238) (see Table 24).

Treatment	FUVSW	Artificial seawater
control	0.96±1.32	1.08±1.47
180 ppb	47.36±8.23	54.09±8.58

Table 24 Survivorship of *E. modestus* nauplii (stage II) from a natural adult population exposed to 180 ppb for 48 h (at 22.5°C and 32 ppt) in either FUVSW or artificial seawater

These results indicate that the organic matter content of the 'Instant Ocean' salts balanced out any added toxicity due to the copper content of the salts. As a result, there was no real need to have incorporate that extra measured copper in the calculation of the LD_{50}, which would have been even lower than the one presented in Table 20.

DISCUSSION

The factors affecting copper toxicity to *E. modestus* nauplii (and probably to any other marine organism) evaluated in this Chapter have been largely biotic with the exception of temperature. Those factors were studied individually to facilitate the experimental procedure. However, in the marine environment they are likely to interact (see Sprague, 1970) and their effects are most probably additive (see Hodson *et al.*, 1979; Mance, 1987).

A summary of the effects of the different parameters follows in order to aid the discussion of the results:

1. Copper concentrations up to 1,000 ppb had no effect on the survivorship and development of algal fed *E. modestus* nauplii in the presence of 40% Algal Culture Medium (ACM)

2. ACM is essential for the normal development of the nauplii

3. Lack of ACM is not directly detrimental to the nauplii, but might increase the rate of mortality through a nutritional deficiency (contributing to the copper-induced stress) and, in turn, an energy limitation (preventing them from coping with the added copper stress)

4. ACM has no effect on 'stationary-phase' algae

5. ACM is necessary for the growth of 'growth-phase' algae

6. Lack of ACM reduces naupliar ingestion rates

7. Reduced temperature from over 20°C-26°C to 18°C reduces copper toxicity to the nauplii

8. There is a relationship between temperature and copper toxicity since within the range tolerated by the species, toxicity increases with temperature

9. Nauplii from a laboratory-maintained population showed a lower LD_{50} than nauplii from a natural population at Menai Bridge

10. The hatching method ('natural' v. 'artificial') has no effect on naupliar fitness

11. There is a relationship between the organic content of seawater and copper toxicity, where toxicity decreases with increasing organic content

12. The 'Instant Ocean' salts used in making up artificial seawater have a significant copper and organic content

13. The organic content of the 'Instant Ocean' salts may function to detoxify their copper content

The first factor examined was the presence of ACM in the culture media of nauplii which were exposed to 25-1,000 ppb copper yet fed on algae throughout the exposure period. Naupliar mortality percentages were very low, even at the highest concentrations tested. This fact suggests that the toxic action of copper was completely masked, probably due to metabolites of *R. reticulata* originating from the ACM and subsequently present in the naupliar culture media. These

organic materials are able to complex cupric ions, the most toxic form of copper in aquatic environments (see Steemann Nielsen & Wium-Andersen, 1970), thus reducing copper toxicity (see Fogg & Westlake, 1955; McKnight & Morel, 1979; Van der Berg *et al.*, 1979; Härdsted-Roméo & Gnassia-Barelli, 1980; Slauenwhite & Wangersky, 1991).

Naupliar mortality values, however, were far below those obtained for *B. amphitrite* nauplii in a previous study where culture conditions were identical (Royo-Gelabert, 1991, unpublished). It could therefore be suggested that *B. amphitrite* nauplii are more sensitive to copper than *E. modestus* nauplii, which might result from their smaller size. Thus, throughout their development, *B. amphitrite* nauplii are always from 0.01 mm to 0.18 mm shorter than *E. modestus* nauplii (see Knight-Jones & Waugh, 1949; Costlow & Bookhout, 1958) and, since one of the ways copper gains access to an organism is through diffusion across the body surface (see review by Depledge & Rainbow, 1990 and references therein), a smaller size presenting a higher surface/volume ratio could result in a comparatively greater copper uptake and hence higher toxicity. It could also be argued, however, that a larger organism needs more food and will hence have higher a copper intake, since another way for copper to access the nauplius is through ingestion of food (see review by Depledge & Rainbow, 1990 and references therein).

There was no evidence that copper delayed or suppressed the moulting or development of the nauplii as suggested by

Lang *et al.* (1981). These authors studied copper effects on survivorship and development of *B. improvisus* nauplii, finding reductions in the development rate from stage II to stage III at copper concentrations above 40 ppb. This discrepancy supports the suggestion that copper toxicity may be masked.

On the other hand, all the nauplii cultured in 0% ACM (seawater), and likewise fed throughout the experimental period died. Thus, copper-exposed larvae survived for a minimum of 6 h and a maximum of 9 days, depending on the concentration, whereas all the 'control' nauplii had died by the day 13. These results differ significantly from observations on *B. amphitrite* nauplii (see Royo-Gelabert, 1991, unpublished) and on the *E. modestus* nauplii from the previous test, fed in both cases on 'unwashed' algae, and where 100% mortality was either not observed until copper reached 1,600 ppb (and there had been 96 h of exposure) or never observed, respectively. In the present case, the algae were 'washed' in a way that greatly reduced any organic ligands, resulting from algal metabolism, present in the Algal Culture Media (ACM), in which the algae were grown in the laboratory (following methods similar to those used by Walne, 1966). Such metabolites would alter the chemical speciation of copper, reducing the amount of cupric ions and, as a result, copper toxicity to the nauplii. Lack of ACM would have prevented or reduced complexation of copper ions hence copper might have been mostly in its ionic form, leading to the progressive reduction in the survivorship of

the nauplii with increasing copper concentration. It could therefore be stated that lack of ACM in the naupliar culture media increased copper toxicity to the nauplii.

There remains, however, the case of the 'control' nauplii, where mortality also reached 100%, which could indicate that the high mortality values observed were caused by the use of an unhealthy larval batch.

Different ACM percentages were then tested in order to determine which was suitable to demonstrate copper effects on *E. modestus* nauplii (without masking or enhancing such effects). The results of the test showed naupliar mortality percentages which reached 100% at 0% ACM, decreasing down to 26% mortality at 40% ACM, where intermediate ACM percentages resulted in intermediate mortalities. In addition, 20% ACM appeared to be a threshold level, above which the nauplii tolerated the added copper. These results could be explained as a function of the amount of organic matter of algal origin present in the naupliar culture media.

Nevertheless, the test showed again that at 0% ACM 'control' (no copper) nauplii did not survive well. This phenomenon was previously justified by suggesting that it could have been caused by a bad larval batch. That explanation is not valid in the present case as nauplii from the same batch survived much better when grown in 1,000 ppb copper using ACM of over 20%. It would seem, therefore, that the cause of the high mortality in the 'control' nauplii originated in the culture medium itself. This was made up of

19.9 ml FUVSW and 100 µl deionised, distilled water, and always contained 'washed' (0% ACM) *R. reticulata* in a concentration of 100 cell.μl^{-1}. Thus, the reason for the death of the nauplii might have been the exclusion of ACM.

Nauplii were then grown in either 0% ACM or 100% ACM to investigate the beneficial effects of ACM. The test, which did not include food or copper, showed that starvation killed all the nauplii in five days without any significant difference in mortality between the treatments (0% ACM and 100% ACM). Therefore, it can be stated that if *R. reticulata* was not present in the naupliar culture media, the effects of 0% ACM and 100% ACM on the survival of the nauplii were identical. Any differences in mortality meant that nauplii at 100% ACM might have been able to obtain nutrition by absorbing organic matter from the culture media (see Baylor & Sutcliffe, 1963). As a result, those nauplii died more slowly, but such was not significant.

Observations on the effects of different ACM percentages were repeated using fed larvae to validate the results of previous tests without stressing the nauplii through lack of food. The test showed that naupliar mortality in the absence of ACM was as high as had been observed previously. Mortality values at 20% ACM and 40% ACM were equivalent and lower than 10%, which can be considered 'normal' or inherent to the larval stock. Both the rate of naupliar development and the percentage of cyprids obtained at the end of the experiment indicated that the use of 20% ACM and 40% ACM was essential for the normal larval development of *E.*

modestus, since most of the nauplii reached the cypris stage on day 9 of the experiment (the temperature ranged from 20°C to 26°C).

The evidence gathered so far shows that at least 20% ACM is necessary for the normal development of the nauplii; indeed that percentage was actually adopted as the most suitable to demonstrate copper effects on naupliar survival with the minimal expected interference from complexing ligands.

The reason for the very high naupliar mortality at 0% ACM was still unclear. It could have been that this percentage had a detrimental effect on the algae and, as a result, on the nauplii that fed on them. Algal survivorship without ACM did not differ significantly from that of algae in a 'stationary-growth phase' (see Massuti & Margalef, 1950) at 40% ACM. The slight, but not significant, increase in mortality of the cells with time and the significant reduction in the percentage of immobile cells for both treatments was probably indicative of a stressed culture, which was beginning to re-enter the 'growth-phase' after removal from the batch culture, centrifugation and resuspension in fresh seawater. The second test with algae clearly in the 'growth phase' was more representative of the usual batch cultures used at Menai Bridge.

That second test showed that significant growth occurred with 40% ACM, but there was no growth when ACM was not used. The percentage of immobile cells remained constant with time

when ACM was used, but the lack of ACM led to an increase in the percentage of immobile cells, which was not, however, significant. Therefore, lack of ACM did not kill the algal population, but prevented its growth, suggesting 'nutritional stress'. Thus, that medium did not fulfil the nutritional requirements of the algae, since it was made up of just 20 ml of FUVSW and nothing else. As a result, it did not contain enough nutrients to maintain an algal population of at least 100 cell.μl^{-1}, which is the density routinely used to grow barnacle nauplii in the laboratory (see Stone, 1988; Neal & Yule, 1994). The concentration of algae, however, did not decrease dramatically over the time period and, some 60-70 cells.μl^{-1} would hence still have been available. Algal concentrations in the sea have never been reported as being much higher than 10 cells.μl^{-1}, even in 'bloom' conditions. Lack of cells would not therefore have been the cause of naupliar mortality.

Naupliar ingestion rates were significantly lower with no ACM in the medium. Those observed when 40% ACM was used were twice as great once allowance was made for 'control' concentrations. At 60 cells.μl^{-1} to 100 cells.μl^{-1} *R. reticulata*, cell ingestion by *E. modestus* nauplii should have been at a maximal level (see Yule, 1986; Harms, 1987), so differences in concentration due to reduced algal growth in the cultures devoided of ACM would not have caused the difference in naupliar ingestion rates. The algae were therefore either unpalatable to the nauplii when grown without ACM, or the reduced quantities of algal metabolites

discouraged maximal feeding activity by the nauplii. Yule (1986) showed how the water handling capacity of *E. modestus* nauplii was increased in the presence of 'food' algae (including ACM), so the lack of ACM in the naupliar culture media may well have prevented this increased feeding response in the current experiments. A reduction in ingestion rate would lead to nauplii which were energy limited such that growth was difficult to maintain and any regulation of external contamination (e.g. copper) would be impossible. The toxic effect of copper on 'nutritionally stressed' nauplii would thus be greater.

Similarly, Winner *et al.* (1977) showed that poorly fed *Daphnia magna* were more sensitive to long-term copper exposure, monitored through longevity, reproductive maturation and instantaneous rate of population growth, than animals that had been properly fed. Those authors concluded that such differences were due to 'nutritional stress' induced by the poor diet which would be cumulative with the copper stress. Furthermore, Lang *et al.* (1980, 1981) found differences in spontaneous swimming speeds between fed and unfed *B. improvisus* nauplii exposed to sublethal copper levels.

The problems of 'nutritional stress', copper complexation through food products and copper toxicity to barnacle larvae are thus intimately intertwined and must be considered in their entirety when interpreting toxicity test results.

The conduct of such tests was investigated through a series of *E. modestus* nauplii toxicity tests, each designed to highlight the need for careful design. Early attempts to establish workable LD_{50}s showed great variability. Such differences could have resulted from the manner in which the naupliar culture media were prepared. Thus, the 100 µl of the copper solution required to produce each of the final copper concentrations in the cultures came from 'intermediate' solutions obtained by dilution of a 10^6 ppb stock. Not all the 'intermediate' solutions were freshly prepared for each test. In addition, the volume of the culture media was relatively small (4 ml), which implies that any systematic error in their formulation (a badly adjusted pipette etc.) would significantly affect the final copper concentrations. Considerable attention to detail was needed to arrive at a working standardisation of test procedures.

Temperature has a direct effect on rate processes in organisms and as such has a considerable influence on the toxicity of pollutants (Sprage, 1970; Eisler, 1979). The current work showed reduced copper toxicity to *E. modestus* nauplii at 18°C versus an average temperature of 24°C. Similarly, Lang *et al.* (1979) had also observed a reduction in the post-exposure mortality of acorn barnacle nauplii grown in copper-containing media at 20°C versus 25°C. The temperature reduction also increased the development time from seven to eight days (see Moyse, 1960; Tighe-Ford *et al.*, 1970).

Increasing temperatures from 12.5°C to 22.5°C showed

increased copper toxicity to the nauplii. The highest temperature was chosen for further tests involving toxicity to ensure adequate characterisation of the effect within the shortest time interval.

The provenance of the parent population and the conditions of their habitat, etc. have been shown to affect the copper tolerance of the first generation (see Bryan & Hummerstone, 1971). The populations used in the current work did not differ in terms of pollution of the surrounding medium, but did so in other, less specific, environmental parameters. Thus, nauplii hatched from a laboratory-maintained population tolerated significantly higher copper concentrations than those from a natural population in the Menai Strait. Environmental conditions between the two populations were very different, the former was kept at temperatures of 22-24°C, fed regularly on a mixed diet of unicellular algae and the nauplii of *Artemia sp.*, and cleaned frequently. They were not under a tidal regime but permanently submerged and so could feed continually. In contrast, the adults at Menai Bridge pier were exposed to lower temperatures (5°-17°C), and had fewer opportunities to feed since they lived under a tidal regime, which also caused regular stress. Therefore, it could be suggested that, since they lived under more favourable conditions, the laboratory population produced nauplii that were somehow physically fitter and able to resist adverse conditions, and so showed higher copper tolerance.

Alternatively, the differences in copper tolerance could be explained solely in terms of the temperature differences existing between the habitats of the two groups of barnacles. The animals from Menai Bridge pier lived at lower temperatures than those used in the present test (22.5°C) and when their nauplii were cultured at such temperatures they could have suffered a thermal shock which would have made them more sensitive to any adverse agent, in this case copper.

Naupliar fitness could be affected by factors such as the nature and amount of naupliar reserves and, in this case, stress/shock resulting from the hatching method. Throughout this study, barnacles from natural populations were forced to hatch, which might have had a detrimental effect on naupliar survivorship. Thus, even if the eggs are ready to hatch, naupliar release is normally withheld until the time is appropriate (see Walker, 1992). Another source of stress for those nauplii could have been the temperature difference between the parent habitat and the laboratory, although in the present case the adults had been maintained under experimental conditions for 3 days prior to the test. Such a time period is insufficient to have changed the field acclimation and the nauplii released would have been conceived and developed at field temperatures.

The test found no significant differences with regard to parent provenance or hatching methods, although the difference between the 'naturally-hatched' field and laboratory populations was very close to being so. Again, the

laboratory population produced larvae which were able to survive slightly better. Such a difference was not evident for 'artificial' hatching conditions.

It is still not clear why nauplii from laboratory-maintained populations are better at tolerating stress. It is known, however, that the quality of yolk material in eggs often has a great influence on the survival of bivalve larvae (Holland, 1978). It has been suggested, however, that barnacles maintain a high egg quality (see Crisp, 1986, 1987) and reduce the number of eggs produced in the face of environmental stress (see Walker, 1992).

The present work clearly demonstrated the influence of the organic content of seawater in reducing the toxicity of copper. In all but one anomalous case (artificial seawater made up of 'Instant Ocean' salts) toxicity was reduced as organic matter increased. These results highlight the dynamics of copper toxicity in seawater, where ionic copper, the most toxic form to aquatic organisms (Steemann Nielsen & Wium-Andersen 1970), is complexed by organic (and inorganic) matter hence reducing copper toxicity (Sunda & Guillard, 1976).

The results of the copper analysis in the samples showed that the 'nominal' value for the copper concentrations used in these tests and the previous one using FUVSW, were not significantly different from the 'actual' value, since copper concentrations in the different waters were below 2 ppb hence, had no import on the measured LD_{50}s. However, such

similarity was not achieved in the artificial seawater, where the average copper concentration was 41.336±1.934 ppb, i.e. 20 times higher than in the other cases (although this was taken into account when calculating the LD_{50}s and did not affect the overall comparison).

The artificial seawater was expected to give the lowest LD_{50}, as it was assumed that little organic material would have been present to reduce the added copper toxicity. This assumption was unfounded, as 18% of the dry weight of the 'Instant Ocean' salts was organic. By comparing the survival of nauplii in the artificial seawater and FUVSW, it was found that the extra copper in the salts was completely complexed by the organic matter present, since naupliar mortality at both 0 ppb and 180 ppb added copper was identical for both types of water.

The balance of organic matter to toxic element shown by the 'Instant Ocean' salts was so good that copper-LD_{50} for *E. modestus* nauplii occupied the place expected of it as if it had no organic content. 'Practical Fishkeeping' magazine (November 1994) pointed out that salts like 'Instant Ocean' can contain an additive to detoxify excess toxic metals (e.g. copper) and it is clear that this additive is an organic complexing agent. Similarly, Snell *et al.* (1991) stated that commercial sea salts used technical grade chemicals, some of which contained impurities that could influence test results; and found that results from acute toxicity tests on rotifers carried out using 'Instant Ocean' differed from those utilising ASPM synthetic seawater (Guillard, 1983).

Treatment:	0% ACM	100% ACM	Total
a	8	5	13
b	6	1	7
c	6	2	8
d	6	6	12
Total	26	14	40

X^2=2.927, p=0.513, df=3

Table 1 Chi-square test on the survival distributions of *E. modestus* nauplii from a natural population after 5 days (at 20.5-26°C and 32 ppt), without food, in media made up of either seawater, 0% ACM, (x4) or Algal Culture Medium, 100% ACM, (x4)

Source of variation	DF	SS	MS	F	P
Time	1	41036	41036	533.80	0.000
Treatment	1	114	0	0.00	0.976
TimexTreat	1	14	14	0.18	0.677
Error	28	2152	77		
Total	31	3316			

Table 2 Two-way Analysis of Variance performed on the regression lines of the percentage surviving with time of *E. modestus* nauplii (stage II) from a natural population after 5 days (at 23°C and 32 ppt), without food, in media made up of either seawater, 0% ACM, (x4) or Algal Culture Medium, 100% ACM, (x4)

ACM	No. obs.	Mean	stdev	S.E.
20%	2	5	1.41	1
40%	2	5.50	0.70	0.50
t=0.45, p=0.73, df=1				

Table 3 t-test on the percentage mortality values for fed *E. modestus* nauplii from a natural population grown for 17 days (at 19.5-23.5°C and 32 ppt) in media made up of either 0% ACM (x2) or 40% ACM (x2)

Source of variation	DF	SS	MS	F	P
Time	3	1550.0	516.7	2.86	0.070
Treatment	1	41.8	41.8	0.23	0.637
TimexTreat	3	642.2	214.1	1.18	0.347
Error	16	2893.6	180.9		
Total	23	5127.7			

Table 4 Two-way Analysis of Variance performed on the mean values for the total concentration (cells.μl^{-1}) of *R. reticulata* cells grown for 57 h (at 22-24°C and 32 ppt) in media made up of either 0% ACM (x3) or 40% ACM (x3)

Source of variation	DF	SS	MS	F	P
Time	3	19018.0	6339.3	41.30	0.000
Treatment	1	0.2	0.2	0.00	0.970
TimexTreat	3	383.9	128.0	0.83	0.495
Error	16	2456.1	153.5		
Total	23	21858.3			

Table 5 Two-way Analysis of Variance performed on the mean values for percentage of immobile *R. reticulata* cells grown for 57 h (at 22-24°C and 32 ppt) in media made up of either 0% ACM (x3) or 40% ACM (x3)

Source of variation	DF	SS	MS	F	P
Time	1	358.0	358.0	5.39	0.049
Treatment	1	7865.6	7865.6	118.34	0.000
TimexTreat	1	918.9	918.9	13.82	0.006
Error	8	531.7	66.5		
Total	11	9674.2			

Table 6 Two-way Analysis of Variance performed on the values for the total concentration (cells.μl^{-1}) of *R. reticulata* cells grown for 48 h (at 23.5°C and 32 ppt) in media made up of either 0% ACM (x3) or 40% ACM (x3)

Source of variation	DF	SS	MS	F	P
Time	1	34.50	34.51	1.60	0.242
Treatment	1	15.17	15.17	0.70	0.426
TimexTreat	1	13.42	13.42	0.62	0.453
Error	8	172.70	21.59		
Total	11	235.80			

Table 7 Two-way Analysis of Variance performed on the values for the percentage of immobile *R. reticulata* cells grown for 48 h (at 23.5°C and 32 ppt) in media made up by 0% ACM (x3) or 40% ACM (x3)

% ACM	[Algae] 0 hrs	20 hrs	Mean [algae]
0 control	89.50 ±1.11	95.55 ±13.64	
0 a	89.50	59.72	
0 b	89.50	80.83	71.05
0 c	89.50	63.33	±9.66
0 d	89.50	80.55	
0 e	89.50	70.83	
40 control	89.76 ±1.85	129.44 ±26.30	
40 a	89.76	69.72	
40 b	89.76	74.72	73.10
40 c	89.76	72.22	±3.04
40 d	89.76	71.38	
40 e	89.76	77.50	

Table 8 Total concentrations (cells.μl^{-1}) of *R. reticulata* cells grown for 20 h (at 26°C and 32 ppt) in media made up of either 0% ACM (x5) or 40% ACM (x5), used in the calculation of *E. modestus* naupliar ingestion rates, cell concentrations are the mean value for 3 replicate counts of the total number of cells in the haemocytometer

Treatment (ppb)	cont.	25	50	100	250	500	1000	Total
No. dead	5	8	11	9	12	8	14	67
No. cyprids	67	62	70	76	63	79	56	473
Total	72	70	81	85	75	87	70	540

$X^2=7.830$, p=0.250, df=6

Table 9 Chi-square test on the survivorship and development of *E. modestus* nauplii from a natural population grown for 17 days (at 18°C and 32 ppt) in different copper concentrations

Added copper (ppb)	N	Number bottom		Number swimming	
		Day 1	Day 2	Day 1	Day 2
control a	28	1	3	27	21
control b	22	2	2	20	20
50 a	26	4	5	22	18
50 b	35	5	5	30	30
100 a	35	9	13	26	18
100 b	18	11	13	7	4
150 a	25	13	16	12	6
150 b	35	24	25	14	7

Table 10 Survivorship of *E. modestus* nauplii from a natural population, exposed for 48 h (at 22.5°C and 32 ppt) to different copper concentrations and subsequently transferred to unpolluted media where they remained for 24 h. Day 1= values after a lapse of 48 h, Day 2=after a further 24 h

Added copper (ppb)	N				Number dead			
	MA	MB	MC	L	MA	MB	MC	L
100 a	-	55	65	71	-	4	7	1
100 b	80	56	77	41	6	2	6	2
100 c	82	55	71	63	11	4	8	3
100 d	44	39	71	64	8	2	8	3
100 e	58	51	54	62	9	5	6	1
200 a	76	46	65	53	51	16	32	6
200 b	60	60	65	53	28	17	35	10
200 c	78	46	59	39	32	17	24	9
200 d	51	-	57	48	29	-	36	7
200 e	55	34	63	46	38	8	34	6
300 a	46	62	35	24	45	61	33	20
300 b	50	56	47	30	39	52	40	15
300 c	52	52	27	23	44	50	24	17
300 d	38	50	43	29	34	48	40	12
300 e	47	43	25	32	42	42	22	19
400 a	*	*	*	36	*	*	*	33
400 b	*	*	*	27	*	*	*	26
400 c	*	*	*	40	*	*	*	36
400 d	*	*	*	52	*	*	*	50
400 e	*	*	*	57	*	*	*	56

Table 11 Mortality of *E. modestus* nauplii (stage II), from natural (MA, MB and MC) and laboratory-maintained (L) populations, exposed to different copper concentrations for 48 h (at 22.5°C and 32 ppt), to establish the LD_{50} (lethal dose for the 50% of the population); values shown are the actual figures used in the Probit analysis, a-e=positions in the culture tray

Adult population		Hatching method	N	Number dead
Laboratory	a	'artificial'	45	2
	b		67	2
	c		61	2
	d		49	5
Laboratory	a	'natural'	63	1
	b		69	2
	c		57	2
	d		84	3
Port Dinorwic	a	'artificial'	52	3
	b		43	3
	c		43	5
	d		48	2
Port Dinorwic	a	'natural'	45	4
	b		36	5
	c		49	7
	d		52	7

Table 12 Mortality of *E. modestus* nauplii (stage II), from either natural (Port Dinorwic) or laboratory-maintained adult populations, hatched by either an 'artificial' or a 'natural' method and grown in the laboratory for 4 days (at 20°C and 32 ppt) until cyprids started to appear; values shown are the actual figures used in the Probit analysis, a-d=replicate cultures

Source of variation	DF	SS	MS	F	P
Adults	1	16.913	16.913	3.79	0.075
Hatching	1	0.328	0.328	0.07	0.791
AdultsxHatching	1	20.862	20.862	4.67	0.052
Error	12	53.607	4.467		
Total	15	91.709			

Table 13 Two-way Analysis of Variance performed on the mean values for the mortality (no. dead) of *E. modestus* nauplii (stage II) from either natural (Port Dinorwic) or laboratory-maintained adult populations, hatched by either an 'artificial' or a 'natural' method and grown in the laboratory for 4 days (at 20°C and 32 ppt) until cyprids started to appear

Added copper (ppb)		N				Number dead			
		U	F	A	M	U	F	A	M
100	a	18	14	29	63	1	1	13	2
100	b	52	13	36	41	3	1	17	2
100	c	45	27	41	43	5	2	23	2
100	d	47	25	45	55	5	2	28	4
100	e	38	14	19	31	4	2	13	2
200	a	46	41	58	59	6	8	46	13
200	b	39	32	56	55	10	8	41	8
200	c	30	46	49	34	6	8	42	6
200	d	74	45	65	29	27	16	49	10
200	e	76	47	49	44	21	7	41	18
300	a	29	52	87	67	17	35	86	52
300	b	56	48	48	56	30	32	47	36
300	c	31	45	67	41	16	33	66	32
300	d	75	34	44	38	45	29	43	31
300	e	45	38	42	38	30	27	-	32
400	a	29	34	73	23	26	33	-	21
400	b	50	45	85	24	45	44	-	-
400	c	44	28	68	23	-	-	-	-
400	d	68	37	82	33	64	-	-	-
400	e	29	22	59	10	-	-	-	-

Table 14 Mortality of *E. modestus* nauplii (stage II), from a natural adult population, exposed to different copper concentrations for 48 h (at 22.5°C and 32 ppt) using different waters, U=unfiltered water from the Menai Strait, F=filtered water from the Menai Strait, A=artificial seawater, M=mixture of unfiltered water from the Menai Strait (50%) and FUVSW (50%), values shown are the actual ones used in the Probit analysis, a-e=positions in the culture tray

Treatment		N	% Mortality
FUVSW control	a	51	0.00
	b	35	0.00
	c	43	2.32
	d	38	0.00
	e	40	2.50
FUVSW 180 ppb	a	28	39.28
	b	26	46.15
	c	35	40.00
	d	30	53.33
	e	31	58.06
Artificial control	a	40	0.00
	b	33	0.00
	c	41	0.00
	d	37	2.77
	e	38	2.63
Artificial 180 ppb	a	26	61.53
	b	25	52.00
	c	28	42.85
	d	25	60.00
	e	24	58.33

Table 15 Survivorship of *E. modestus* nauplii (stage II), from a natural adult population, exposed to 180 ppb for 48 h (at 22.5°C and 32 ppt) in either FUVSW or artificial seawater, a-e=positions in the culture tray

Source of variation	DF	SS	MS	F	P
Copper	1	7696.3	7696.3	560.29	0.000
Water	1	27.5	27.5	2.00	0.176
CopperxWater	1	20.6	20.6	1.50	0.238
Error	16	219.8	13.7		
Total	19	7964.2			

Table 16 Two-way Analysis of Variance performed on the mean values for the percentage mortality of *E. modestus* nauplii (stage II), from a natural adult population, exposed to 180 ppb copper for 48 h (at 22.5°C and 32 ppt) in either FUVSW or artificial seawater

CHAPTER FOUR

EFFECTS OF COPPER ON THE RESPIRATION OF *Elminius modestus* NAUPLII

CONTENTS

Oxygen consumption in barnacle nauplii, as with any other organism, reflects the metabolic demand (see Crisp, 1976; Holland, 1978) and can be affected by several exogenous parameters, such as temperature (Southward, 1955b; Newell & Northcroft, 1965; Barnes & Barnes, 1969; Belman & Childress, 1973; Harms, 1987; Kurmaly *et al.*, 1989b; see also review by Newell, 1973), salinity (Bhatnagar & Crisp, 1965), photoperiod (see Newell, 1973) and food availability (see Newell, 1973), as well as endogenous factors such as activity level and stage of development (see review by Newell, 1973; also Bridges & Brand, 1980). Exogenous influences should also include pollutant exposure. Thus, depending on the concentration, a pollutant such as copper can increase, reduce or even inhibit barnacle larval respiration (see Bernard & Lane, 1963). Increments in larval oxygen uptake would result from the energetic cost of coping with increased copper levels (see Bernard & Lane, 1963). Naupliar respiration rate could also be reduced by copper interfering with the respiratory processes involved in gas exchange and transport (see review by Spicer & Weber, 1991). Beyond certain threshold concentrations, copper could even inhibit the respiration process through its toxic action on respiratory enzymes (see reviews by Lewis & Cave, 1982; White & Rainbow, 1985) (see also review by Spicer & Weber, 1991).

Bhatnagar & Crisp (1965) first studied the oxygen consumption of *Elminius modestus* stage II nauplii using a micro-Winkler technique for determining oxygen concentrations (for review of techniques see Davenport, 1976b). Their data was later used by Crisp (1976), who calculated the respiration rate as $2.2x10^{-6}$ $ml O_2.h^{-1}.nauplius^{-1}$ at 16°C. More recently, Harms (1987), who also used the Winkler method to determine oxygen concentration, estimated the respiration rate of *E. modestus* stage II nauplii at three different temperatures: 12°, 18° and 24°C as $0.8x10^{-6}$, $1.6x10^{-6}$ and $2.7x10^{-6}$ $ml O_2.h^{-1}.nauplius^{-1}$ respectively.

The present Chapter investigates the effects of copper on the oxygen consumption of *E. modestus* stage II nauplii, both over short-term and long-term exposure periods, and aims to determine whether this follows the response pattern described in the literature for other marine invertebrate organisms.

The oxygen consumption of *E. modestus* stage II nauplii was measured throughout this study in both copper-contaminated and uncontaminated media. Barnacle nauplii have a high surface-volume ratio, with a total length ranging from 0.36 mm to 0.43 mm in the case of *E. modestus* (Knight-Jones & Waugh, 1949), so their oxygen consumption is generally low (Bhatnagar & Crisp, 1965). According to Davenport (1976b), the techniques for the measurement of the oxygen consumption of small aquatic organisms respiring in the range of 10^{-10} to 10^{-3} $ml O_2.h^{-1}.individual^{-1}$ were characterised by a low rate of experimental productivity. As a result, he developed a method for larvae based on the use of an oxygen electrode (Kanwisher, 1959) in a small volume (1.5-2 ml) of seawater which employed several hundred individuals for each measurement.

In the current study, naupliar oxygen consumption was measured by means of a polarographic oxygen electrode, and 40 or 50 nauplii in 100 µl of seawater following the procedure outlined in Kurmaly *et al.* (1989a, 1989b).

The polarographic oxygen electrode (Model 1302, Strathkelvin Instruments, Glasgow, UK) was fitted to the base of a glass receptacle whose wall invaginated at the top to form the experimental chamber, which was surrounded by a water jacket (RC 100 Respirometer, Strathkelvin Instruments,

Glasgow, UK) (see Figure 5). The receptacle was connected to a thermostatic recirculating water bath allowing a continuous water flow in the chamber's water jacket and regulating the temperature of the seawater in the experimental chamber.

The volume of water in the experimental chamber was kept constant by means of an adjustable plunger inserted at the top. The plunger was made of perspex and had a hollow centre to allow any excess liquid to flow out. A nylon thread was pushed into this opening to prevent the larvae finding access to the hole.

Oxygen tension was monitored by an oxygen meter (Model 781, Strathkelvin Instruments, Glasgow, UK) and the output was recorded continuously on a chart recorder (CR 6525 Recorder, JJ Instruments, Southampton, UK).

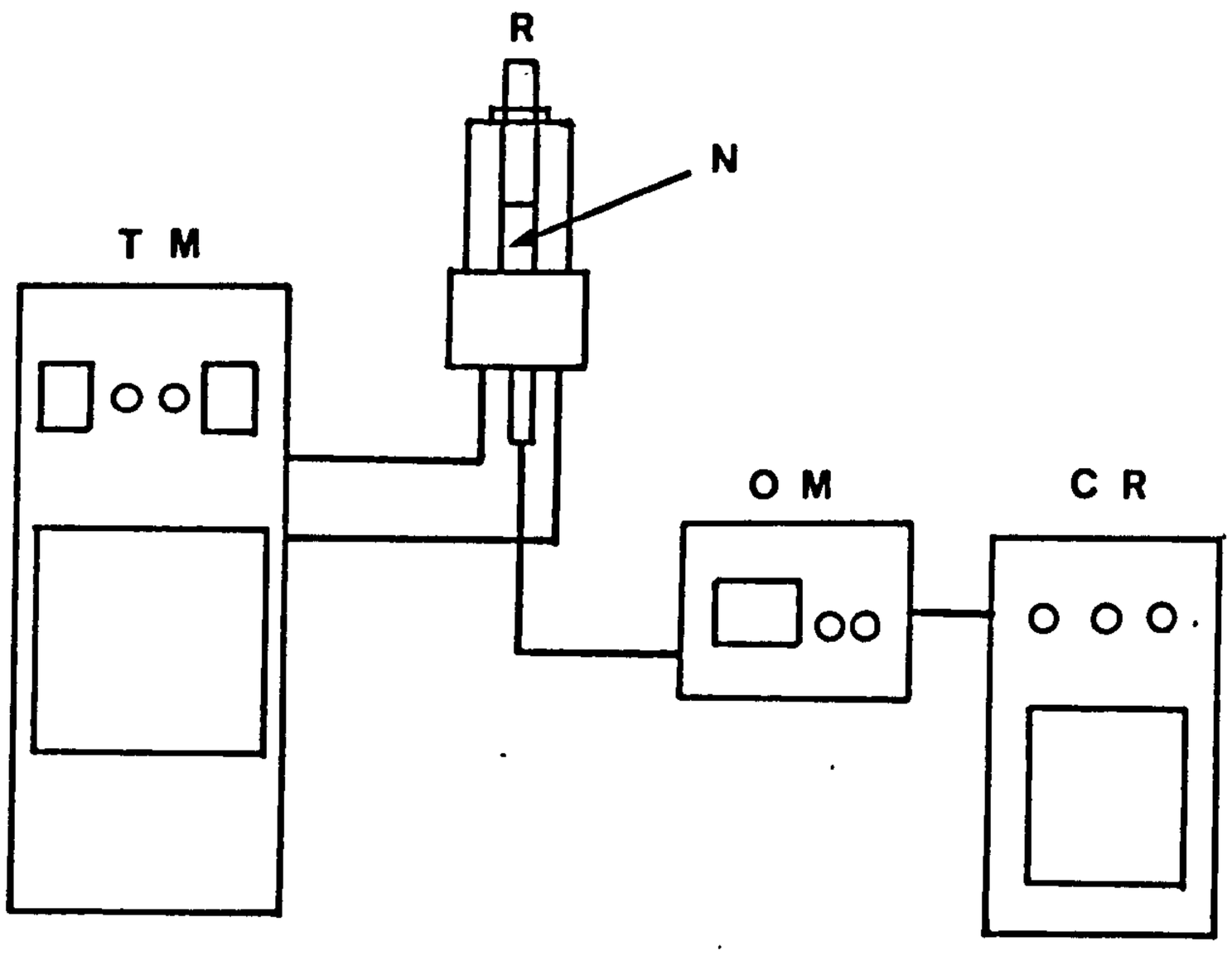
Prior to any measurements, the chamber was cleaned for 2 min with dilute sodium hypochlorite and rinsed thoroughly with distilled water.

The oxygen content of the water in the experimental chamber was measured within 100-80% air saturation to prevent concentration-dependent respiration (Belman & Childress, 1973; Crisp, 1984). The electrode was calibrated to zero oxygen tension by using distilled water with a little sodium dithionite (strong oxidising agent). Subsequently, the chamber was filled with air-saturated FUVSW (obtained by constant aeration over 40 min) to calibrate for 100% oxygen saturation.

A series of three, 20-min 'blank runs' was conducted each day, where the chamber was filled with air-saturated

Figure 5

Respirometry apparatus used to measure the oxygen consumption of *Elminius modestus* nauplii (stage II), TM= thermocirculator, N= nauplii, R=respirometer, OM=oxygen meter, CR=chart recorder



FUVSW and no nauplii, to determine the electrode consumption *per se* and the respiration of any microorganisms left in the experimental chamber (see Crisp, 1984).

The naupliar oxygen consumption rate measurements were made with roughly 50 nauplii suspended in 100 µl of air-saturated FUVSW (or equivalent at the required copper concentration) and the 100% calibration point was re-established prior to each measurement. The plunger was then inserted and the oxygen tension monitored until a constant-slope trace had been produced on the chart recorder (20-25 min). The number of nauplii in the chamber was subsequently recorded and the chamber rinsed, first with distilled water and then with FUVSW.

The slope of the trace between the initial and the final times relative to the calibration points (distance between 0% and 100% saturation) on the chart recorder paper, together with the volume of liquid in the chamber, chart speed, salinity, temperature, number of nauplii and oxygen consumption of the electrode ('blank runs') were used to calculate naupliar respiration rates.

Prior to measuring the respiration rate of *E. modestus* nauplii under copper exposure, it was necessary to determine whether the oxygen consumption of the polarographic electrode ('blank run') varied with either copper concentration or time throughout the day.

Test copper concentrations were 25, 35, 50, 75 and 100 ppb made up by serial dilution of a 500 ppb 1 litre initial

solution (see Chapter Two: General Materials and Methods). The volume of copper solution in the experimental chamber was 100 µl at 32 ppt, and the experimental temperature was 22.5°C.

Two x20-min measurements, with a 5-hour interval, were carried out for each of the above copper concentrations. The electrode was calibrated at the beginning of the session, and readjusted for 100% saturation between measurements.

In preparation for the study of the effects of copper on the respiration of *E. modestus* nauplii, it was necessary to assess the natural variability in naupliar oxygen consumption. The nauplii were produced by adults taken from a natural population living on Menai Bridge pier. Newly hatched stage I nauplii were maintained for 2 h and 30 min in FUVSW, to guarantee the metamorphosis to stage II (see Harms, 1987) before they were used. Test nauplii were not fed during the exposure time.

The electrode was calibrated before any oxygen consumption measurements were taken and readjusted for 100% saturation after each measurement. The experimental chamber was bleached with diluted hypochlorite solution to reduce the effects of microbial respiration. Three 'blank runs' were performed at the beginning of the session, which consisted of seven, 20-min oxygen measurements carried out in 100 µl of air-saturated FUVSW at 32 ppt and 22.5°C.

Short-term effects of copper on the respiration rate of *E. modestus* nauplii were determined over three days for a exposure period of 20 min, using 25, 35, 50, 75, 100, 125

and 150 ppb copper. Test copper concentrations were used in groups of either 2 or 3, with each concentration replicated 3 times each day. Thus, the respiration rate of the nauplii at 50 and 125 ppb was estimated on day 1, that at 25, 75 and 150 ppb on day 2, and the rate at 35 and 100 ppb on day 3. High and low concentrations were tested on each day in order to eliminate any day-to-day differences arising from the use of different larval batches. The nauplii were hatched from adults living on Menai Bridge pier and left to metamorphose to stage II. Stage II larvae, which were not fed throughout the experimental period, were then kept for 20 min at the required air-saturated copper concentration before the measurements were made.

The electrode was calibrated before any measurements were taken and readjusted for 100% saturation after each measurement. Up to 3 'blank runs' were performed at the beginning of each daily session. The sessions consisted of between six and nine oxygen measurements, carried out over 25 min in 100 µl of air-saturated FUVSW at 32 ppt and 22.5°C.

The effects of temperature on the respiration rate of *E. modestus* nauplii were assessed in order to determine whether the experimental temperature (22.5°C) used in testing the oxygen consumption of nauplii under short-term copper exposure was adequate.

Test temperatures were 12°, 13°, 14°, 15°, 16°, 17°, 18°, 19°, 20°, 21°, 22°, 23° and 24°C. The nauplii were

hatched from Menai Bridge adults and left to metamorphose to stage II.

The electrode was calibrated and 8 'blank runs' were performed at intermediate temperatures before any measurements (carried out in the usual manner) were taken. At temperatures at which no 'blank runs' were carried out, 'blank' respiration rates were calculated from the regression lines of the measured 'blanks'.

Long-term effects of copper on the respiration rate of *E. modestus* were determined over exposure periods ranging from 20 min to about 6 h, at copper concentrations of 50, 75, 500 and 1,000 ppb. Test copper doses were used in pairs and duplicated on consecutive days, successive naupliar oxygen consumption measurements being carried out each day at 30 min intervals.

The nauplii were hatched from adults living on Menai Bridge pier, left to metamorphose to stage II, and then transferred to the appropriate copper concentration were they would remain (unfed) until the measuring session, and also between measurements. Both the naupliar copper cultures and the experimental chamber were kept at 16°C. The electrode was calibrated and 3 'blank runs' were performed at the beginning of the session, which consisted of between five and eight x25-min respiration measurements carried out in 100 µl of the required air-saturated copper concentration, where 100% oxygen saturation was readjusted as usual.

RESULTS

Oxygen consumption of the electrode with time and copper

The oxygen consumption of the electrode was rather high, ranging from $7.44\pm0.37x10^{-5}$ $mlO_2.h^{-1}$ (at 75 ppb) to $9.82\pm0.08x10^{-5}$ $mlO_2.h^{-1}$ (at 35 ppb), and was probably a result of microbial respiration within the experimental chamber, since the chamber had not been bleached prior to the measurements. Friedman's test, an equivalent of the parametric Analysis of Variance for randomised block designs, which had to be used because of the extreme differences in variance, showed that there were no significant differences in the mean oxygen consumption with time (S=9.09, p=0.107, df=5) (see Table 25).

Added copper (ppb)	Oxygen consumption t=0 h	t=5 h	Mean oxygen consumption
control	6.64	8.66	7.65 ± 1.43
25	7.81	8.66	8.24 ± 0.60
35	9.76	9.88	9.82 ± 0.08
50	8.99	9.19	9.09 ± 0.14
75	7.17	7.70	7.44 ± 0.37
100	8.96	8.66	8.81 ± 0.21

Table 25 Oxygen consumption ($x10^{-5}$ $mlO_2.h^{-1}$) of the polarographic electrode measured at different copper concentrations and times of day (at 22.5°C and 32 ppt)

Since the presence of copper did not affect the electrode's oxygen consumption, the time involved in measuring the respiration rate of copper-exposed *E. modestus*

nauplii could be reduced by using the same 'blank run' for all copper concentrations. In addition, it was not necessary to carry out 'blank runs' with any specific frequency, as one 'blank run' could cover the whole measuring session.

Natural variability in naupliar respiration rate

Natural variability in the oxygen consumption of *E. modestus* nauplii ranged from $2.35x10^{-6}$ $ml O_2.h^{-1}.nauplius^{-1}$ to $4.05x10^{-6}$ $ml O_2.h^{-1}.nauplius^{-1}$. The 'blank run' values were lower than in previous observations, giving a more representative idea of the oxygen consumption of the electrode (see Table 26).

Measurement	N	Oxygen consumption	Mean oxygen consumption
Blank a	-	$1.79x10^{-5}$	
Blank b	-	$1.54x10^{-5}$	$1.75x10^{-5}$
Blank c	-	$1.92x10^{-5}$	$(\pm 0.19x10^{-5})$
a	22	$2.35x10^{-6}$	
b	15	$3.01x10^{-6}$	
c	20	$2.91x10^{-6}$	
d	27	$3.19x10^{-6}$	$3.13x10^{-6}$
e	29	$3.20x10^{-6}$	$(\pm 0.50x10^{-6})$
f	15	$4.05x10^{-6}$	
g	24	$3.22x10^{-6}$	

Table 26 Oxygen consumption ($ml O_2.h^{-1}.nauplius^{-1}$) of *E. modestus* nauplii (stage II) from a natural adult population, in FUVSW (at 22.5°C and 32 ppt)

Observed naupliar respiration values were of the same order of magnitude as that determined from Bhatnagar & Crisp's (1965) data at 16°C, $2.2x10^{-6}$ $ml O_2.h^{-1}.nauplius^{-1}$. However, the mean figure obtained here ($3.13\pm0.50x10^{-6}$ $ml O_2.h^{-1}.nauplius^{-1}$) was comparatively higher, probably as a result of using a higher experimental temperature (see Newell & Northcroft, 1967). The value was also of the same

order of magnitude, but a little higher than that reported by Harms (1987), who estimated *E. modestus* stage II naupliar respiration rates as $2.7x10^{-6}$ $mlO_2.h^{-1}.nauplius^{-1}$ at 24°C.

Nevertheless, the figure was considered to be an adequate estimate of the respiration rate of the nauplii and could be used as a reference when studying copper effects on the naupliar respiration rate. Furthermore, the numbers of nauplii used in each run ranged from 15 to 29 and showed no correlation between individual rates per nauplius and the number of nauplii used in the chamber ($r=-0.252$, $p=0.293$).

Short-term effects of copper on naupliar respiration rate

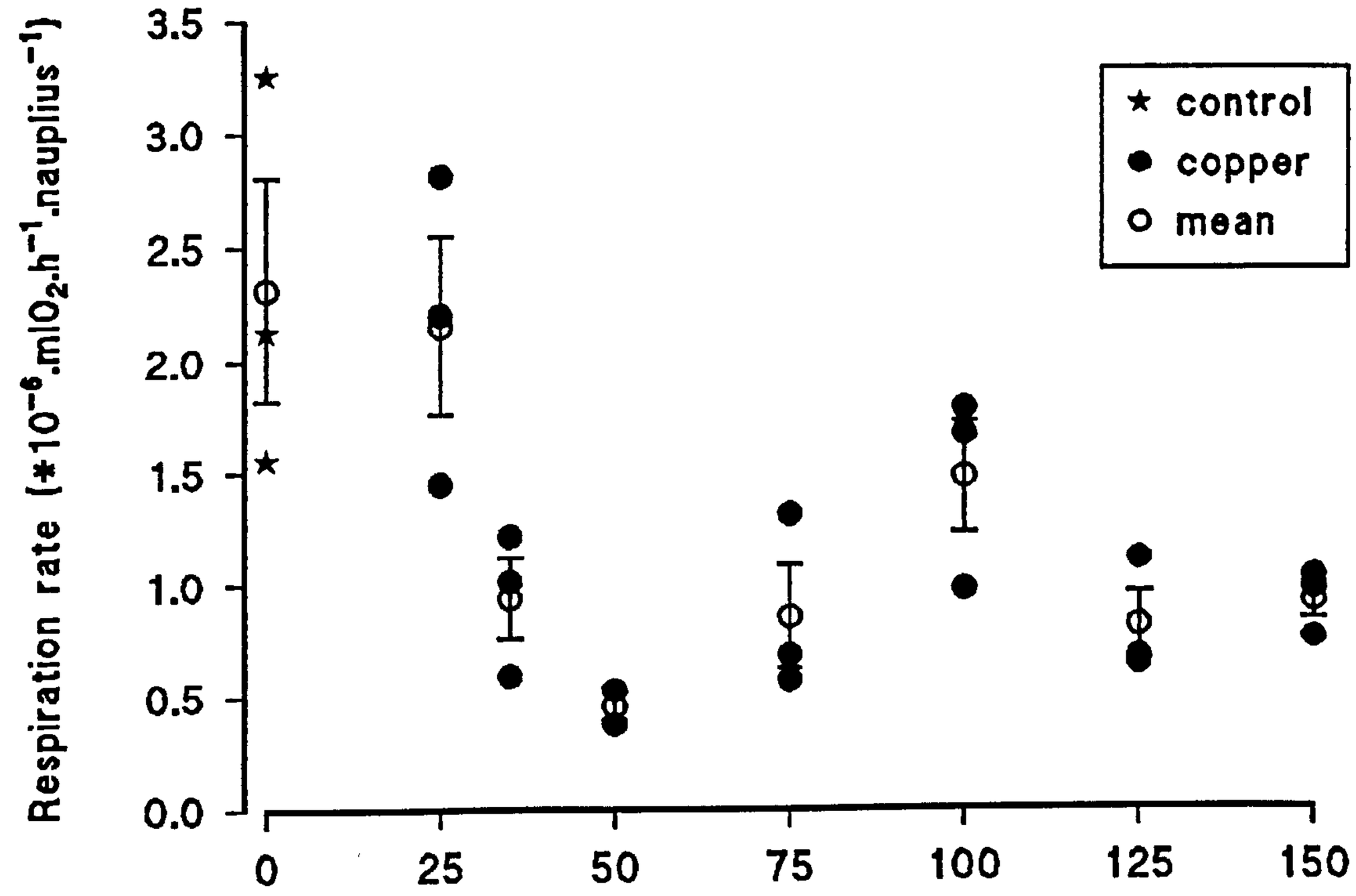
Naupliar oxygen consumption appeared to be affected by copper exposure (see Table 27 and Figure 6).

Added copper (ppb)		N	Oxygen consumption	Mean oxygen consumption
Blank	a b	-	-	$2.83 \times 10^{-5} \pm 0.40 \times 10^{-5}$
	a b c	-	-	$3.18 \times 10^{-5} \pm 0.53 \times 10^{-5}$
	a	-	-	2.75×10^{-5}
control	a	51	3.26×10^{-6}	2.31×10^{-6} ($\pm 0.86 \times 10^{-6}$)
	b	26	2.13×10^{-6}	
	c	41	1.56×10^{-6}	
25	a	62	2.21×10^{-6}	2.16×10^{-6} ($\pm 0.68 \times 10^{-6}$)
	b	21	2.81×10^{-6}	
	c	30	1.45×10^{-6}	
35	a	34	1.22×10^{-6}	0.94×10^{-6} ($\pm 0.32 \times 10^{-6}$)
	b	24	1.02×10^{-6}	
	c	23	0.59×10^{-6}	
50	a	63	0.38×10^{-6}	0.46×10^{-6} ($\pm 0.11 \times 10^{-6}$)
	b	35	0.53×10^{-6}	
75	a	42	0.69×10^{-6}	0.86×10^{-6} ($\pm 0.40 \times 10^{-6}$)
	b	27	1.32×10^{-6}	
	c	20	0.57×10^{-6}	
100	a	13	1.79×10^{-6}	1.48×10^{-6} ($\pm 0.44 \times 10^{-6}$)
	b	26	0.98×10^{-6}	
	c	47	1.68×10^{-6}	
125	a	35	0.66×10^{-6}	0.82×10^{-6} ($\pm 0.26 \times 10^{-6}$)
	b	23	0.68×10^{-6}	
	c	31	1.12×10^{-6}	
150	a	82	0.76×10^{-6}	0.93×10^{-6} ($\pm 0.14 \times 10^{-6}$)
	b	41	0.98×10^{-6}	
	c	43	1.04×10^{-6}	

Table 27 Oxygen consumption ($ml O_2 . h^{-1} . nauplius^{-1}$) of *E. modestus* nauplii (stage II) from a natural population, exposed to different copper concentrations for 20 min (at 22.5°C and 32 ppt)

Figure 6

Oxygen consumption ($ml O_2.h^{-1}.nauplius^{-1}$) of *Elminius modestus* nauplii (stage II) from a natural population, exposed to different copper concentrations for 20 minutes (at 22.5°C and 32 ppt), mean=mean values ± standard error



In general, values decreased from the mean 'control' figure of $2.31x10^{-6}\pm0.86x10^{-6}$ ml $O_2.h^{-1}.nauplius^{-1}$ down to the $0.93x10^{-6}\pm0.14x10^{-6}$ ml $O_2.h^{-1}.nauplius^{-1}$ of nauplii exposed to 150 ppb (see Table 27 and Figure 6).

An Analysis of Variance (homogeneous variance at Bartlett's statistic, $X^2=7.739$, $p=0.356$, $df=7$) carried out on these values showed that there were significant differences in the mean naupliar oxygen consumption between copper concentrations ($F=5.38$, $p=0.003$).

Subsequently, a Newman Keuls test, a sequential variant of the Tukey method for pairwise comparisons, showed that the respiration rate of 'control' nauplii was equivalent to that at 25 ppb (mean difference=0.16). In addition, the test showed that both these values were significantly higher than those at the remaining copper concentrations, which did not differ significantly amongst themselves (see Table 28).

Comparison	$Q_{0.05}$	Difference for 0.05	Mean difference	
cont.- 50 ppb	4.94 (8, 15)	1.68	1.86	s
cont.-125 ppb	4.78 (7, 15)	1.34	1.50	s
50 - 25 ppb			-1.70	s
cont.- 35 ppb	4.59 (6, 15)	1.29	1.38	s
50 -150 ppb			-1.02	ns

Table 28 Sequential Newman Keuls procedure for multiple comparisons of the oxygen consumption values ($x10^{-6}$ ml $O_2.h^{-1}.nauplius^{-1}$) of *E. modestus* nauplii (stage II) from a natural population, exposed to different copper concentrations for 20 min (at 22.5°C and 32 ppt), average S.E.=0.28, ns/s=(non) significant differences

Naupliar respiration rates increased with temperature over the range 12-19°C. At above 19°, the rate remained essentially constant indicating maximum metabolic activity over that temperature range. In contrast, the 'blank runs' exhibited an inverse linear relationship with temperature (see Table 29 and Figure 7).

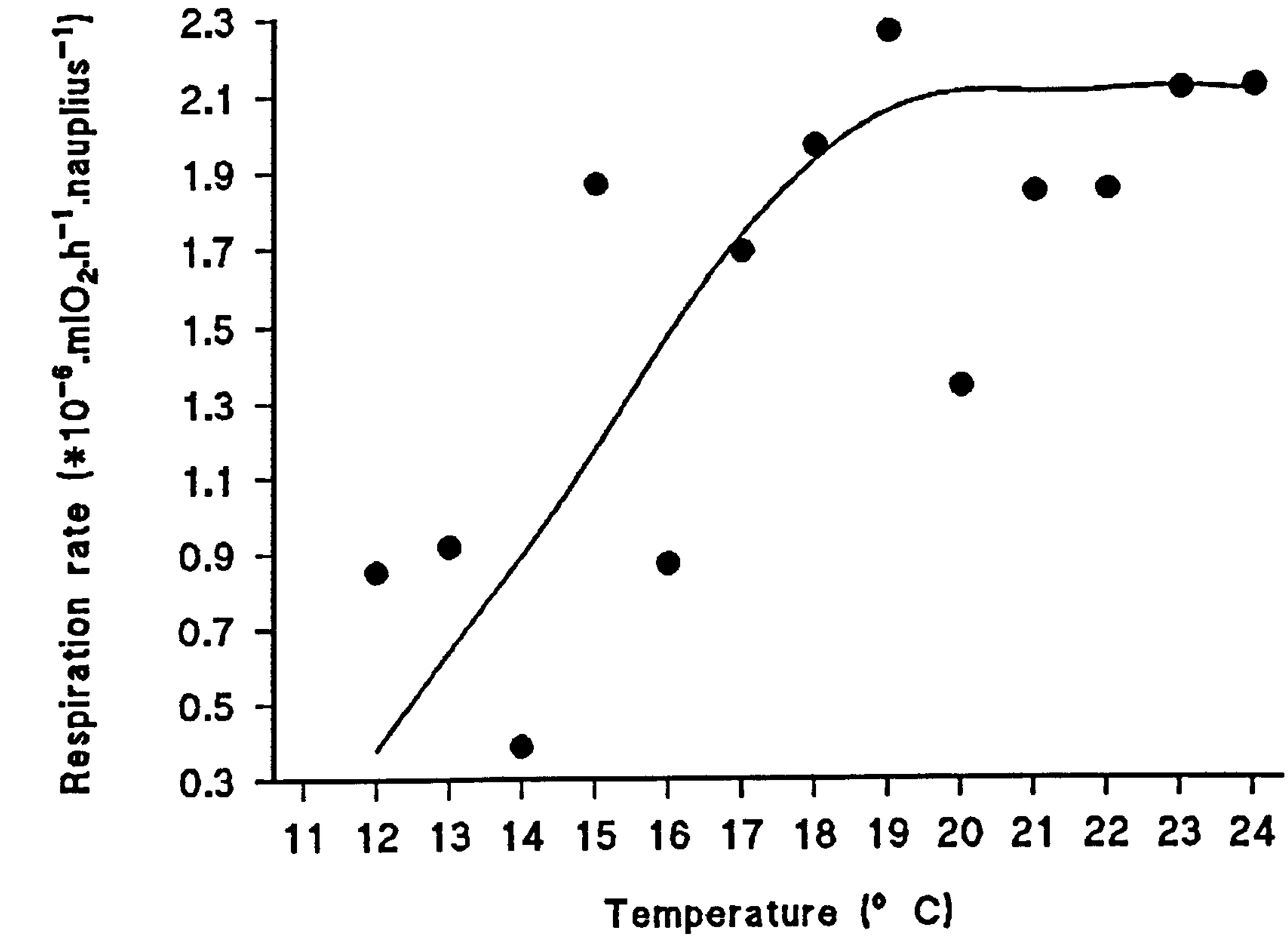
T°C	N	'Blank runs'	Oxygen consumption
12°	118	7.85×10^{-5}	0.85×10^{-6}
13°	97	7.48×10^{-5}	0.92×10^{-6}
14°	51	$*6.47 \times 10^{-5}$	0.39×10^{-6}
15°	94	5.88×10^{-5}	1.87×10^{-6}
16°	39	$*5.30 \times 10^{-5}$	0.87×10^{-6}
17°	41	$*4.72 \times 10^{-5}$	1.69×10^{-6}
18°	77	3.92×10^{-5}	1.97×10^{-6}
19°	42	1.00×10^{-5}	2.27×10^{-6}
20°	34	$*2.98 \times 10^{-5}$	1.35×10^{-6}
21°	54	2.40×10^{-5}	1.85×10^{-6}
22°	48	$*1.82 \times 10^{-5}$	1.85×10^{-6}
23°	59	1.98×10^{-5}	2.12×10^{-6}
24°	70	0.88×10^{-5}	2.13×10^{-6}

Table 29 Oxygen consumption ($ml O_2 . h^{-1} . nauplius^{-1}$) of *E. modestus* nauplii (stage II) from a natural adult population, at different temperatures and 32 ppt, *=calculated 'blank run'

Thus, measuring naupliar oxygen consumption at 22.5°C would only demonstrate a reduction in rate as caused by an endogenous variable, but was unlikely to demonstrate any increase in rate if the maximum metabolic activity had already been reached.

Figure 7

Oxygen consumption ($mlO_2.h^{-1}.nauplius^{-1}$) of *Elminius modestus* nauplii (stage II) from a natural population, at different temperatures and 32 ppt



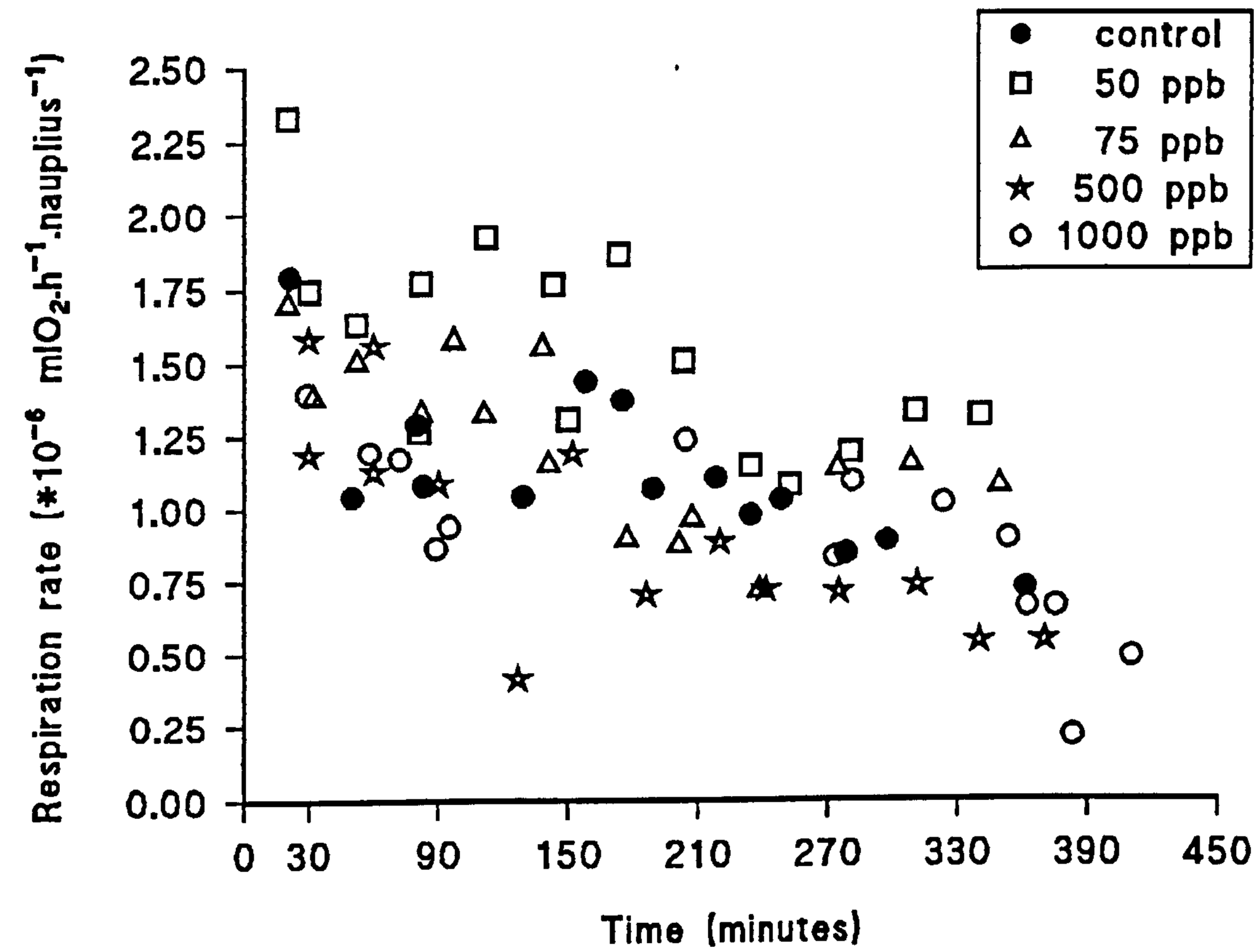
Long-term effects of copper on naupliar respiration rate

Naupliar respiration rates appeared to be affected by both the duration of the experimental period, which ranged from 20 to 400 min (about 6 h), and copper concentration. Thus, in general, oxygen consumption of both copper-exposed and 'control' nauplii decreased with time. In addition, the respiration rates of copper-exposed nauplii seemed to be set at a different level to that of 'controls'; such levels appeared to be highest at the lowest copper concentrations (see Figure 8).

There were significant linear correlations between respiration rates at each copper concentration and time (50 ppb, 75 ppb and 500 ppb=-0.677; 1,000 ppb=-0.700). An Analysis of Variance comparing the lines showed that there were no significant differences between the slopes (F=0.26, p=0.903), whereas the adjusted mean respiration rates exhibited significant differences (F=4.26, p=0.004). The average slope was then calculated as -0.0020 ± 0.0003 $ml O_2 . h^{-1} . nauplius^{-1} . m^{-1}$. Differences between adjusted means were subsequently tested for significance by an Analysis of Covariance (see Table 30).

Copper exposure at 50 ppb resulted in a significantly higher adjusted mean rate than any of the other treatments. There were no other significant differences, although 75 ppb copper showed an almost identical mean to the 'control,'

Figure 8
Oxygen consumption ($mlO_2.h^{-1}.nauplius^{-1}$) of *Elminius modestus* nauplii (stage II) from a natural population that had been exposed to different copper concentrations for about 6 hours (at 16°C and 32 ppt)



while the adjusted means for 500 ppb and 1,000 ppb were slightly lower (see Table 30).

Added copper (ppb)	control	50	75	500	1000
Adjusted means	1.12	1.50	1.18	0.96	1.02

Comparisons	Mean differences	
control - 50 ppb	0.39	s
control - 75 ppb	0.06	ns
control - 500 ppb	-0.16	ns
control - 1000 ppb	-0.10	ns
50 ppb - 75 ppb	-0.32	s
50 ppb - 500 ppb	-0.54	s
50 ppb - 1000 ppb	-0.49	s
75 ppb - 500 ppb	-0.22	ns
75 ppb - 1000 ppb	-0.16	ns
500 ppb - 1000 ppb	0.06	ns

Table 30 Analysis of Covariance performed of the adjusted means for the respiration rates ($x10^{-6}$ $mlO_2.h^{-1}.nauplius^{-1}$) of *E. modestus* nauplii (stage II) from a natural population that had been exposed to different copper concentrations for about 6 h (at 16°C and 32 ppt), average S.E.=0.064, difference for 0.05=0.257, ns/ s=(non) significant differences

DISCUSSION

Naupliar oxygen consumption at 22.5°C was affected by short-term copper exposure, all the concentrations tested being sublethal within the experimental period. In general, respiration rates of nauplii exposed to copper were lower than those of the 'controls'. However, at 25 ppb copper, oxygen consumption was essentially the same as that of 'control' nauplii. There hence appeared to be a threshold concentration between 25 and 35 ppb, beyond which respiration was reduced. The expected increase in metabolic rate at low concentrations resulting from the energetic cost of copper regulation (see Bernard & Lane, 1963) was not demonstrated. However, nauplii exposed to copper levels above 25 ppb were not able to maintain their 'normal' metabolic rate. Thus, copper's action on *E. modestus* nauplii was to inhibit metabolism in a manner similar to that shown by Corner & Sparrow (1956) for *Artemia sp.*, where a 25% reduction in respiration rate was found in response to copper contamination without significant effects on mortality.

The failure to show increased metabolic rates may have resulted from the choice of experimental temperature, set at 22.5°C to highlight copper toxicity to the nauplii (see Chapter Three: Factors affecting copper toxicity to *Elminius modestus* nauplii). However, the relationship between temperature and oxygen consumption in *E. modestus* nauplii was

such that, within the range of temperatures tolerated by the species, the metabolic rate increased with temperature to a maximum level after which no further increase was possible (see also Southward, 1955; Newell & Northcroft, 1965; Barnes & Barnes, 1969; Belman & Childress, 1973; Harms, 1987; Kurmaly *et al.*, 1989b). Thus, at 22.5° the nauplii could well have been working at near maximum metabolic rates and were hence given little opportunity to show a further increase in response to other imposed variables, although decreases should have been obvious. Therefore, 22.5°C was not the most suitable temperature for studying variations in the naupliar respiration rate and this was why a lower experimental temperature (16°C) was chosen for subsequent measurements.

The observed response to temperature was equivalent to that shown by Harms (1987), who calculated the energy budget of *E. modestus* stage II nauplii, estimating the naupliar oxygen consumption at 12°, 18° and 24°C and showing that the temperature influence between 12°-18°C was higher than that between 18° and 24°C. In addition, and despite having used a different method, the oxygen consumption values estimated by Harms (1987) were very similar to those obtained in the present study.

Reducing the experimental temperature implied that a longer time period was necessary in order to allow differences in the naupliar respiration rates to be expressed. In addition, the long-term copper exposure test showed an unexpected linear decrease in metabolic rate with naupliar development time, which had undoubtedly increased

variability in the earlier observations.

All copper concentrations tested proved sublethal within the exposure time (up to 6 h), during which the nauplii were always seen swimming inside the experimental chamber. However, the slopes of the lines representing respiration rate as a function of time were negative, indicating a reduction in oxygen consumption. In addition, the adjusted mean respiration rates showed significant differences. Thus, nauplii at the lowest copper concentration (50 ppb) were respiring significantly faster than the rest. Although there were no significant differences, the mean adjusted rate for nauplii at 75 ppb was virtually identical to the 'control' rates and those at 500 ppb and 1,000 ppb copper were generally lower.

Nauplii exposed to 50 ppb copper were most probably 'regulating' the excess copper, either through storage or excretion, and consequently incurring an energetic cost which was translated into increased oxygen consumption. At 75 ppb copper, expenditure on regulation could have balanced the inhibition due to copper, thus explaining absence of any difference in relation to the 'controls'. At the highest copper levels, the regulatory capacity could have been exceeded and general metabolism reduced, coincidentally close to 'control' values, due to copper inhibition of certain physiological processes such as pyruvate metabolism (Huggins & Munday, 1971).

The actual lack of significant differences between these

copper concentrations above 50 ppb and the overall reduction in respiration rates with time, however, suggests that the metabolic rate had declined in response to starvation as remaining yolk from the egg was utilised.

The long-term exposure test one again highlighted the effect of temperature on naupliar respiration rates, since the reduction in temperature from 22.5°C to 16°C increased the threshold copper concentration beyond which respiration was reduced, from 35 ppb to 50 ppb.

Bernard & Lane (1963) found a similar pattern of larval oxygen uptake response with long-term copper exposure in cyprids of *B. amphitrite niveous*, although those larvae had been exposed to a much higher range of copper concentrations (0.50-500 ppm).

Source of variation	DF	SS	MS	F	P
Treatment	7	8.8862	1.2695	5.38	0.003
Error	15	3.5390	0.2359		
Total	22	12.4252			

Table 1 Analysis of Variance performed on the mean values for the oxygen consumption ($mlO_2.h^{-1}.nauplius^{-1}$) of *E. modestus* nauplii (stage II) from a natural population, exposed to different copper concentrations for 20 min (at 22.5°C and 32 ppt)

Source of variation	DF	SS	MS	F	P
Time	1	4.07016	3.09839	54.24	0.000
Treatment	4	2.59931	0.24329	4.26	0.004
TimexTreat	1	0.05921	0.01480	0.26	0.903
Error	62	3.54185	0.05713		
Total	71	10.27053			

Table 2 Two-way Analysis of Variance performed on the regression lines of the values for the oxygen consumption of *E. modestus* nauplii (stage II) from a natural population, exposed to different copper concentrations for about 6 h (at 16°C and 32 ppt)

Added copper (ppb)	Time (min)	N	Oxygen consumption	Time (min)	N	Oxygen consumption
	21	106	$1.79x10^{-6}$	189	72	$1.07x10^{-6}$
	50	80	$1.04x10^{-6}$	218	77	$1.11x10^{-6}$
	80	47	$1.29x10^{-6}$	234	71	$0.98x10^{-6}$
control	83	54	$1.08x10^{-6}$	248	103	$1.04x10^{-6}$
	129	96	$1.05x10^{-6}$	278	48	$0.85x10^{-6}$
	158	60	$1.44x10^{-6}$	297	35	$0.90x10^{-6}$
	175	76	$1.37x10^{-6}$	361	44	$0.73x10^{-6}$
	20	77	$2.33x10^{-6}$	173	96	$1.86x10^{-6}$
	30	73	$1.74x10^{-6}$	203	73	$1.51x10^{-6}$
	52	132	$1.64x10^{-6}$	234	69	$1.15x10^{-6}$
50	81	89	$1.27x10^{-6}$	252	166	$1.09x10^{-6}$
	82	94	$1.77x10^{-6}$	280	120	$1.20x10^{-6}$
	112	65	$1.93x10^{-6}$	310	143	$1.34x10^{-6}$
	143	78	$1.76x10^{-6}$	340	108	$1.32x10^{-6}$
	150	103	$1.31x10^{-6}$			
	20	103	$1.70x10^{-6}$	177	120	$0.89x10^{-6}$
	32	64	$1.38x10^{-6}$	201	33	$0.87x10^{-6}$
	52	93	$1.50x10^{-6}$	207	132	$0.96x10^{-6}$
	82	158	$1.33x10^{-6}$	238	119	$0.72x10^{-6}$
75	97	65	$1.58x10^{-6}$	274	56	$1.14x10^{-6}$
	111	127	$1.33x10^{-6}$	308	140	$1.16x10^{-6}$
	138	114	$1.55x10^{-6}$	349	210	$1.08x10^{-6}$
	141	144	$1.15x10^{-6}$			
	30	98	$1.18x10^{-6}$	186	82	$0.70x10^{-6}$
	30	115	$1.58x10^{-6}$	220	130	$0.88x10^{-6}$
	60	96	$1.12x10^{-6}$	260	96	$0.88x10^{-6}$
500	60	147	$1.56x10^{-6}$	275	59	$0.71x10^{-6}$
	90	108	$1.08x10^{-6}$	311	114	$0.74x10^{-6}$
	127	64	$0.41x10^{-6}$	340	90	$0.55x10^{-6}$
	152	97	$1.19x10^{-6}$	370	123	$0.86x10^{-6}$
	29	131	$1.40x10^{-6}$	281	95	$1.10x10^{-6}$
	58	70	$1.19x10^{-6}$	323	94	$1.03x10^{-6}$
	72	120	$1.17x10^{-6}$	353	89	$0.90x10^{-6}$
1000	89	87	$0.87x10^{-6}$	362	96	$0.84x10^{-6}$
	95	130	$0.94x10^{-6}$	375	27	$0.67x10^{-6}$
	204	115	$1.24x10^{-6}$	383	55	$0.22x10^{-6}$
	273	110	$1.03x10^{-6}$	410	132	$0.50x10^{-6}$

Table 3 Oxygen consumption ($ml O_2.h^{-1}.nauplius^{-1}$) of *E. modestus* nauplii (stage II) from a natural adult population, exposed to different copper concentrations for about 6 h (at 16°C and 32 ppt)

CHAPTER FIVE

EFFECTS OF LONG-TERM COPPER EXPOSURE ON A LABORATORY POPULATION OF *Elminius modestus*

CONTENTS

There is abundant literature regarding the effects of copper on a variety of behavioural, physiological, morphological and genetic parameters in barnacles (see, for example, Clarke, 1947; Pyefinch & Mott, 1948; Weiss, 1947, 1948; Nevo *et al.*, 1978; Powell & White, 1989; Patarnello *et al.*, 1991). Long-term chronic exposure studies in the laboratory are scarce, however, even where time is an important factor determining the impact of copper on barnacle biology.

Amongst the biological parameters affected by copper, two of special relevance to continued survival are somatic growth and reproduction, both of which require net energy accumulation (see Bourget & Crisp, 1975a, 1975b; Crisp & Bourget, 1985). If a barnacle is exposed to continuous low levels of copper contamination, some of the animal's energy might be directed towards coping with the associated stress (e.g. detoxification) and cannot therefore be used for either growth or reproduction.

Barnacle growth is influenced by several environmental factors, many of which determine food availability (see reviews by Bourget & Crisp, 1975a; Crisp & Bourget, 1985). Feeding, involving both food-capture and particle filtration, is achieved through cirral activity (see Southward, 1955a; Crisp & Southward, 1961; Southward & Crisp, 1965; Anderson &

Southward, 1987), which depends mainly on the barnacle's size and the prevailing temperature (Southward, 1955b; Newell & Northcroft, 1965; Ritz & Crisp, 1970). Furthermore, barnacle feeding behaviour can be specifically influenced by variables such as salinity, pH and oxygen tension (Southward & Crisp, 1965; Foster, 1970, Davenport, 1976a), which are conditions that have to be coped with even in the absence of pollution.

The cirral beating rate of *E. modestus* at 15°C-25°C reaches a maximum of 111 beats.min^{-1} to 132 beats.min^{-1} at 24°C, declines sharply at 30°C and ceases completely at 33-35°C (Southward, 1955b). This rate is faster than that of any other British species, making *E. modestus* more efficient in terms of food diverted to growth and reproduction (Crisp, 1958). In addition, this species produces several broods of larvae per year (Crisp, 1958), making it an ideal organism in which to study copper effects on reproductive output. *E. modestus* tolerates silt and pollution better than any other species in Britain (Crisp, 1958). However, copper and cadmium can reduce its overall activity, affect the cirral movement by increasing pumping (for types of cirral activity see Crisp & Southward, 1961) and decrease beat frequency, where these effects depend on both the metal concentration and the duration of the exposure period (see Powell & White, 1989).

This Chapter studies the effects of long-term copper exposure on the growth and reproduction of *E. modestus*, aiming to assess whether a reduction in those parameters might be explained, in part, by a reduction in ingestion rate which would result from copper-induced alterations on the

barnacle's feeding behaviour, as reported for several other species in the literature.

MATERIALS AND METHODS

The overall macroscopic effects of copper on *E. modestus* growth and reproduction were monitored over time periods ranging from one month to a little over four months in specimens kept on glass panels in the laboratory from spat to full adulthood. Growth was assessed by reference to variations in the lateral width. Effects on reproduction were studied by visually examining the development of the gonads following Crisp & Davies' (1955) descriptions of the stages in the maturation of *E. modestus* ovaries, and of the evaluation of the fertilised ovary as well as embryo development, which is detected by the progressive darkening of the egg masses from pale cream through ochre, fawn to brown.

Attempts to collect natural *E. modestus* spat-fall (July-August, 1993) failed due to poor recruitment during that period. Spat were therefore obtained from the bulk culture of nauplii in the laboratory (see Moyse, 1960; Tighe-Ford *et al.*, 1970; Yule, 1984) and the settling of the resulting cyprids. Thus, a 70 litre black polystyrene bin was filled with 60 litre of FUVSW (32 ppt) and approximately 50,000 stage I *E. modestus* nauplii produced by adults collected from Menai Bridge pier (see Chapter Two: General Materials and Methods). The larvae were fed on a mix of the diatom *Skeletonema costatum* [Greville] (Cleve), to give a

concentration of 50 cell.µl $^{-1}$, and the flagellate *R. reticulata*, to give a concentration of 35 cell.µl^{-1}.

The bin was covered with a lid, gently aerated with a thin air pipe and left overnight for the water to acquire laboratory temperature. Next day the medium was replaced by water at 22°C, the animals were fed again and an immersion aquarium heater was placed in the bin to maintain the temperature at 22°C. From then onwards, the water was replaced and the animals were fed every other day.

Cyprids started to appear after 10 days and were transferred to a 500 ml crystallising dish filled with FUVSW where they were kept, at laboratory temperature, for four days to increase the chances of settlement (see Crisp & Meadows, 1962). The water in the culture bin was then changed every day.

The settlement procedure consisted of transferring the four day old cyprids to another 500 ml crystallising dish filled with FUVSW and covered with 2 mm thick glass panels (approx. 3x6 cm) (see Pyefinch & Mott, 1948; Crisp & Davies, 1955) that had been coated with a thin layer of Arthropodin to enhance settlement (see Crisp & Meadows, 1962). A strong air jet was directed onto the water surface to produce a water flow in the dish, in order to prevent the cyprids from being trapped in the water surface film. Settled spat were kept in the crystallising dish to induce further settlement and fed on *R. reticulata* (100 cell.µl^{-1}).

After day 12, the culture bin was stopped and the last cyprids were transferred to the crystallising dish. Only 244

of the several hundred cyprids produced actually settled successfully.

Once they were a week old, the settled spat were split into three groups of about 80 animals each. The panels separating each group were then slotted into supports resting on the bottom of small 3 litre fish tanks filled with 2 litre of FUVSW, which was gently aerated and kept at laboratory temperature (13.2°C-22.5°C, with an average of 18.2°C). The barnacles were fed to excess on a 50:50 mix of *S. costatum* and *R. reticulata* taken straight from the batch culture ('unwashed' algae, 40% ACM) (see Chapter Two). Every third day, the water in the tanks was changed and the animals were rinsed with clean FUVSW and fed.

After 13 days, the barnacles had grown to a diameter of 2-3 mm and were thereafter fed on either rotifers (5-10 per ml) or *Artemia sp.* nauplii (5-10 per ml), both routinely grown in the laboratory for feeding invertebrates. In addition, the tanks were allocated a copper concentration as follows: A=500 ppb, B=200 ppb and C=0 ppb (the 'control'). The copper originated from a 10^6 ppb stock solution and was added in quantities which produced a 50 ppb concentration in the tanks. Every other day the water in the tanks was changed, the barnacles were cleaned to remove any organic matter deposited on them (see Pyefinch & Mott, 1948) and fed with *Artemia sp.* (5-10 per ml), and the copper dose was increased as needed.

The temperature in the tanks, the number of dead

animals, any *Artemia* remaining in them, barnacle cirral activity and feeding behaviour, inseminating activity, presence of faecal pellets, gut condition and presence of larvae were noted with every water change. At intervals, the barnacle lateral width was measured with a micrometer eyepiece and gonad maturation was assessed with the aid of a stereoscope (see Crisp & Davies, 1955) and recorded.

Four days after the first copper addition, when the animals were 17 days old, it was observed that those exposed to copper (150 ppb at that point) were noticeably smaller than those in the 'control' tank. Once copper levels reached 200 ppb and the barnacles were 19 days old, having spent 6 days in copper-containing media, it was decided to stop increasing the concentration in tank B (200 ppb) and maintain it at that level. However, after 12 days, when the barnacles were 25 days old, the concentration had to be reduced to 150 ppb, since the condition of the barnacles was deteriorating. Copper increase in tank A was also stopped, after 8 days of copper exposure (animals 21 days old), when the concentration had reached 250 ppb. After both groups of barnacles had spent 13 days in media containing copper, *R. reticulata* was also added to give a concentration of 100 cell.μl^{-1} and so supplement the diet.

Over half of the animals in tank A (250 ppb) had died after 26 days of copper exposure, at which stage they were a little over a month old. The experiment was therefore terminated and the barnacles at the lower copper concentration (tank B, 150 ppb) were transferred to a

copper-free medium, where they were fed with algae and *Artemia sp.* to assess possible recovery. The 'control' barnacles were separated into two groups, one of which was maintained with no added copper (C), while the other was allocated a copper concentration of 50 ppb (C*). The copper was introduced in quantities sufficient to produce 10 ppb in the culture, every third day, when the water was cleaned and the animals were fed. Once the concentration reached 50 ppb the tanks were changed every other day.

The experiment ran for 127 days, by which time the animals were 166 days old.

Two 750 ml water samples were taken from tank C* (50 ppb) after one of the 48 hour periods and analysed for copper following the method outlined in Chapter Two.

Potential copper-induced reductions in the ingestion rate of *E. modestus* were assessed by comparing the ingestion rates in copper-exposed barnacles with those in uncontaminated animals. The barnacles were exposed to 10 ppb for 2.5 days and then to 20 ppb for the following 4.5 days. Subsequently, the animals were transferred to clean media (FUVSW), where they were kept for an additional week.

Test barnacles (n=40) were originally from a natural population living on slate panels at Port Dinorwic and were chosen because the panels fitted into the experimental dishes. The rostro-carinal diameter of the barnacles was measured on arrival in the laboratory, where the animals were kept in a tank of FUVSW, with aeration and food, until the

water acquired the ambient temperature (approximately 22°C). The panels with the attached barnacles were then evenly distributed in 500 ml transparent polystyrene containers filled with 250 ml of a culture medium made up of 249.99 ml FUVSW and 10 µl of a 10^6 ppb stock copper solution. The barnacles were fed on *Artemia sp.* nauplii to give an initial density of 20 nauplii.ml^{-1}. The containers were constantly stirred with an impeller at 32 r.p.m. (see Crisp & Southward, 1961) (see Plate 6).

The experiment involved three treatments: A=barnacles in FUVSW with *Artemia*; B=barnacles in FUVSW, copper and *Artemia*; and C=FUVSW and *Artemia*, where each treatment was duplicated. The salinity in the culture media was 32 ppt and the containers were kept in the laboratory under natural light at an ambient temperature (21.8°C average). The culture media were changed initially after 12 h and every 24 h thereafter. The numbers of *Artemia* remaining at the end of each time period were estimated by pipetting 4 x1 ml water samples from each container and counting the number of nauplii in a Bogorov tray with the aid of a stereoscope. The disappearance of *Artemia* from the cultures was estimated at the time of each water change during the week the barnacles were in copper-containing media. In addition, *Artemia* numbers were also measured over the first and last 24 h of the one-week recovery period.

During the sampling at Port Dinorwic and also when sorting the animals in the laboratory, a special effort was made to select animals of roughly the same size. However, an

Analysis of Variance performed on the barnacle rostro-carinal diameters (homogeneous variance, Bartlett's statistic=3.974, p=0.264, df=3) showed that there were significant differences between the mean values for each treatment (Aa,'control' barnacles, 5.3±0.8 mm), (Ab, idem, 5.4±0.7 mm), (Ba, 'copper' barnacles, 5.8±0.9 mm), (Bb, idem, 6.3±0.7 mm) (F=8.10, p≤0.001). Nevertheless, these differences would not affect the results of the experiment, since the copper treatments (Ba and Bb) under which the barnacles were expected to eat less, were those involving the biggest animals.

The experiment included two flasks (Ca and Cb) that contained only FUVSW and *Artemia* without barnacles, to assess whether the *Artemia* were sedimenting to the bottom of the containers and consequently not being sampled (see Crisp, 1984).

Reductions in the ingestion rate on copper-exposed *E. modestus* could result from either reduced cirral activity or prolonged cirral withdrawal inside the opercular plates. The validity of these hypotheses was tested through two independent experiments. The first, test A, examined the effects of 5, 10, 15 and 20 ppb copper on opercular plate closure over a short-term exposure period (20 min). The other, test B, involved a single copper concentration, 20 ppb, since that was the concentration which had previously shown a particularly noticeable reduction in barnacle ingestion rate. This second test was designed to confirm the results from test A, as well as to examine the effects of

copper exposure on the above parameters over a longer period of time.

The barnacles came from a natural population living on mussels at Conway, since mussel shells were the most suitable substratum for the experimental dishes. The mussels were brought back to the laboratory and cleaned, opened and emptied to leave just the shells with the barnacles, which were then left to acclimate to laboratory conditions. Subsequently, the mussel shells with attached barnacles were divided into groups of 20 barnacles each and placed in a 1,000 ml crystallising dish filled with 500 ml of FUVSW, plus the required volume of a 10^6 ppb stock copper solution, with a final salinity of 32 ppt. The crystallising dish was then placed on a magnetic stirrer, creating a water current to encourage the animals to open (see Southward & Crisp, 1965). The dish was kept in the laboratory under artificial lightning, which was continuous during the experimental period, at an ambient temperature of approximately 21°C in test A and approximately 25°C in test B. The culture medium and the animals were changed between measurements at the different copper concentrations.

In test A, the barnacles were fed on the nauplii of *Artemia sp.* 12 h before they were used. After that period, they were placed in the crystallising dish and left for 20 min, when the number of open barnacles was recorded for a one-minute period. This procedure was repeated three times for each copper concentration.

In test B, the animals were fed on *Artemia* 5 h before

they were used. Afterwards, they were placed in the crystallising dish and left for 20 min, when the number of open animals and the type of cirral activity, either beating or pumping (see Crisp & Southward, 1961), were recorded. Subsequently, the animals were observed for 10 min to determine which of the types of cirral activity was dominant. In addition, the beat frequency of one animal (of the same size for both treatments) was measured by counting the number of beats in one minute (see Southward, 1955a; Southward & Crisp, 1965). Five replicate measurements of beat frequency were made each time on the same barnacle. The animals were then left for 20 h, after which the above procedure was repeated. *Artemia sp.* nauplii were then added to the culture medium to give a concentration of 2.5 nauplii.ml^{-1}. After 30 min the test parameters were recorded again and the number of *Artemia* remaining in the dish counted using 4 x1 ml water samples. The same amount of *Artemia* was subsequently added again and 30 min later, the above measurements were repeated for the final time.

RESULTS

Long-term effects of copper on barnacle growth and reproduction

E. modestus spat mortality from settlement to the moment copper was added to the cultures was quite considerable, ranging from about 20% to a little over 30%. Once copper at 50 ppb was introduced in the tanks, mortality increased further, and by the end of the experimental period reached 60% for tank A (250 ppb) and 40% for B (150 ppb). This showed that the original target copper concentrations chosen for the test (A=500 ppb, B=200 ppb) were unrealistic for small spat within the time scale available for acclimation (see Table 31).

Tank	Added copper (ppb)	Time in copper medium	Age	N	% Mortality
A	200			59	27.10!
B	200	8 days	21 days	63	30.86!
C	control			53	35.36!
A	250			24	59.32*
B	150	26 days	39 days	34	39.28*
C	control			53	0.00*

Table 31 Survivorship of *E. modestus* spat grown (at 18.8°C average temperature and 32 ppt) for approximately 1 month in different copper concentrations, !=since settlement, *=from 8 to 26 days in copper-containing media

Size differences between copper and 'control' animals were evident after the former had spent only four days in copper. No further increase in copper concentration occurred

beyond day 6 in tank B (200 ppb). After 8 days in copper-containing media, when the animals were 21 days old, the copper concentration in tank A was stabilised at 250 ppb. The size of the animals in each of the tanks was measured for the first time on that day, showing that the mean lateral width of those exposed to copper was about half that of the 'controls' (A=0.832±0.189 mm, B=0.921±0.252 mm, C=1.820±0.520 mm) (see Table 32).

Tank	Added copper (ppb)	Time in copper medium	Age	N	Mean width	Min. width	Max. width
A	200			59	0.832±0.189	0.437	1.250
B	200	8 d	21 d	63	0.921±0.252	0.250	1.312
C	control			53	1.820±0.520	0.437	2.625
A	250			24	0.958±0.154	0.562	1.250
B	150	26 d	39 d	34	1.051±0.206	0.437	1.375
C	control			52	4.290±0.785	1.187	5.937

Table 32 Size of *E. modestus* spat grown (at 18.8°C average temperature and 32 ppt) for approximately 1 month in different copper concentrations, expressed as the lateral width in millimetres; d=days

The state of the copper-exposed barnacles continued to decline even after the concentration in tank B was reduced to 150 ppb and their diet was supplemented with *R. reticulata*. There were always high numbers of *Artemia* nauplii left after the 48 h and there were no visible faecal pellets on the bottom of the tanks, hence the animals were hardly eating, let alone growing.

Eventually, after 26 days of copper exposure, when the animals were 39 days old, the experiment was stopped, since

the copper concentrations were clearly in excess of the levels appropriate for the aim of the experiment. The barnacles were measured for a final time and this showed that the mean lateral width of those exposed to copper was about one quarter that of the 'controls' (A=0.958±0.154 mm, B=1.051±0.437 mm, C=4.290±0.785 mm) (see Table 32).

A Kruskal-Wallis test performed on the lateral width values for each treatment at the different times, showed that at least one median differed significantly from at least one other (H=198.71, $p \leq 0.001$, df=5).

Subsequently, Dunn's procedure for multiple comparisons (standard normal deviate at 0.95, Z=2.94) determined that, after 8 days in copper-containing media, the barnacles in tank A were significantly smaller than those in tank B (median difference=0.062 mm), which may reflect the initial distribution of spat to treatments, since at that time they were both exposed to the same copper concentration (200 ppb). In addition, both groups of animals were significantly smaller than the 'controls' (median differences=0.937 mm for A and 0.875 mm for B) (see Table 33).

The differences became more accentuated with time, and after 26 days in copper-containing media barnacles in tank A (250 ppb) had just reached the size previously attained by those in tank B (then 200 ppb) (median=0.937 mm). However, they were still significantly smaller than the barnacles currently in tank B (150 ppb) (median difference=0.125 mm). Again, the two groups of barnacles in tanks with copper-containing media were much smaller than the 'controls'

(median differences=3.437 mm for A and 3.312 mm for B) (see Table 32).

Level	N	Median	Mean Rank		Comparisons (Z) 1	2	3	4	5
1	59	0.8750	70.2	2	5.40	-	-	-	-
2	63	0.9375	95.7	3	4.86	*0.83	-	-	-
3	24	0.9375	100.9	4	10.05	5.57	3.69	-	-
4	34	1.0625	126.6	5	25.80	20.95	15.06	12.34	-
5	53	1.8125	197.5	6	37.78	33.13	24.34	22.77	11.79
6	52	4.3750	257.5						
tot	285		143.0						

Table 33 Dunn's test performed on the median values for the lateral width (in mm) of *E. modestus* spat grown (at 18.8°C average temperature and 32 ppt) for approximately 1 month in media containing copper. Levels: 1,2= 200 ppb after 8 days, 3=250 ppb after 26 days, 4=150 ppb after 26 days, 5,6='control' tank after 8 days and 26 days respectively, tot=total, minimum average rank differences for significance Z=2.94, *=no significant differences

The experiment was continued by separating a copper-treatment group from the surviving 'controls', which would have a maximum copper concentration of 50 ppb added in 10 ppb aliquotes every 3 third day.

Only one barnacle from tank B (150 ppb) recovered from the copper exposure and was afterwards maintained with the continuing 'control' animals.

Differences arose between 'control' (C) and 'copper' (C*, 50 ppb) animals in all the parameters that were routinely recorded even though after any of the 48-hour periods the copper concentration in tank C* (50 ppb) was down to 43% (to 28±0.9 ppb) due to adsorption to the walls, etc.

After 48 h exposure to 10 ppb, some *Artemia* nauplii

could be seen in tank C* (50 ppb) but not in the 'control' tank, indicating that copper-exposed barnacles ingested less. Initial differences in size ('controls' were significantly larger, t=2.65, p=0.012) and/or in the total number of animals (the 'control' group had 5 more barnacles) might have been responsible for the difference in feeding rates. However, this difference was consistently recorded throughout the experiment, as were a lack of identifiable faecal pellets, gut emptiness and reduced cirral activity (only the pneumostome was visible for most of the time) in the barnacles from C* (50 ppb).

Mortality constituted another major difference between C ('control') and C* (50 ppb) barnacles. Thus, 'control' mortality over the 166-day experimental period was 10%; whereas the mortality of copper-exposed barnacles was five times greater at 50% (see Table 34).

Tank	Added copper (ppb)	Time in copper medium	Age	N	% Mortality
C	control	35 days	74 days	29	0.00
C*	50			24	0.00
C	control	127 days	165 days	26	10.34
C*	50			12	50.00

Table 34 Survivorship of *E. modestus* spat grown (at 18.8°C average temperature and 32 ppt) for a little over 4 months in 50 ppb copper-containing medium

Size also differed between the C ('control'), C* (50 ppb) and B (previously 150 ppb) animals. After a little over

one month without added copper, the latter (one individual) had grown to five times its initial width (5.5 mm from 1.1 mm) and it kept on growing, surpassing the average size of C* (50 ppb) animals after approximately two months in the absence of copper).

Size differences between C ('control') and C* (50 ppb) specimens had existed by chance from the start, but they became accentuated with exposure time. Thus, lateral width was always significantly greater in the 'control' barnacles (0.5 mm difference, see Table x). After 35 days in copper-containing medium (age=74 days) the difference between the groups was around 2 mm in lateral width, whereas after 62 days (age=101 days) it had reached almost 3 mm (see Table 35 and Plates 2-3).

Tank	Added copper (ppb)	Time in copper medium	Age	N	Mean width	Min. width	Max. width
C	control	0 d	39 d	29	4.530±0.583	3.437	5.937
C*	10			24	3.997±0.912	1.187	5.000
C	control	35 d	74 d	29	7.215±1.037	4.462	9.231
C*	50	(≈1 m)		24	5.147±0.925	2.308	6.154
C	control	62 d	101 d	28	8.412±0.931	6.923	10.615
C*	50	(≈2 m)	(≈3.5 m)	19	5.433±0.852	3.538	6.923

Table 35 Size of *E. modestus* spat grown (at 18.8°C average temperature and 32 ppt) for a little over 4 months in 50 ppb copper-containing medium, expressed as the lateral width in millimetres; d=days, m=months

A Two-way Analysis of Variance (homogeneous variance at Barttlet's statistic, X^2=9.282, p=0.098, df=5) with time as a covariate determined that growth rates over the 101 days of

the exposure period differed significantly, the value for copper-exposed barnacles being approximately one-third that of the 'controls' (interaction $F=44.57$, $p \leq 0.001$) (see Table 36).

Tank	Added copper (ppb)	Slope	stdev	t-value	p
C	control	0.063	0.003	16.50	0.000
C*	50	0.024	0.004	5.38	0.000

Table 36 Growth rate ('slope' in $mm.day^{-1}$) differences in *E. modestus* spat grown (at 18.8°C average temperature and 32 ppt) for a little over 4 months in copper-containing media

Reproductive organ development was assessed for the first time after two months of copper exposure, when the barnacles were approximately three and a half months old. All C ('control') animals (100%) showed well-developed ovaries, whereas the percentage of barnacles with ovaries in C* (50 ppb) was lower (79%) and in about half (32%) of those cases the ovaries were poorly developed. In addition, C* (50 ppb) never produced eggs, whereas a high proportion (61%) of C ('control') barnacles produced fertilised eggs, the majority of them (43%) being well advanced, with eye spots clearly visible (see Table 37 and Plates 2, 4-5).

Inseminating activity was initially observed in both groups of barnacles, although from approximately three and a half months onwards this behaviour was noted only in the 'controls'. At the end of the experiment, the percentages of

barnacles with ovaries and eggs had decreased in both tanks. The single animal in B (previously 150 ppb) recovered and matured sexually, but eggs were never fertilised, since it was placed too far from any possible inseminant. 'Control' barnacles produced larvae, since those that had eggs showing eye spots at one of the 'checking' times did not contain eggs the next time or, if they did, no eyes were evident. However, nauplii were never detected in the water.

Tank	Added copper (ppb)	Time in copper medium	Age	N	%Ovary	%Eggs	%Eggs +eyes	Larvae
C	control	62 d	101 d	28	100.00	60.71	42.85	-
C*	50	(≈2 m)	(≈3.5 m)	19	78.94	0.00	0.00	-
C	control	83 d	122 d	28	100.00	17.85	10.71	25%
C*'	50	(≈2.5 m)	(≈4 m)	19	84.21	0.00	0.00	-
C	control	127 d	165 d	26	91.66	25.00	12.50	-
C*	50	(≈4 m)	(≈5.5 m)	12	44.44	0.00	0.00	-

Table 37 Reproductive organ development of *E. modestus* spat grown (at 18.82°C average temperature and 32 ppt) for a little over 4 months in media containing 50 ppb copper; d=days, m=months, %Ovary, %Eggs etc. refer to the number of animals showing these characteristics

Towards the end of the experiment, after approximately three months, 'control' barnacles (C) did not appear to be as fit as they were at the beginning. They started to eat less and the percentages of animals with developed ovaries and eggs decreased.

The ingestion rates in copper-exposed barnacles varied with time and copper concentration. There was a considerable natural variability in the 'control' animals, where the value decreased from 40 *Artemia*.h^{-1}.$barnacle^{-1}$ obtained after the first 12 h of the exposure period to 19 *Artemia*.h^{-1}.$barnacle^{-1}$ after 60 h (see Table 38).

Treatment	Added copper	N	Time	Ingestion rate	Mean ingestion
Aa	control	39	12 hrs	37.065	39.912
Ab		40		42.759	±4.026
Ba	10 ppb	41	12 hrs	35.563	33.580
Bb		37		31.598	±2.804
Aa	control	39	36 hrs	24.631	23.877
Ab		40		23.124	±1.065
Ba	10 ppb	41	36 hrs	19.726	21.166
Bb		37		22.606	±2.037
Aa	control	39	60 hrs	20.339	19.191
Ab		40		18.042	±1.624
Ba	10 ppb	41	60 hrs	15.952	16.766
Bb		37		17.580	±1.151

Table 38 Ingestion rate (*Artemia*.h^{-1}.$barnacle^{-1}$) of *E. modestus* from a natural population grown (at 21.8°C and 32 ppt) for 60 h in media containing 10 ppb copper

Copper reduced the ingestion rate, even at 10 ppb, the reduction being evident after the first 12 h of the experiment. A Two-way Analysis of Variance (homogeneous variance at Barttlet's statistic, X^2=1.628, p=0.897, df=5)

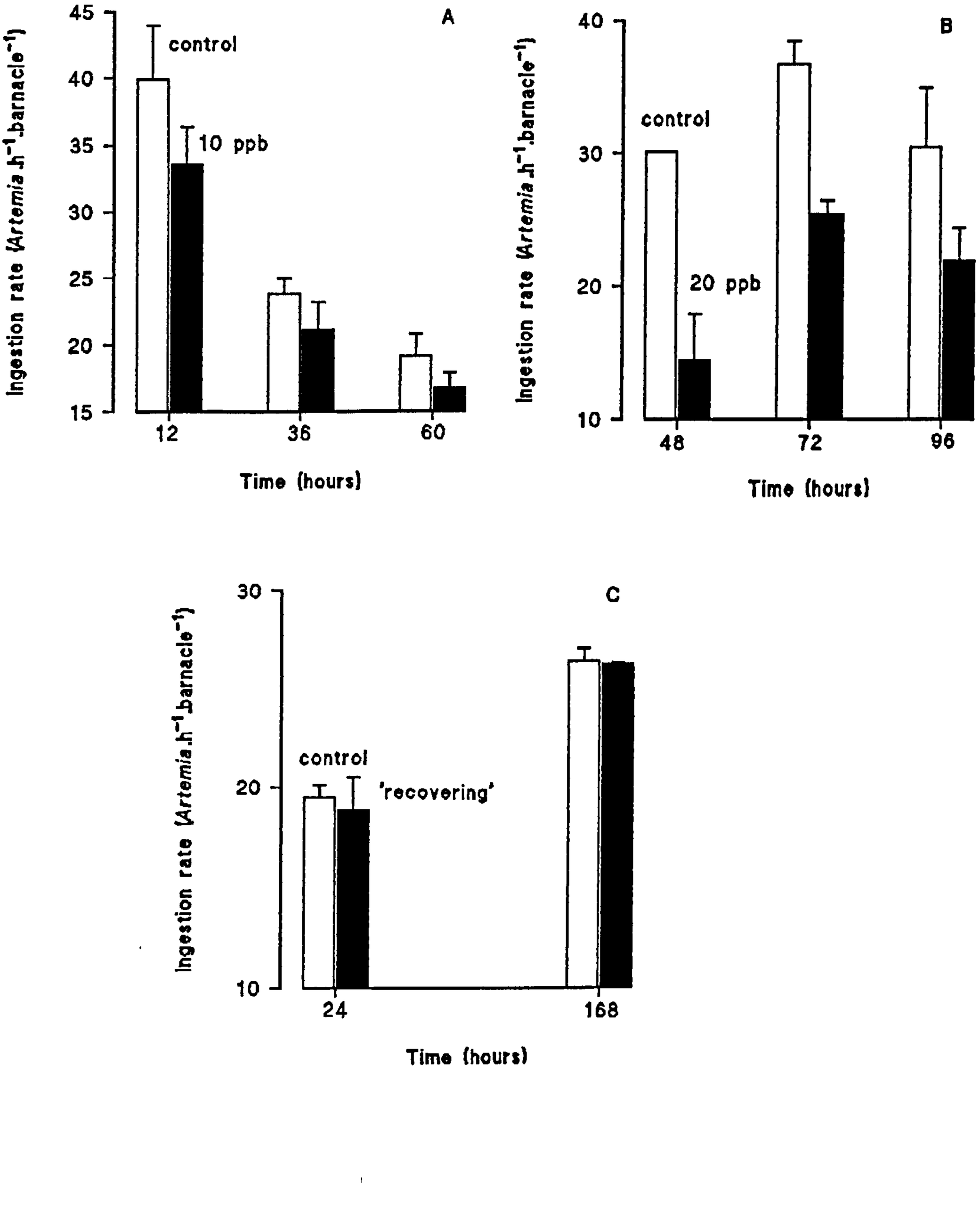
performed on the ingestion rates calculated for each replicate showed that, in general, there had been a significant decrease in ingestion rate with time. Barnacles exposed to 10 ppb (Ba and Bb) showed significantly lower ingestion rates than those of the 'controls' (time $F=69.06$, $p \leq 0.001$; treatment $F=7.89$, $p=0.031$) with no significant interaction ($F=0.85$, $p=0.472$) (see Table 38 and Figure 9).

An increase in the copper concentration to 20 ppb made the differences between rates even more obvious. After 48 h there were no *Artemia* left in the 'control' containers and, as a result, their ingestion rate had to be estimated by reference to the 'minimum ingestion rate' (total number of *Artemia* divided by the number of barnacles times the time difference). The value obtained was 30 *Artemia*.h^{-1}.barnacle^{-1}, an obvious underestimate. In contrast, the number of *Artemia* in the containers where the barnacles were exposed to copper was still approximately 20 nauplii.ml^{-1} (see Table 39 and Figure 9).

Over the first 48 h, 'control' barnacles showed an ingestion rate which was certainly higher than that in the copper-exposed animals, but was undeterminable. The rates were therefore analysed statistically only for the 72- hour and 96-hour values. A Two-way Analysis of Variance (homogeneous variance at Barttlet's statistic, $X^2=1.475$, $p=0.687$, $df=3$) showed that, although the overall ingestion rates of 'control' barnacles had decreased after 96 h relative to 72 h, the differences were not quite

Figure 9

Ingestion rate (mean ± stdev *Artemia*.h^{-1}.barnacle^{-1}) of *Elminius modestus* from a natural population grown (at 21.8°C and 32 ppt) for different time periods in media containing either 10 ppb (A) or 20 ppb (B) copper, which were then maintained for a week in unpolluted media (C), 'recovering'= barnacles previously exposed to copper



significant (F=6.45, p=0.064). However, the ingestion rates in copper-exposed barnacles were consistently lower than those in the 'controls' (F=26.73, p=0.007); here again no significant interaction was evident (F=0.48, p=0.526).

Treatment	Added copper	N	Time	Ingestion rate	Mean ingestion
Aa	control	39	48 hrs	minimum	ingestion
Ab		40		rate	30.078
Ba	20 ppb	41	48 hrs	12.131	14.516
Bb		37		16.902	±3.373
Aa	control	39	72 hrs	35.435	36.666
Ab		40		37.898	±1.741
Ba	20 ppb	41	72 hrs	24.765	25.471
Bb		37		26.177	±0.998
Aa	control	39	96 hrs	27.404	30.492
Ab		40		33.579	±4.366
Ba	20 ppb	41	96 hrs	20.206	21.946
Bb		37		23.686	±2.461

Table 39 Ingestion rate (*Artemia*.h^{-1}.$barnacle^{-1}$) of *E. modestus* from a natural population grown (at 21.8°C and 32 ppt) for 96 h in media containing 20 ppb copper, after having spent 60 h in media containing 10 ppb copper

After they had been in clean media for 24 h, copper-exposed barnacles showed ingestion rates equivalent to those in the 'control' animals and those rates persisted until the end of the additional week (see Table 40 and Figure 9).

A Two-way Analysis of Variance (homogeneous variance at Barttlet's statistic, X^2=3.806, p=0.283, df=3) confirmed that there were no significant differences with either time (F=116.26, p≤0.001) or treatment (F=0.28, p=0.624), and that

there was no significant interaction (F=0.14, p=0.726)

Treatment	Added copper	N	Time	Ingestion rate	Mean ingestion
Aa	control	39	24 hrs	18.984	19.452
Ab		40		19.920	±0.662
Ba	0 ppb	41	24 hrs	17.685	18.848
Bb		37		20.010	±1.644
Aa	control	39	168 hrs	25.943	26.402
Ab		40		26.861	±0.649
Ba	0 ppb	41	168 hrs	26.353	26.299
Bb		37		26.245	±0.076

Table 40 Ingestion rate (*Artemia*.h^{-1}.$barnacle^{-1}$) of *E. modestus* from a natural population grown (at 21.8°C and 32 ppt) for a week in clean media after having spent about a week in media containing copper

At the end of the 20-minute experimental period for test A, there were no significant differences in the number of open animals between the treatments, either amongst the different copper concentrations or between any of these and the 'controls' (X^2=5.278, p=0.259, df=4) (see Table 41).

Copper (ppb)	N	% Open	Mean % open
control a	20	95.00	
control b	22	95.45	95.15
control c	20	95.00	±0.25
5 a	20	100.00	
5 b	28	96.42	98.80
5 c	20	100.00	±2.06
10 a	20	90.00	
10 b	20	100.00	91.66
10 c	20	85.00	±7.63
15 a	20	95.00	
15 b	20	85.00	93.33
15 c	20	100.00	±7.63
20 a	20	95.00	
20 b	20	100.00	98.33
20 c	20	100.00	±2.88

Table 41 Test A: Opercular plate closure response in *E. modestus* from a natural population exposed to different copper concentrations for 20 min (at 21.1°C and 32 ppt)

In test B (21-hour exposure period) the number of open animals in 20 ppb copper was identical after 20 min to that in the 'control' group thus confirming the results of test A. Furthermore, 20 h later there were still no

significant differences in the number of open animals between the 'copper' and 'control' cultures (Fisher's exact test p=0.329). This absence of differences between cultures was maintained throughout the remaining exposure period and the number of open animals was the same at both 20 h 30 min, and 21 h (Fisher's exact test p=0.243 for both) (see Table 42).

In contrast to the above results, the type of cirral activity was found to differ between the copper-exposed and 'control' animals. The comparison was made on two of the five types of activity distinguished by Crisp & Southward (1961), viz. normal beat and pumping. Thus, the overall number of 'pumping' animals in 20 ppb copper was significantly higher than in the 'controls' (X^2=8.314, p=0.003, df=1) throughout the experiment. Furthermore, the relative distribution of pumping barnacles between cultures did not change over the 21 hour exposure period (X^2=1.277, p=0.734 , df=3) (see Table 42).

After 20 h, 2,500 *Artemia sp.* nauplii were introduced in the barnacle culture dish. Barnacle ingestion rates were measured over the succeeding 30 min to monitor the feeding behaviour of copper-exposed animals. Subsequently, and in anticipation of higher rates from hungry barnacles, a further 2,500 *Artemia* nauplii were added to each culture. A second ingestion rate was then measured over the next 30 min (see Winner *et al.*, 1977; Lang & Marcy, 1982).

Added copper (ppb)	Time	N	% Open	Movement B	Movement P	Type of activity	Mean [*Artemia*]
	20m	20	100.0	13	7	Beating	-
control	20h	20	90.0	13	5	Beating	2.50±1.00
	20h 30m	20	100.0	16	4	Fast beat	0.00
	21h	20	100.0	9	11	Fast beat	1.75±0.95
	20m	20	100.0	6	14	Fast beat	-
20	20h	20	85.0	6	11	Beating	2.50±0.50
	20h 30m	20	90.0	4	1	Fast beat	1.75±0.95
	21h	20	90.0	0	18	Pumping	4.00±0.81

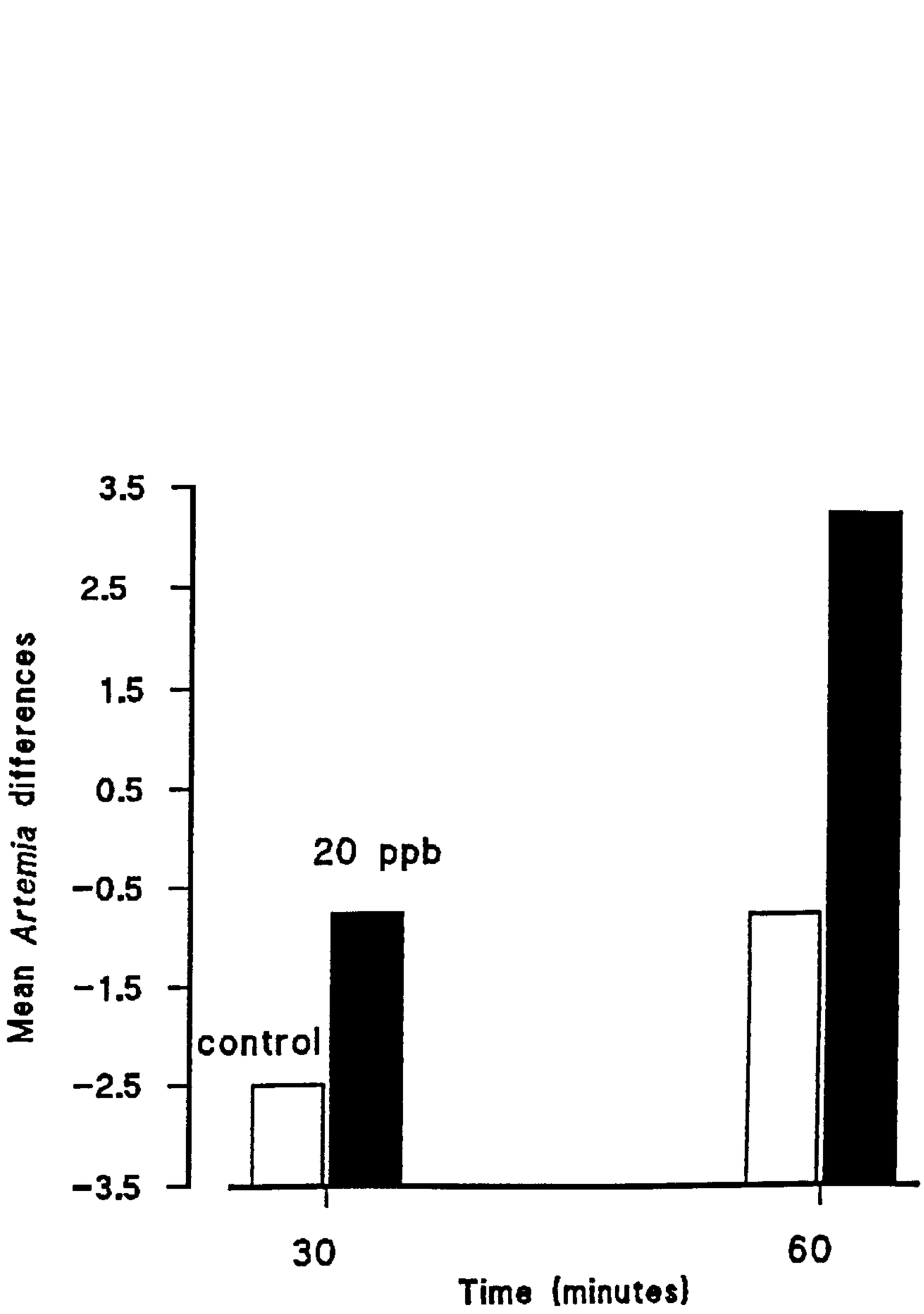
Table 42 Test B: Opercular plate closure response and cirral activity in *E. modestus* from a natural population exposed to 20 ppb copper (at 25°C and 32 ppt) for 21 h, B=no. of animals beating, P=idem pumping, m= minutes, h=hours, Mean [*Artemia*]=mean number of nauplii.ml^{-1} in the cultures, Beating=different animal

Figure 10 shows a plot of the differences in the mean numbers of nauplii present in the test medium 30 min after the *Artemia* additions. The bar graph indicates that 'control' barnacles ate more than the copper-exposed animals after the first feeding trial and that the difference was even more evident after the second 30-minute period, where copper-exposed barnacles were mostly pumping and ate hardly any *Artemia*. In addition, 'control' animals ate less than they had in the first food presentation, but were still generally beating at the end of the experimental time (see Table 42 and Figure 10).

The beat frequency per minute also varied with treatment, where fast beat (another of the five types of cirral activity described by Crisp & Southward 1961) was invariably observed in the 'control' animals when they were

Figure 10

Ingestion rate (*Artemia*.h^{-1}.barnacle^{-1}) of *Elminius modestus* from a natural population exposed to 20 ppb copper for 20 hours (at 25°C and 32 ppt), which was calculated from the differences in the number of *Artemia* nauplii measured twice over a 60-minute period in the barnacle culture media



presented with food. However, this type of activity was also exhibited by the copper-exposed barnacles from the beginning of the experimental period. Unfortunately, continuity was lost, since the animal chosen to monitor type and beat frequency in the copper culture did not open after the 20 h. The animal was then substituted by another of similar size (see Table 42).

Nevertheless, it appeared that the copper-exposed animals were beating at a higher rate than the 'controls'. However, the large variability in the results data could not be stabilised by transformation and consequently parametric statistics could not be employed. The non-parametric Kruskal-Wallis test was therefore used to compare beat frequencies between 'copper' and 'control' groups, showing the existence of significant differences with time ($H=25.14$, $p \leq 0.001$, $df=5$). Subsequently, Dunn's procedure for multiple comparisons (standard normal deviate at 95%, $Z=2.93$) determined that the introduction of food had increased the beating rate of 'control' barnacles (median difference=59 beats.min^{-1}). Copper-exposed barnacles, however, did not beat significantly faster in the presence of food (medians=62 beats.min^{-1} and 95 beats.min^{-1}) (see Table 43).

However, the number of observations at each time was low, so the power of Dunn's test to identify significant differences was low as well. Differences were therefore outlined by plotting the mean beat rates ± 95% confidence intervals against time. The plot showed that the chosen 'control' barnacle beat at a lower rate than the copper-

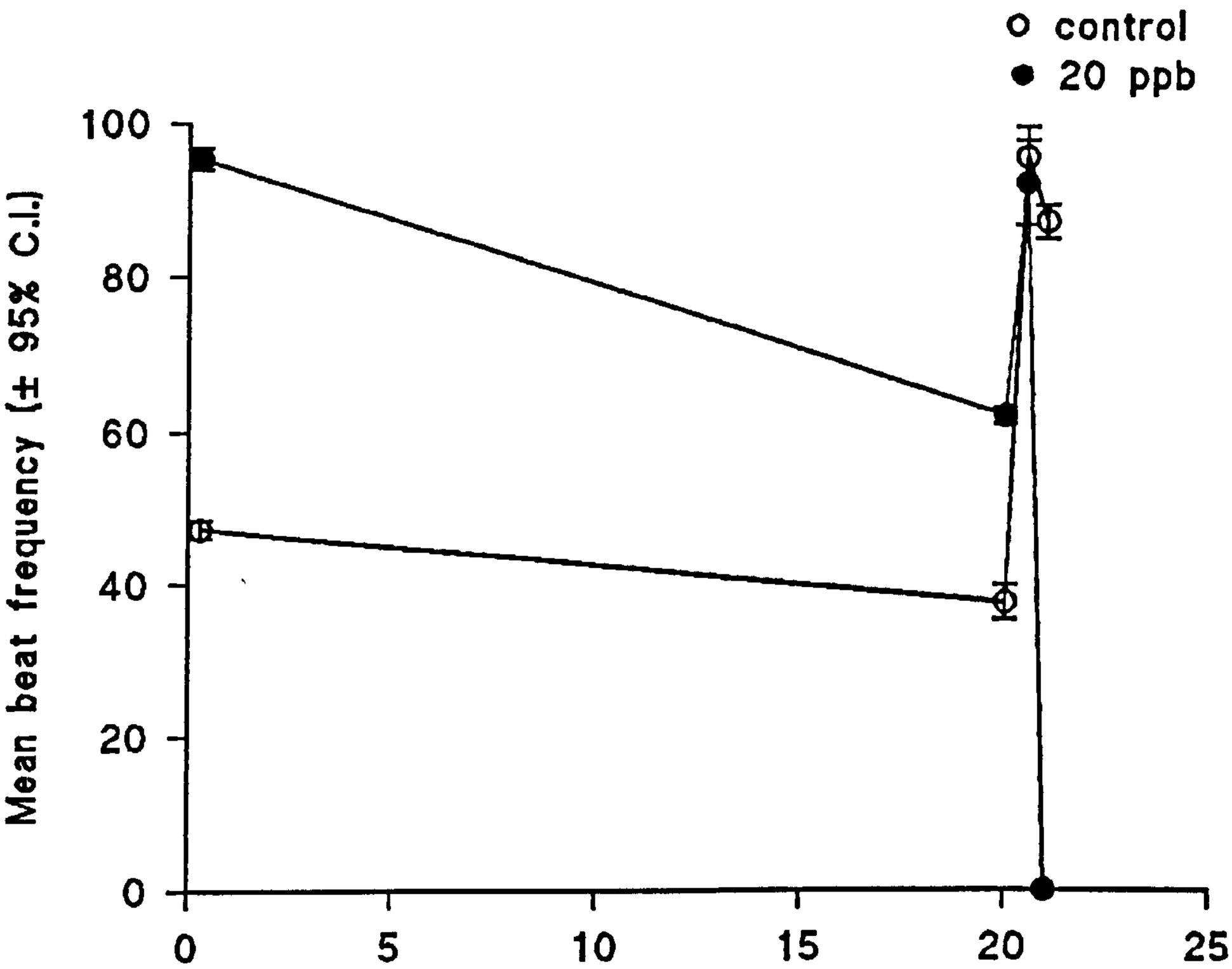
exposed animal throughout the exposure period, with the exception of the measurement taken at 20 h and 30 min, after the addition of food, where both animals beat at the same, fast rate (see Figure 11).

Level	N	Median	Mean Rank		Comparisons (Z) 1	2	3	4	5
1	5	47.00	8.0	2	0.89	-	-	-	-
2	5	38.00	3.0	3	2.87	*3.71	-	-	-
3	5	97.00	24.0	4	2.64	*3.53	0.23	-	-
4	5	95.00	22.7	5	0.89	1.79	1.97	1.74	-
5	5	62.00	13.0	6	2.56	*3.46	0.30	0.07	1.67
6	5	95.00	22.3						
total	30								

Table 43 Dunn's test performed on the median values for the beat frequency (beats.min^{-1}) of *E. modestus* from a natural population exposed to copper (at 25°C and 32 ppt) for 21 h. Levels: 1='control' after 20 m, 2='control' after 20 h, 3='control' after 20 h 30 m., 4=20 ppb after 20 m, 5=20 ppb after 20 h, 6=20 ppb after 20 h 30 m., minimum average rank difference for significance Z=2.93, *=significant differences

Figure 11

Mean beat frequency (no. beats.min^{-1}) ± 95% Confidence Interval in *Elminius modestus* from a natural population exposed to 20 ppb copper (at 25° C and 32 ppt) for 21 hours



DISCUSSION

Several parameters of the biology of *E. modestus* spat were affected when these were grown under copper exposure for time periods ranging from one month to a little over four months. Copper-exposed barnacles showed diminished survivorship, impaired growth and reproductive development, and reduced ingestion rates.

When 13-day-old *E. modestus* spat were exposed to copper (A=250 ppb, B=210/150 ppb) for approximately a month (26 days), the mortality was very high (over 50%), even when the final copper concentrations in the cultures had been reached by gradual addition. However, earlier studies with both *E. modestus* and *B. amphitrite* exposed to copper from spat to adulthood under very similar culture conditions to those in the present study, showed that *E. modestus* survived for up to 80 days at 1,000 ppb and for 23 days at 1,200 ppb (Royo-Gelabert & Yule, 1994). The main difference between those early studies and the present one is that those barnacles were fed on a mixed diet of the unicellular algae *R. reticulata* and *Artemia* nauplii, where the algal presence in the culture media reduced copper toxicity to the barnacles through adsorption to algal surfaces and complexation to algal exudates (see Steemann Nielsen & Wium-Andersen, 1970; Andrew *et al.*, 1977; Sunda & Guillard, 1976; Sanders *et al.*, 1983; Ahsanullah & Florence, 1984). Here, *E. modestus* spat

were fed only on *Artemia* nauplii and the comparatively high mortality observed possibly reflects the lack of algae in the culture media and consequently reduced copper complexation.

Reducing the copper concentration to 50 ppb (C*) meant that, after almost five months, the percentage mortality was reduced compared to the previous value, but was still five times greater than that of the 'control' culture (50 % versus 10%).

Copper also seemed to interfere with cirral activity and the feeding process. The former was reduced compared with that in 'control' barnacles for most of the testing time and must have led to a reduction in the ingestion rate. More *Artemia* were always left over, faecal pellet production was always low and the guts of copper-exposed barnacles were always emptier than those of the 'controls'.

Size differences between the copper-exposed and 'control' groups were noticeable after four days at 200 ppb (A and B) and became increasingly obvious until over half of the copper-exposed animals had died after 26 days of exposure. Barnacles at 50 ppb (C*) were also smaller throughout the four-month experimental period and their growth rate was one-third that of the 'controls'. A major factor in the growth difference must be connected with the inability of barnacles exposed to copper to ingest *Artemia* at an adequate rate.

Reproductive organ development also differed between the 'control' and copper-exposed barnacles, the number of animals

showing developed ovaries always being lower in the latter. In addition, copper-exposed animals never showed any egg masses. Barnacle egg size shows little variation (see Crisp, 1986, 1987) and it is the number of eggs, not their size or biochemical composition, that is reduced when reproductive energy is low (Walker, 1992). In the copper-exposed barnacles, energy from ingestion was reduced, leaving so little extra energy available that no eggs were produced. In contrast, 'control' barnacles did produce nauplii, since mature egg masses were evident, even if they later disappeared, presumably through hatching. The nauplii were never seen and must have been eaten by their parents.

'Control' barnacles appeared less fit after the fourth month of the experimental period. Similarly, Powell & White (1989) found marked reductions in both the level of cirral activity and the regularity of the activity rhythm, over time in the laboratory for both *B. (Semibalanus) balanoides* and *B.crenatus*. Southward & Crisp (1965) had also reported decreased activity in *B. nubilus* and *B perforatus* kept in the laboratory.

Copper at 10 ppb and 20 ppb reduced the ingestion rate in *E. modestus*, which was probably due to inhibition of the barnacle's feeding activity. The reduction, however, was neither complete (total) nor irreversible, since the animals did still eat while exposed to copper, and once transferred to clean media they showed ingestion rates identical to those of the 'control' animals.

At the end of the ingestion rate test periods (up to 96 h), both 'control' and copper-exposed barnacles tended to eat comparatively less than at the start, probably because they were satiated.

With regards to the mechanism whereby the ingestion rate was reduced in copper-exposed barnacles in relation to that in the 'controls', there were never any significant differences in the number of open animals between those in 'copper' (5-20 ppb) and 'control' treatments after 20 min exposure. Thus, in agreement with Powell & White's (1989) observations on the influence of copper and cadmium on the behaviour of *B. (Semibalanus) balanoides* and *B. crenatus*, sublethal copper levels did not induce opercular plate closure. Closure of the shell valves is typical of bivalve molluscs in the presence of lethal levels (e.g. 500 ppb) of toxic metals as a way of protecting their tissues (Davenport, 1977). Barnacles are also capable of isolating their bodies from the surrounding medium (Davenport, 1976a), but, according to Powell & White (1989), copper-exposed *B. (Semibalanus) balanoides* and *B. crenatus* did not close the opercular plates even when copper solutions of up to 500 ppb were pipetted directly onto them. In this study, not even an increase in the copper exposure period to 21 h provoked any increase in the frequency of opercular plate closure.

Both copper-exposed and uncontaminated open animals were always active, showing either normal beat, fast beat or pumping (see Crisp & Southward (1961). In contrast, Powell & White (1989), found significant differences in the mean

percentage activity of *B. (Semibalanus) balanoides* and *B. crenatus* exposed for 20 h to, what they established to be, sublethal copper concentrations of 80 ppb and 100 ppb.

Powell & White (1989) also observed changes in the type of cirral activity, in the form of increased pumping, but in only less than 20% of cases, when copper concentrations were used that they considered to be sublethal (80-100 ppb). In contrast, they found that in the case of lethal (500 ppb) copper levels, there was an increase in pumping activity with time. In the present study, sublethal copper levels of 20 ppb invariably gave rise to increased pumping activity (to the detriment of beating), whereas 'control' barnacles showed high levels of beating activity throughout the exposure period.

The beat frequency per minute also appeared to differ between copper-exposed (20 ppb) and 'control' barnacles. Fast beat (see Crisp & Southward, 1961) was observed in 'control' barnacles when they were presented with *Artemia* nauplii, which was most probably a food triggered reaction, since they had by the starved for over 24 h. Crisp & Southward (1961) suggested that the beating rate increased when food was added to a barnacle culture because the cirri had to be withdrawn more often to deal with the food captured. When barnacles exposed to copper were beating at all, it was always with a fast rhythm.

Although only one barnacle in each category was examined, individual variability in beat rate was evident

(see Table 12 in Appendix). In the absence of food the two animals exposed to copper had a higher beat rate than the one which was not exposed. Upon the introduction of food, however, the 'control' barnacle was capable of doubling its beat rate, while the barnacles exposed to copper showed no increase at all.

Powell & White (1989) found no real increase in fast beat relative to normal beat in *B. (Semibalanus) balanoides* exposed to sublethal copper levels. However, the majority of their barnacles which were exposed to lethal levels did show reductions in beat duration with time in copper-containing medium, but only after 6 to 26 h of increased beat frequency.

Differences in the threshold copper concentrations for altered cirral activity between Powell & White's (1989) and the present study were probably caused by differences in the experimental temperatures, 10±0.5°C and 25°C respectively, where the higher temperature would have increased copper toxicity to *E. modestus* relative to *B. (Semibalanus) balanoides* and *B. crenatus*.

Reduced ingestion rates in barnacles exposed to 20 ppb copper, which were observed throughout the current tests, resulted from changes in the type of activity from beating to pumping and an inability to increase beat frequency in the presence of food. As a consequence, barnacles which were exposed to copper for a period slightly exceeding four months ate relatively less and their growth rate consequently reduced or growth stopped altogether. Sufficient sustenance

for survival must have been obtained, perhaps through absorption of dissolved organic matter (see Baylor & Sutcliffe, 1963), unless the barnacles were able to survive the extended periods of starvation by drawing on the reserves accumulated in the three weeks prior to copper treatment.

Short-term copper exposure (21 h-1 month) effects on barnacle feeding were reversible. In contrast, long-term exposure proved fatal due to the prolonged inability to feed as required and only one animal recovered after approximately one month of copper exposure.

It may therefore be concluded that copper had a direct effect on the barnacle's ingestion mechanism by preventing normal feeding activity. Thus, long-term copper exposure effects on barnacles reduced their gross energy input irrespective of any energetic cost of coping with the copper stress. It follows that even short-term exposure to low levels of copper (particularly if periodic in nature) will have an impact on the energy input to individual barnacles, thus reducing the organisms' ability to grow and ultimately the fecundity of the population.

PLATES

Plate 2

Long-term copper exposure experiment on *Elminius modestus*, barnacles exposed to copper (left) and uncontaminated barnacles (right), view from below showing gonad development (top) and view from above showing size differences (bottom)

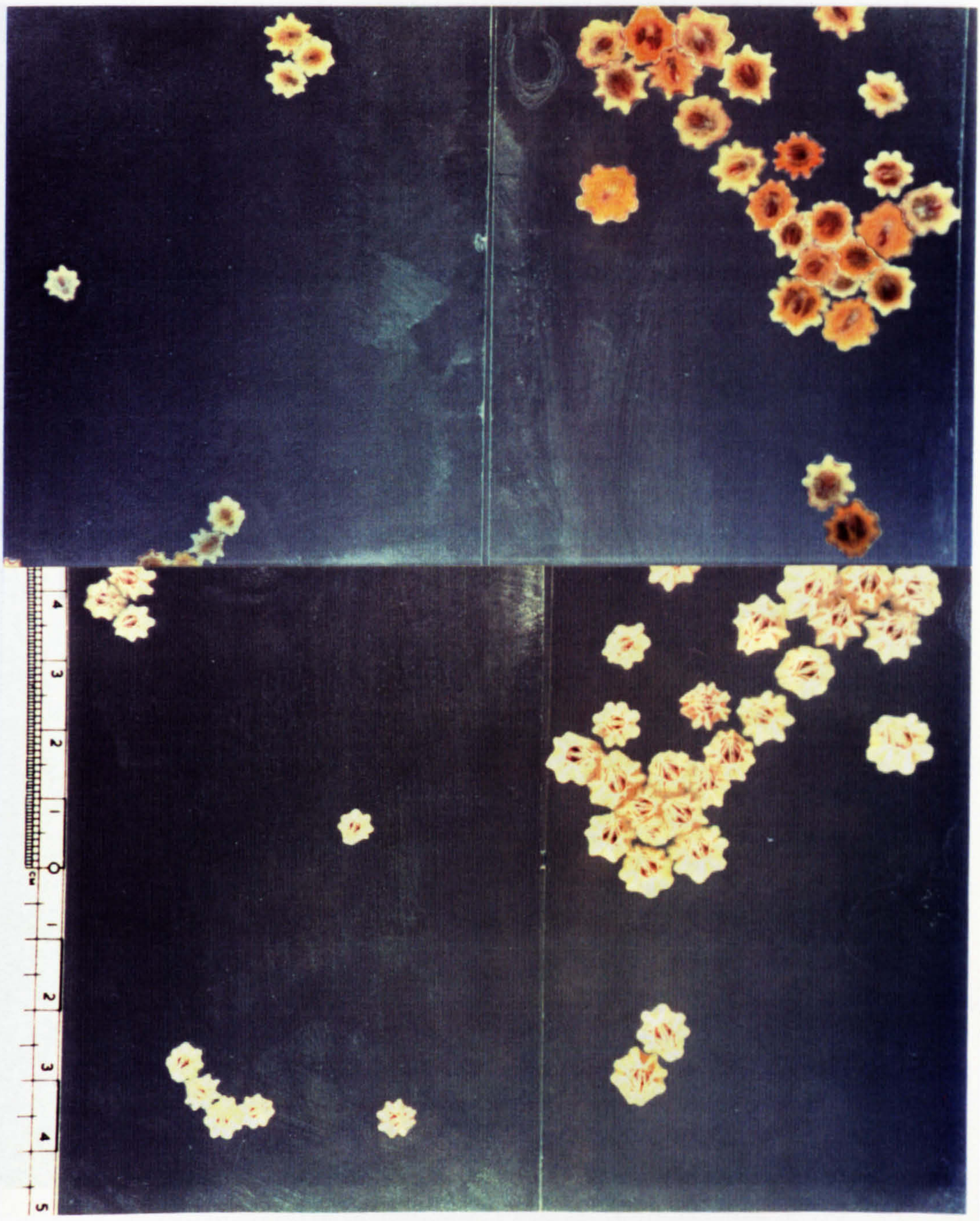


Plate 3
Long-term copper exposure experiment on *Elminius modestus*, barnacles exposed to copper (top) and uncontaminated barnacles (bottom), view from above showing size differences

Plate 4
Long-term copper exposure experiment on *Elminius modestus*, barnacles exposed to copper (top) and uncontaminated barnacles (bottom), view from below showing gonad development

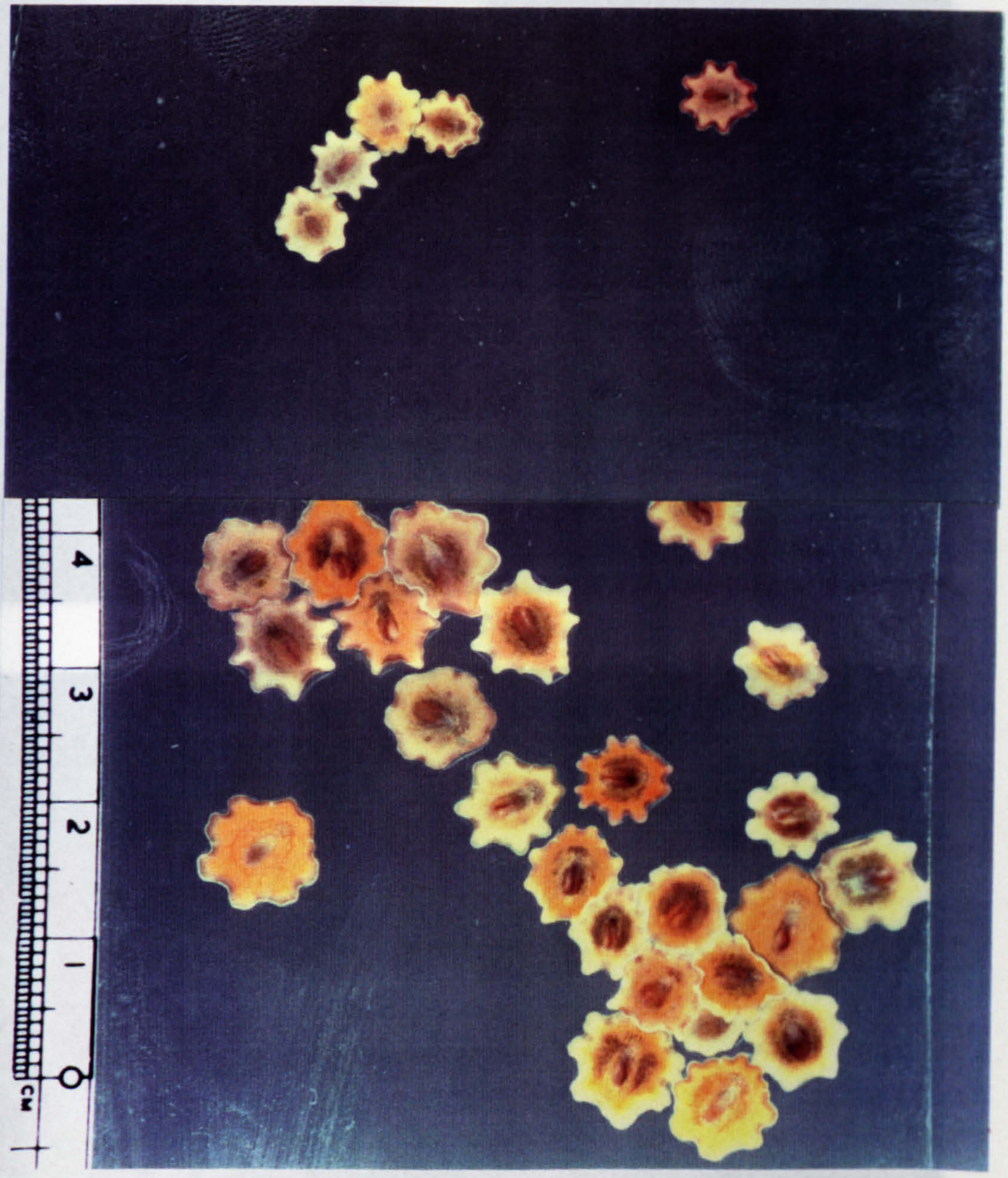


Plate 5

Long-term copper exposure experiment on *Elminius modestus*, barnacle exposed to copper (top) and uncontaminated barnacle (bottom), view from below showing differences in ovary development

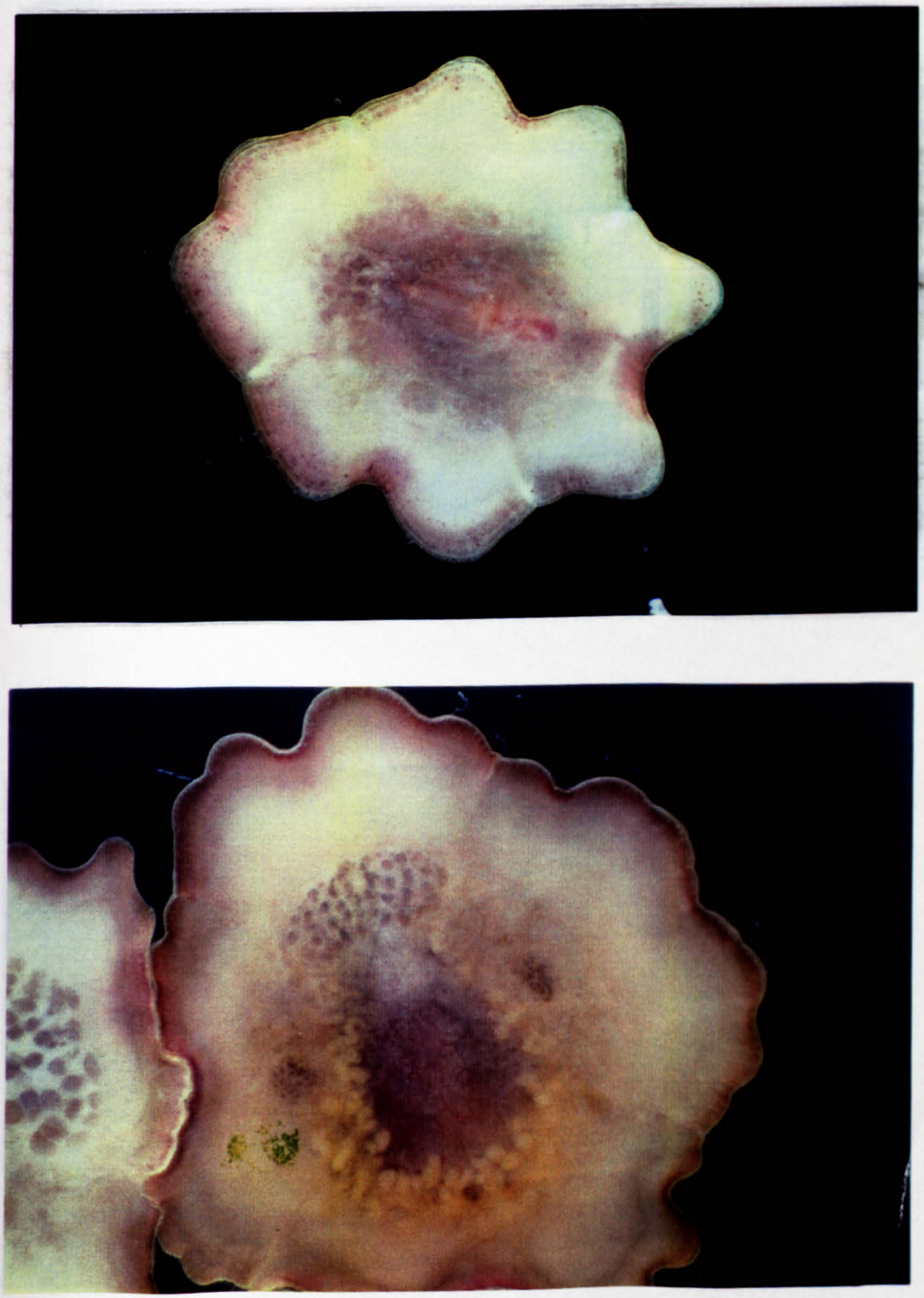
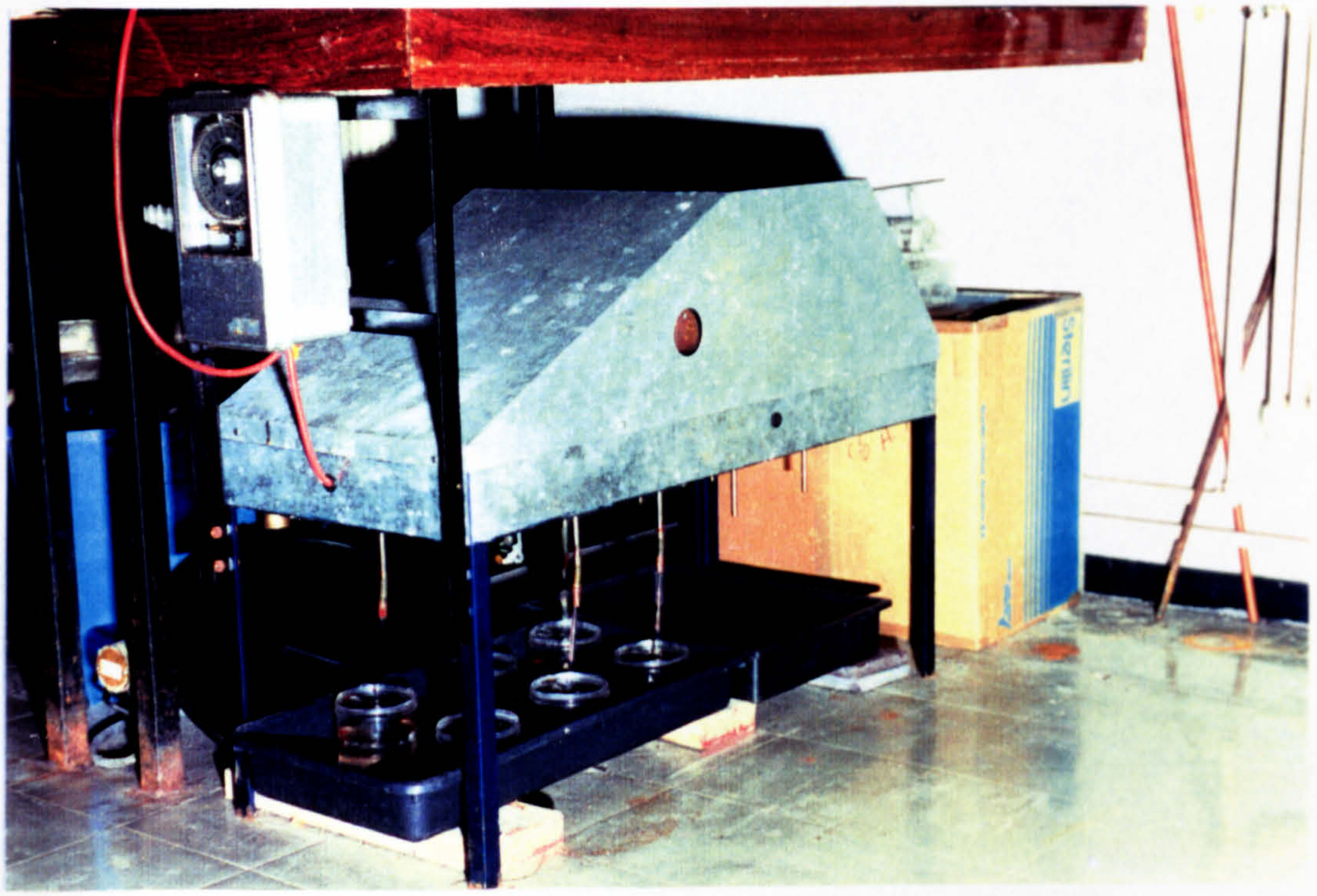
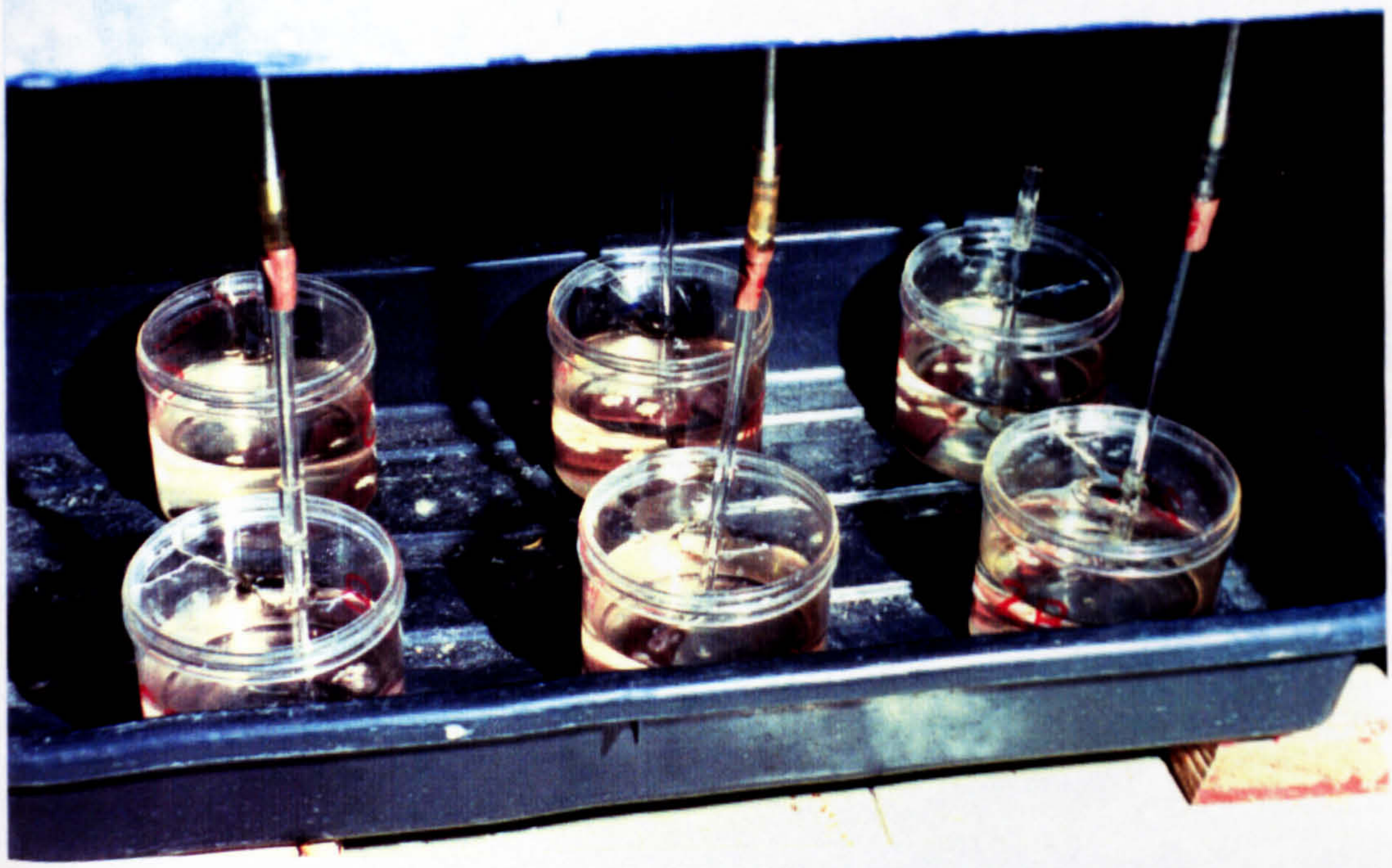


Plate 6
Impeller used to stir *Elminius modestus* culture media during assessment of ingestion rates





Source of variation	DF	SS	MS	F	P
Time	1	208.93	179.13	220.08	0.000
Treatment	1	130.65	4.89	6.01	0.015
TimexTreat	1	36.93	36.28	44.57	0.000
Error	148	120.46	0.81		
Total	151	496.31			

Table 1 Two-way Analysis of Variance, with time as a covariate, performed on the mean values (in mm) for the lateral width of *E. modestus* spat grown (at 18.8°C average temperature and 32 ppt) for a little over 4 months in media containing copper (C*, 50 ppb)

Source of variation	DF	SS	MS	F	P
Time	2	766.96	383.48	69.06	0.000
Treatment	1	43.83	43.83	7.89	0.031
TimexTreat	2	9.48	4.74	0.85	0.47
Error	6	33.32	5.55		
Total	11	853.59			

Table 2 Two-way Analysis of Variance performed on the mean values for the ingestion rate (*Artemia*.h^{-1}.barnacle^{-1}) of *E. modestus* from a natural population grown (at 21.8°C and 32 ppt) for 60 h in media containing 10 ppb copper

Source of variation	DF	SS	MS	F	P
Time	1	47.040	47.040	6.45	0.064
Treatment	1	194.844	194.844	26.73	0.007
TimexTreat	1	3.513	3.513	0.48	0.526
Error	4	29.154	7.289		
Total	7	274.550			

Table 3 Two-way Analysis of Variance performed on the mean values for the ingestion rate (*Artemia*.h^{-1}.barnacle^{-1})of *E. modestus* from a natural population grown (at 21.8°C and 32 ppt) for 96 h in media containing 20 ppb copper, after having spent 60 h in media containing 10 ppb copper

Source of variation	DF	SS	MS	F	P
Time	1	103.702	103.702	116.26	0.000
Treatment	1	0.250	0.250	0.28	0.624
TimexTreat	1	0.126	0.126	0.14	0.726
Error	4	3.568	0.892		
Total	7	107.646			

Table 4 Two-way Analysis of Variance performed on the mean values for the ingestion rate (*Artemia*.h^{-1}.barnacle^{-1})of *E. modestus* from a natural population, grown (at 21.8°C and 32 ppt) for a week in clean media after having spent about a week in media containing copper

Added copper (ppb)	N	Number open	Number closed
control a	20	19	1
control b	22	21	1
control c	20	19	1
5 a	20	20	0
5 b	28	27	1
5 c	20	20	0
10 a	20	18	2
10 b	20	20	0
10 c	20	17	3
15 a	20	19	1
15 b	20	17	3
15 c	20	20	0
20 a	20	19	1
20 b	20	20	0
20 c	20	20	0

Table 5 Test A: Opercular plate closure response in *E. modestus* from a natural population exposed to different copper concentrations for 20 min (at 21.1°C and 32 ppt)

Added copper (ppb)	Time	N	Number open	Number closed
control	20m	20	20	0
	20h	20	18	2
	20h 30m	20	20	0
	21h	20	20	0
20	20m	20	20	0
	20h	20	17	3
	20h 30m	20	18	2
	21h	20	18	2

Table 6 Test B: Opercular plate closure response in *E. modestus* from a natural population exposed to 20 ppb copper (at 25°C and 32 ppt) for 21 h, m= minutes, h=hours

Added [Cu] (ppb)					
	control	5	10	15	20	Total
Open	59	67	55	56	59	296
Closed	3	1	5	4	1	14
Total	62	68	60	60	60	310
X^2=5.278, p=0.259, df=4						

Table 7 Chi-square test of Test A: Opercular plate closure response in *E. modestus* from a natural population exposed to different copper concentrations for 20 min (at 21.1°C and 32 ppt)

Added [Cu] (ppb)			
	control	20	Total
Open	18	17	35
Closed	2	3	5
Total	20	20	40
p=0.329			

Table 8 Fisher's exact test of Test B: Opercular plate closure response in *E. modestus* from a natural population exposed to 20 ppb copper (at 25°C and 32 ppt) for 20 h

Added [Cu] (ppb)			
	control	20	Total
Open	20	18	38
Closed	0	2	2
Total	20	20	40
p=0.243			

Table 9 Fisher's exact test of Test B: Opercular plate closure response in *E. modestus* from a natural population exposed to 20 ppb copper (at 25°C and 32 ppt) for both 20 h and 30 min and 21 h

Added [Cu] (ppb)			
	control	20	Total
Pumping	27	57	84
Beating	78	73	151
Total	105	130	235
X^2=8.314, p=0.003, df=1			

Table 10 Chi-square test of Test B: Pumping activity in *E. modestus* from a natural population exposed to 20 ppb copper (at 25°C and 32 ppt) for 20 h

Added	Time				
[Cu] ppb	20 m	20 h	20 h 30 m	21 h	Total
control	7	5	4	11	27
20	14	11	14	18	57
Total	21	16	18	29	84
X^2=1.277, p=0.734, df=3					

Table 11 Chi-square test of Test B: Pumping activity in *E. modestus* from a natural population exposed to 20 ppb copper (at 21.1°C and 32 ppt) for 21 h, m=minutes, h=hours

Added copper (ppb)	Time	No beats.min^{-1} 1	2	3	4	5	Mean No beats.min^{-1}
control	20m	45	47	48	50	46	47.2±1.92
	20h	43	38	39	33	36	37.8±3.70
	20h 30m	87	92	102	97	100	95.6±6.10
	21h	82	85	90	90	89	87.2±3.56
20	20m	96	99	95	93	94	95.4±2.30
	20h	64	64	62	60	61	62.2±1.78
	20h 30m	100	85	81	100	95	92.2±8.75
	21h	-	-	-	-	-	-

Table 12 Test B: Beat frequency (beats.min^{-1}) in *E. modestus* from a natural population exposed to 20 ppb copper (at 25°C and 32 ppt) for 21 h, m=minutes, h=hours, in bold=different animal

CHAPTER SIX

EFFECTS OF LONG-TERM COPPER EXPOSURE ON A NATURAL POPULATION OF *Elminius modestus*

CONTENTS

Dulas Bay on the east coast of Anglesey, North Wales, UK, is a well known copper 'hot spot' (Foster & Hunt, 1975; Hunt, 1978; Foster *et al.*, 1978; Rutherford, 1991 unpublished). Copper and other transition metals reach the bay dissolved in the waters of the Afon Goch, which drains Parys Mountain, an old copper mine. Barnacles, *Elminius modestus* (Royo-Gelabert, 1993, per. obs.) and *Balanus (Semibalanus) balanoides* (Foster *et al.*, 1978) live on rocks and boulders both at the mouth of the bay and along the channel that leads from the bay to the sea (see Foster *et al.*, 1978).

Watson (1993, unpublished) sampled the *B. (Semibalanus) balanoides* population and found that individual sizes (rostro-carinal diameter) ranged from almost 6 millimetres to about 13 millimetres. The *E. modestus* population is sparse, consisting of large, presumably old, individuals (about 10 mm in rostro-carinal diameter) relatively isolated from each other and interspersed with very young ones, which are probably the result of the last settlement. The presence of barnacles within the bay results from their tolerance to high levels of metals, which is achieved by means of a detoxification mechanism involving the synthesis of granules, with a matrix made up of metals in a non-toxic, insoluble, inert form (Bernard & Lane, 1961; Walker *et al.*, 1975a,

1975b; Walker, 1977; Thomas & Ritz, 1986; Pullen & Rainbow, 1991) (see Chapter One: General Introduction).

This Chapter aims, firstly, to analyse copper levels both in Dulas Bay waters and in the Afon Goch, in order to estimate the copper input into the bay and to determine the range of copper concentrations available to the barnacles.

Secondly, to assess how the larvae produced by those adults respond to metal pollution. As the most sensitive stage of the barnacle life cycle, it is likely that the larvae hold the key to understanding the success of these organisms in such a contaminated environment. Dulas Bay larvae could be resistant to copper (one of the main metal loads in that environment) as a result of pre-exposure or acclimation to the metal during their development within the adult mantle cavity (see Hodson *et al.*, 1979; Mance, 1987). Such potential resistance is investigated by comparing the survivorship of Dulas Bay nauplii to that of nauplii which originated from a natural adult population living in the relatively unpolluted Menai Strait area, North Wales, UK (see Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994).

Such potential tolerance, however, could be genetic in origin, in which case the copper resistance of the adult barnacle population in Dulas Bay would be genetically controlled and their larvae would consequently also show it (see Bryan & Hummerstone, 1971). A further test is, therefore, necessary comparing the survivorship of nauplii from Dulas Bay to that of nauplii produced by a laboratory-acclimated population which had also originated from Dulas

Bay.

Finally, the Chapter evaluates whether, despite their survival, *E. modestus* from Dulas Bay had been affected by the prevailing copper contamination and general metal loads through alterations in opercular plate morphometry, as seen in an earlier study using barnacles from polluted and unpolluted locations on the west coast of Britain (see Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994).

SITE DESCRIPTION: DULAS BAY

Dulas Bay is an estuarine habitat unsuitable for many marine organisms because of the high concentrations of transition metals, namely copper, iron, zinc and manganese, which occur in both its water and sediments (Foster & Hunt, 1975; Hunt, 1978; Foster *et al.*, 1978; Rutherford, 1991 unpublished). In addition, metal loads in the bay water are mainly in solution (Foster & Hunt, 1975; Hunt, 1978; Foster *et al.*, 1978), which increases their environmental toxicity (see Steemann Nielsen & Wium-Andersen, 1970; Sunda & Guillard, 1976). Hunt (1978) measured the concentrations of dissolved metals at three different locations within the bay and quoted the following ranges: iron 40-200 ppb, copper 10-1,100 ppb, zinc 120-2,000 ppb and manganese 75-1,200 ppb, the concentration being dependent on both salinity and pH values.

Transition metal loads are supplied by the Afon Goch a stream that drains Parys Mountain, which was actively mined for copper until the late nineteenth century (Rowlands, 1966). Weathering leads to oxidation of the sulphide-containing mineral waste materials present in large amounts throughout the area giving rise to sulphuric acid. As a result, the river at its source has a pH of 2.5, which enables the dissolution and transport of high concentrations of the metals present in those minerals (Foster *et al.*, 1978). Despite the contribution of non-metalliferous tributary waters of higher pH (6.6-6.8), river water entering

the estuarine system is still enriched with zinc, copper, iron and manganese by at least two orders of magnitude above uncontaminated river water concentrations (Foster & Hunt, 1975; Foster *et al.*, 1978).

Dulas Bay is restricted at its seaward end by a narrow channel, with the result that rapid dispersion of the metalliferous waters on entry into the marine regime does not occur (Foster & Hunt, 1975). Chemical and physical interactions between the river and the sea are, therefore, confined to the bay, where metal loads are partially transferred from solution to solid phase and deposited into the sediments (Foster *et al.*, 1978). Deposited materials, however, are potentially mobile and so able to return to solution. Such solubilisation occurs first in the interstitial waters of the sediment (Foster & *et al.*, 1978; Rutherford, 1991 unpublished) and secondly, in the water column (Foster *et al.*, 1978).

As a result of the contamination, only organisms resilient to high levels of dissolved metals, especially copper and zinc, are able to live either in the bay or at its mouth. Thus, only seven species (Foster *et al.*, 1978; Royo-Gelabert pers. obs.) are present in significant numbers within the bay, all of which would have some detoxification mechanism preventing the free circulation of high concentrations of metals through their bodies.

Two species of algae, *Fucus vesiculosus* and *Ascophyllum nodosum*, occur at the mouth of the bay (Foster *et al.*, 1978).

Bryan (1969) first pointed out the linear relationship between metal concentrations in the water and those in macroalgae. Foster (1976) reported high concentrations of copper and zinc in both species of algae, which take up dissolved metals from the surrounding water and store them in a non-toxic form in their tissues. The green algae *Ulva sp.* also occurs within the bay on boulders and in the narrow channel that leads to the sea (Royo-Gelabert pers. obs.).

The polychaete *Nereis diversicolor* is able to live in the polluted sediments of the bay (Foster *et al.*, 1978) since it regulates dissolved and particulate copper and zinc. Copper is accumulated in an innocuous form in the epidermis and parts of the nephridia. The permeability of the cuticle to zinc is low and tissue concentrations are controlled by active zinc excretion (Bryan & Hummerstone, 1971; Phillips, 1980).

The amphipod *Corophium volutator* is present within the bay (Foster *et al.*, 1978) and regulates copper and zinc loads through both sequestration and excretion (Bryan, 1968).

Barnacles *E. modestus* (Royo-Gelabert pers. obs.) and *B. (Semibalanus) balanoides* (Foster *et al.*, 1978) live on rocks at both the mouth of the bay and along the channel. Walker *et al.* (1975a, 1975b) observed a correlation between zinc concentrations in body tissues of barnacles from Dulas Bay and those present in the surrounding water. This was the result of accumulation of the metal in the form of discrete inert granules in the tissues associated with the gut. In addition, Walker (1977) reported the presence of copper-

containing granules in the same tissues. Watson (1993, unpublished) measured increased levels of copper and zinc in the shell of *B. (Semibalanus) balanoides* from Dulas Bay, probably reflecting environmental concentrations.

MATERIALS AND METHODS

Copper levels in Dulas Bay waters were assessed through 2x 1 litre water samples taken from the bay at the site were barnacles were usually collected, i.e. the channel leading from the bay to the sea (see Walker, 1977a; Foster *et al.*, 1978). Sampling was carried out, in the winter of 1993, at ebbing tide, low water and flooding tide for neap tides, and at low water and high water for spring tides (neap and spring tides were separated by one month). Three additional 1 litre water samples were collected from the Afon Goch at the point were the river joins the bay at different times of day on three consecutive days. The salinity of the samples was recorded with a hand refractometer (American Optical Corporation) as soon as they were back in the laboratory, where they were left to sediment and acquire the ambient temperature (18-23°C) before being analysed for copper.

The samples were diluted either with FUVSW prior to copper extraction or with carbon tetrachloride prior to spectrophotometry (see Strickland & Parsons, 1972) (see Chapter Two: General Materials and Methods). The copper analysis was performed in accordance with a modified version of the method described by Strickland & Parsons (1972) (see Chapter Two).

Tolerance comparisons between *E. modestus* nauplii from Dulas Bay and those from Menai Bridge were based on their respective LD_{50}s (Lethal Doses for 50% of the population),

when both were exposed to an identical range of copper concentrations.

In the case of the Dulas Bay nauplii, the LD_{50} test was duplicated (DA and DB) and the replicates were carried out with an interval of two months (July and October 1993). For the Menai Bridge nauplii, the test was performed only once (MB) in October 1993, since Menai Bridge data had already been obtained in the previous year (October 1992) and could be used in the LD_{50} comparison. The range of copper concentrations tested was 100, 200, 300, 400 and 500 ppb, although the second replicate of the test run on Dulas Bay nauplii eliminated 500 ppb and included 250 ppb. After hatching, both groups of nauplii were maintained for 2 h and 30 min in FUVSW to guarantee the metamorphosis to stage II (see Harms, 1987) (see Chapter Three: Factors affecting copper toxicity to *Elminius modestus* nauplii), and then approximately 50 of them were used in the tests. The nauplii, which were not fed during the experiment, were cultured in polystyrene trays. The culture media were made up by serial dilution of a 500 ppb 1 litre initial solution, which was produced by diluting 0.5 ml of the stock solution (10^6 ppb) in FUVSW, the final salinity being approximately 32 ppt (see Chapter Two). Five replicate cultures were then distributed within the culture tray in a 5x5 Latin square (see Chapter Two). The tests lasted 48 h, after which the number of dead larvae was assessed using the criterion described in Chapter Three.

The nature of the tolerance observed in *E. modestus* nauplii from Dulas Bay was investigated by comparison of their copper-LD_{50} to that of nauplii produced by adults which had been collected from Dulas Bay in July 1993, four months prior to the test. These adults, which were attached to small rocks, had been slowly acclimated to 18°C, then maintained in the laboratory in a tank filled with FUVSW which was recirculated continuously by means of a pump, at a constant temperature of 18°C under 24-hour cycle artificial lighting. The barnacles were fed regularly on a diet of mixed unicellular algae and the nauplii of *Artemia sp.*. The tank and the animals were cleaned twice a month. Nauplii were obtained during the fourth month (November 1993) by 'natural' hatching (see Chapter Two). The test lasted 48 h, using 100, 200, 250, 300 and 400 ppb copper concentrations, and was performed in the usual manner per duplicate (LA and LB) in two consecutive weeks.

The effects of copper contamination on the morphometry of the opercular plates of the *E. modestus* population at Dulas Bay were evaluated on 50 adult specimens collected (in the winter of 1993) from approximately mid-tidal level. The barnacles were taken back to the laboratory where they were dried at 60°C for 24 h and then weighed. The opercular plates from all the specimens were then removed and cleaned in a strong sodium hydroxide solution for 24 h. Any remaining organic matter was later brushed off under the stereoscope.

The opercular opening of *E. modestus* is symmetrical and

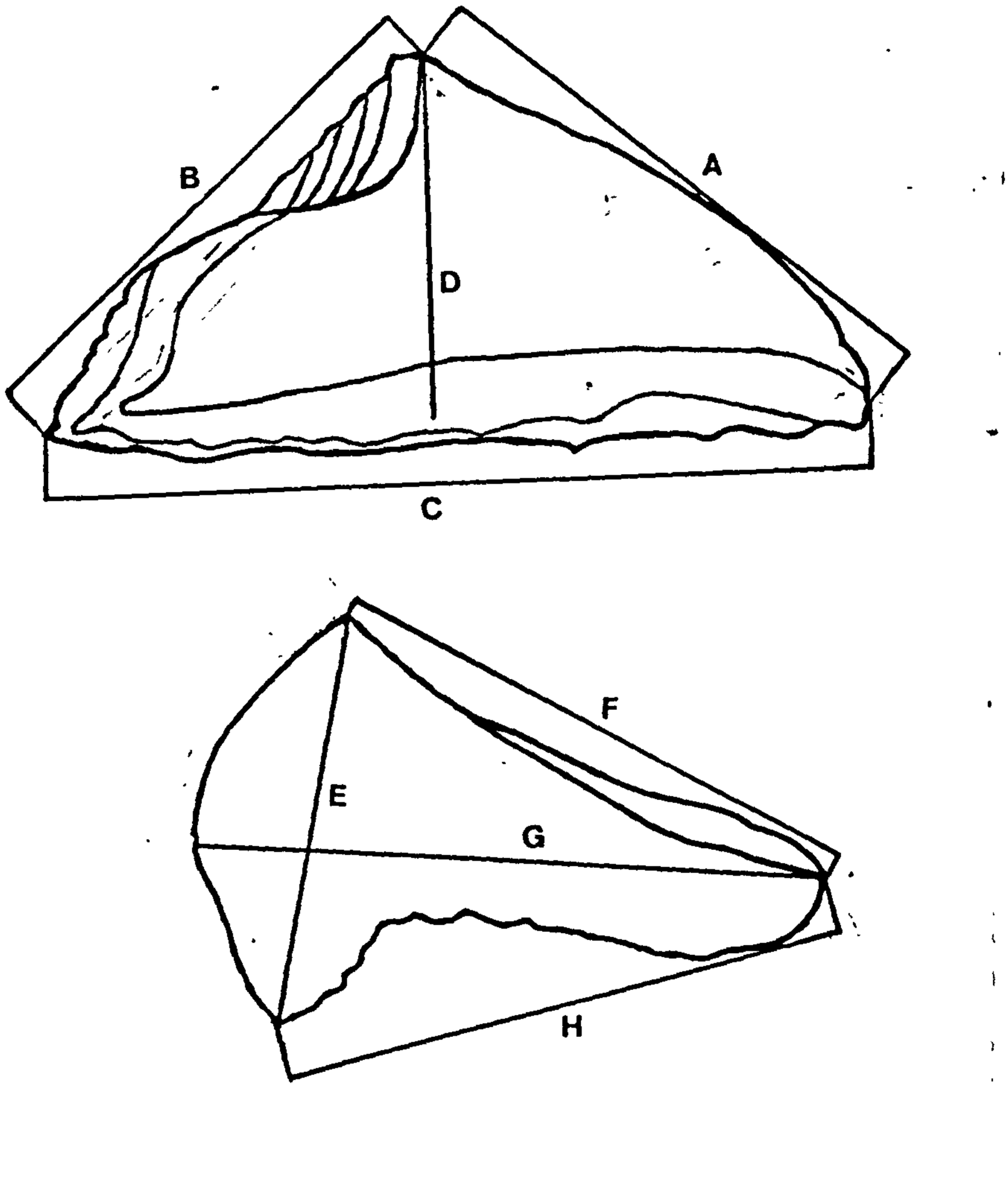
sealed by paired shell plates, the terga and the scuta. An earlier study, using barnacles from Menai Bridge, had confirmed the existence of such symmetry, thereby allowing a significant reduction in the time required for the analysis of the samples (see Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994). Accordingly, drawings were made of only the right side opercular plates and four dimensions, similar to those used by Furman (1989), were measured to the nearest 0.01 mm on each drawing using Vernier callipers (see Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994) (see Figure 12).

In that same study, no significant evidence of allometric growth was shown by the relationship between the rostro-carinal diameter and the dry weight of the barnacles (Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994). The effect of size was therefore eliminated from subsequent statistical analyses by dividing each dimension by the cube root of the dry weight of the animals.

The dimensions of the opercular plates of the Dulas Bay animals were then compared with those from the previous study (see Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994). The comparison was carried out using the dimensions of the right-side terga and scuta only, except where these were broken or missing, in which case the left-side valves were used without loss of precision.

Figure 12

Right–hand side scutum (top) and tergum (bottom) of *Elminius modestus* showing the eight dimensions measured



RESULTS

Copper levels in Dulas Bay waters

The results of the copper analysis showed that levels ranged from 1.75±0.07 ppb at high water in the case of spring tides, where the volume of water covering the animals was larger, to 56.50±6.36 ppb during the ebbing of the neap tide. For neap tides, copper concentrations throughout the tidal cycle were very similar and around 50 ppb, whereas for spring tides the differences between low and high water values were considerable (see Table 44).

Neap tide						Spring tide					
Water	S	ET	LW	FT	Mean	Water	S	HW	LW		Mean
Aa	6.5	-	52.5	-	53.25	Da	32.0	1.8	-		1.75
Ab	6.5	-	54.0	-	±1.06	Db	32.0	1.7	-		±0.07
Ba	30.0	52.0	-	-	56.50	Ea	25.0	-	45.0		46.00
Bb	30.0	61.0	-	-	±6.36	Eb	25.0	-	47.0		±1.41
Ca	31.9	-	-	48.0	53.75	-	-	-	-		-
Cb	31.9	-	-	59.5	±8.13	-	-	-	-		-
Ra	2.0		560			Rd	6.0		940		
Rb	0.0		1,480								
Rc	0.0		4,100								

Table 44 Copper levels (ppb) in water samples from Dulas Bay (A-E) and Afon Goch (R) at different tide stages for both neap and spring tides (ET=ebbing tide; LW=low water; FT=flooding tide; HW=high water; S=salinity in ppt), a-d= replicates

The constancy of the copper values for neap tides is somehow surprising since, as stated by Foster *et al.* (1978), the rates of the various chemical and physical interactions

which combine to decrease the dissolved metal loads within Dulas Bay vary with time, both over a tidal cycle and from day to day due to varying contributions from both fresh and seawater sources. Nevertheless, 8 out of 10 water samples here had copper concentrations of approximately 50 ppb and it would not be unreasonable to use this value as a 'working figure' when referring to the level of copper to which the barnacles were exposed.

Copper concentrations in samples taken from the river mouth ranged from 560 ppb (showing some marine influence at 2 ppt) to 4,100 ppb (showing no marine influence at 0 ppt) (see Table 44). However, high-copper-content river samples Ra (560 ppb) and Rb (1,480 ppb) were not diluted with FUVSW prior copper extraction, but with carbon tetrachloride prior to spectrophotometry of the extracts. This probably led to underestimation of their actual copper content, since the sodium diethyldithiocarbamate might have not been able to complex all the available copper (see Strickland & Parsons, 1972) (see Chapter Two).

Samples Rc (4,100 ppb) and Rd (940 ppb) were both diluted with FUVSW prior to copper extraction and showed no marine influence (0 ppt), reflecting more adequately the copper input of the river into the bay. Differences between the copper content of those samples were due either to the time period separating them (1 month), or the fact that the former was taken during neap and the latter during spring tides.

Naupliar copper-LD_{50} tolerance tests

The percentage mortality of nauplii from Dulas Bay, and for both replicates (DA, DB), increased with copper concentration ranging from 1.05±0.59% (DB) to 3.00±3.92 (DA) at 100 ppb, and reaching 90.55±4.34% at 400 ppb (DB), and 100% at 500 ppb (DA). For the Menai Bridge nauplii, the percentage mortality also increased with copper concentration, ranging from 6.01±2.05% at 100 ppb, to 100% at 400 ppb (see Table 45).

Nevertheless, the percentages for Menai Bridge nauplii were considerably higher than those for Dulas Bay nauplii, which is clear from the concentration that caused 100% mortality, viz. 500 ppb in the previous case, whereas here it was 400 ppb.

A Latin Square Analysis of Variance of the percentage mortality of each of the 5 replicates of the 5 copper concentrations used in each case was carried out in order to check for systematic errors due to the positions of the test chambers. The results of the test showed that in only one case was there evidence of a slight effect attributable to the culture tray row, and this was of minor importance to the calculations of the LD_{50}s (DA, column $F=1.62$, $p=0.233$; row $F=3.54$, $p=0.039$), (DB, column $F=0.24$, $p=0.911$; row $F=3.09$, $p=0.058$), (MB, column $F=0.52$, $p=0.720$; row $F=0.50$, $p=0.739$).

Added copper (ppb)	N DA	N DB	N MB	% Mortality DA	% Mortality DB	% Mortality MB	% Mortality mean DA	% Mortality mean DB	% Mortality mean MB
100 a	67	68	45	0.00	1.47	6.68			
100 b	34	83	67	0.00	1.20	7.46			
100 c	53	75	61	9.43	1.33	3.27	3.00	1.05	6.01
100 d	57	70	49	1.75	0.00	8.16	±3.92	±0.59	±2.05
100 e	52	79	46	3.84	1.26	4.54			
200 a	51	77	63	13.72	22.07	34.92			
200 b	68	75	69	30.88	18.66	46.37			
200 c	40	71	57	42.50	19.71	56.14	24.04	20.17	46.00
200 d	44	77	84	15.90	14.28	47.61	±12.31	±4.37	±7.57
200 e	58	65	60	17.24	26.15	45.00			
250 a	-	74	-	-	37.83	-			
250 b	-	83	-	-	34.93	-			
250 c	-	63	-	-	41.26	-	-	39.86	-
250 d	-	60	-	-	41.66	-		±3.45	
250 e	-	55	-	-	43.63	-			
300 a	66	82	52	84.84	63.41	98.07			
300 b	68	61	43	94.11	65.57	93.02			
300 c	56	52	43	98.21	75.00	95.34	88.29	68.38	95.78
300 d	49	78	48	81.63	67.94	97.91	±7.41	±4.45	±2.17
300 e	79	80	37	82.27	70.00	94.59			
400 a	59	57	45	98.30	85.96	100			
400 b	63	53	36	98.41	87.71	100			
400 c	53	62	49	98.11	91.93	100	98.45	90.55	100
400 d	46	59	52	100	89.83	100	±0.94	±4.34	
400 e	39	71	51	97.43	97.18	100			

Table 45 Survivorship of *E. modestus* nauplii (stage II) from natural adult populations at Dulas Bay (DA, DB) and Menai Bridge (MB), exposed for 48 h (at 22.5°C and 32 ppt) to different copper concentrations; a-e=positions in the culture tray

The LD_{50}s were then calculated according to the method of Finney (1971) and the values for *E. modestus* nauplii (stage II) produced by Dulas Bay adults exposed to copper for 48 h at 22.5°C and 32 ppt were 217 ppb (DA) and 257 ppb (DB). In contrast, the value for the Menai Bridge nauplii was 190 ppb (see Table 46).

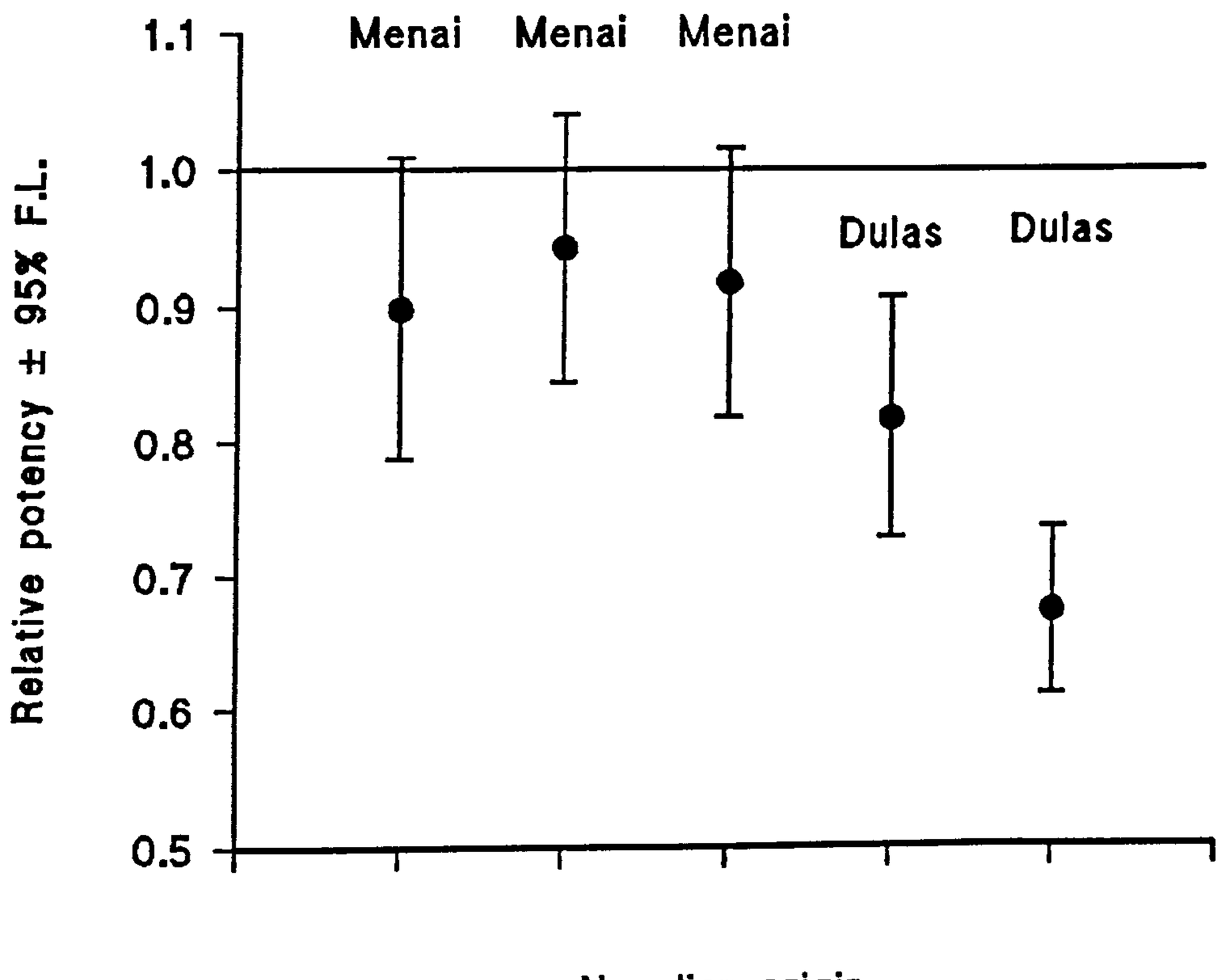
	Replicate	LD_{50} (ppb)	C.I.(95%)
Menai Bridge nauplii (October 92)	A	172	165-181
	B	195	187-202
	C	185	177-192
Dulas Bay nauplii (July-October 93)	DA	217	210-224
	DB	257	251-263
Menai Bridge nauplii (October 93)	MB	190	184-197

Table 46 LD_{50}S (lethal doses for 50% of the population) and 95% confidence intervals (C.I.), determined from the naupliar mortality percentages for *E. modestus* nauplii (stage II) from either Menai Bridge or Dulas Bay, exposed for 48 h to 100-500 ppb copper (at 22.5°C and 32 ppt)

The LD_{50}s were then compared, for which purpose those obtained previously from Menai Bridge (October 1992, see Chapter Three) were also used. The comparison was performed using the method of Relative Potencies described in Finney (1971), taking the LD_{50} for Menai Bridge nauplii of 172 ppb obtained in the previous year as the 'reference' LD_{50} (see Finney, 1971) (see Chapter Two).

In this case there were no significant differences between the relative potencies of any of the LD_{50}s for Menai Bridge nauplii, regardless of the year, demonstrating an absence of time-linked variability, whereas the LD_{50}s for Dulas Bay nauplii were significantly higher (i.e. the relative potencies were lower) (see Table 46 and Figure 13).

Figure 13
Relative potencies and 95% Fiducial Limits of the LD_{50}s of *Elminius modestus* nauplii (stage II), from natural populations at either Menai Bridge (Menai) or Dulas Bay (Dulas), exposed to 100–500 ppb copper for 48 hours (at 22.5°C and 32 ppt)



Naupliar copper-LD_{50} acclimation tests

The percentage mortality of nauplii from a laboratory-acclimated population originally from Dulas Bay, and for both replicates (LA and LB), increased with copper concentration ranging from 1.31±1.86% (LB) to 1.82±1.64% (LA) at 100 ppb, and reaching 63.31±8.87% (LB) and 77.66±0.62% (LA) at 400 ppb. These percentages were similar, but lower than those obtained using nauplii from Dulas Bay adults which had not been kept in the laboratory (see Table 47).

Prior to the calculation of the LD_{50}s, a Latin Square Analysis of Variance of the percentage mortality of each of the 5 replicates of the 5 copper concentrations tested was performed in order to check for systematic errors due to the positions of the test chambers. The test showed that, for each concentration, there were no significant differences between cells (LA, column $F=0.57$, $p=0.690$; row $F=0.75$, $p=0.577$), (LB, column $F=0.80$, $p=0.549$; row $F=2.44$, $p=0.103$).

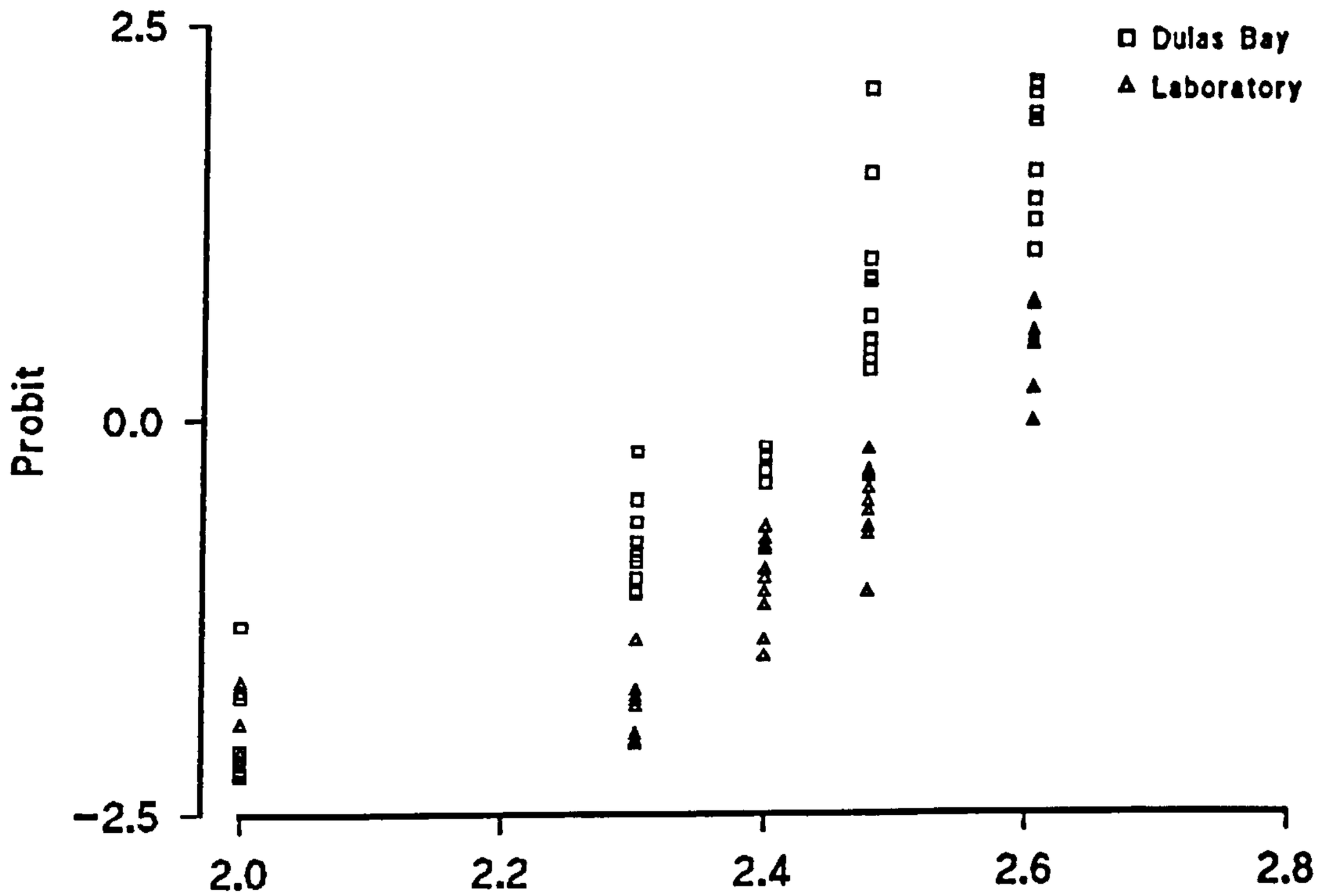
The LD_{50}s were then calculated according to Finney's (1971). Stage II nauplii of *E. modestus* barnacles from Dulas Bay, which were maintained in a clean medium for 4 months and then exposed to copper for 48 h at 22.5°C and 32 ppt, had LD_{50}s of 349 ppb (LA) and 383 ppb (LB). In addition, a combined LD_{50} was calculated for both natural and laboratory-maintained populations by combining the naupliar mortalities of the two replicates for each test (see Table 48).

Added copper (ppb)	N LA	N LB	% Mortality LA	% Mortality LB	% Mortality mean LA	% Mortality mean LB
100 a	69	37	1.44	0.00		
100 b	64	22	0.00	0.00		
100 c	67	39	1.49	2.56	1.82	1.31
100 d	66	24	4.50	0.00	±1.64	±1.86
100 e	59	25	1.69	4.00		
200 a	48	34	4.16	0.00		
200 b	52	41	1.92	0.00		
200 c	45	26	2.22	3.84	2.72	3.08
200 d	50	25	2.00	8.00	±0.98	±3.31
200 e	60	28	3.33	3.57		
250 a	75	37	13.79	8.10		
250 b	58	25	11.72	12.00		
250 c	58	29	15.87	20.68	14.13	16.01
250 d	63	28	6.66	21.42	±5.85	±5.76
250 e	53	28	22.64	17.85		
300 a	80	36	37.50	13.88		
300 b	66	34	30.30	23.52		
300 c	47	30	36.17	30.00	36.77	24.12
300 d	56	39	42.85	28.20	±4.46	±6.26
300 e	54	32	37.03	25.00		
400 a	45	48	77.77	58.33		
400 b	42	26	78.57	69.23		
400 c	22	28	77.27	50.00	77.66	63.31
400 d	39	37	76.92	70.27	±0.62	±8.87
400 e	36	32	77.77	68.75		

Table 47 Survivorship of *E. modestus* nauplii (stage II) from an adult population, originally from Dulas Bay and subsequently maintained in clean medium for 4 months, exposed for 48 h (at 22.5°C and 32 ppt) to different copper concentrations; a-e=positions in the culture tray, LA and LB= replicates carried out in 2 consecutive weeks

The combined LD_{50}s were compared through their Relative Potencies (see Finney, 1971), but omitting those naupliar mortality percentages at 100 ppb copper, since these valucs did not fit the linear trend in probits versus log concentration shown at higher copper levels (see Figurc 14).

Figure 14
Probit versus logarithm concentration for the combined mortalities (2 replicates) of *Elminius modestus* nauplii (stage II), from populations at Dulas Bay, or originally from Dulas Bay but kept in the laboratory for 4 months (Laboratory), exposed for 48 hours to 100-400/500 ppb copper (at 22.5°C and 32 ppt)



	Replicate	LD_{50} (ppb)	C.I.(95%)
Dulas Bay nauplii, adults kept in the laboratory for 4 months	LA	349	336-365
	LB	383	361-421
Combined	LA-LB	340	330-352
Combined	DA-DB	251	244-256

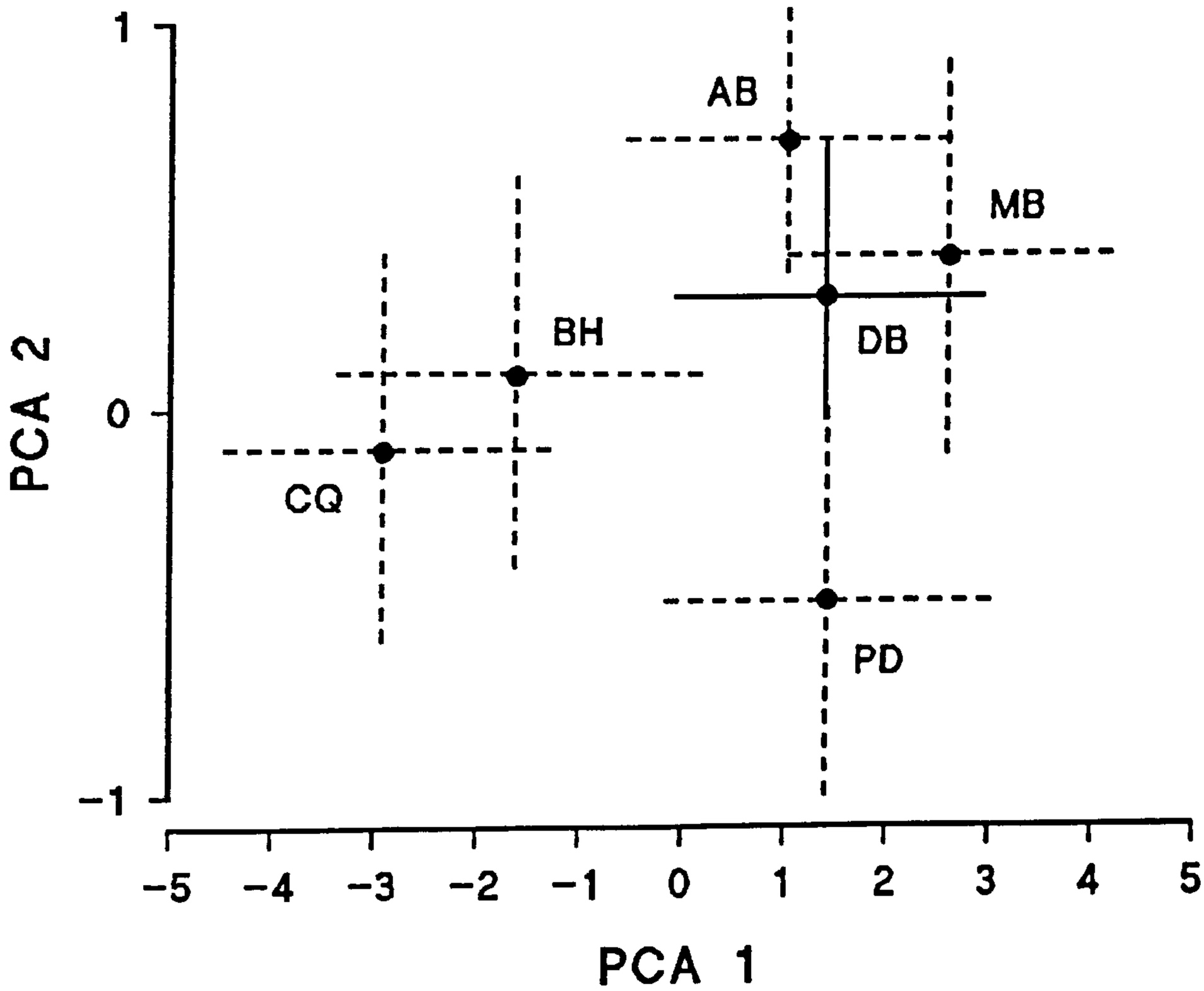
Table 48 LD_{50}S (lethal doses for 50% of the population) and 95% confidence intervals (C.I.), determined from the naupliar mortality percentages for *E. modestus* nauplii (stage II) from Dulas Bay (DA, DB), or originally from Dulas Bay but kept in the laboratory for 4 months prior to use (LA, LB), exposed for 48 h to 100-400/500 ppb copper (at 22.5°C and 32 ppt); Combined=value obtained by pooling the data for both replicates

Clearly, the major effect of copper on larval survival occurred for both sets of nauplii at concentrations in excess of 100 ppb. Mortalities at 100 ppb (and less) would equate to natural mortality as would be expected in the absence of added copper. The test showed that there were significant differences between the relative potencies of the LD_{50}s obtained here and those in the test run on nauplii from Dulas Bay adults which had not spend time in the laboratory.

Effects of copper on barnacle opercular plate morphometry

The first two principal components found by Royo-Gelabert (1991 unpublished) and Royo-Gelabert & Yule (1994) were used to assign Principal Component Analysis (PCA) scores to the Dulas Bay barnacles based on the normalised dimensions for terga and scuta. Figure 15 shows a plot of the mean scores ± stdev on PCA 1 and PCA 2 for the original data of Royo-Gelabert (1991 unpublished) and Royo-Gelabert & Yule (1994) with the present data for Dulas Bay included. The mean value for Dulas Bay on PCA 1 (the axis most closely related to contamination, see Royo-Gelabert & Yule, 1994) is very close to that of Aberystwyth and Pembroke Dock. On the PCA 2, Dulas animals are more similar to Menai Bridge and Aberystwyth barnacles than those from Pembroke Dock.

Figure 15
Principal Component Analysis (PCA) comparing the dimensions of the opercular plates of *Elminius modestus* from Dulas Bay with those of other populations living along a 200 mile range on the west coast of Britain, CQ=Connah's Quay, BH=Birkenhead, AB=Aberystwyth, MB= Menai Bridge, DB=Dulas Bay, PD=Pembroke Dock. Mean PCA scores ± stdev on each of the 1st and 2nd principal components identified by Royo-Gelabert & Yule (1994) are given, trace line= reference study, full line= present study



The water survey carried out in the current study demonstrated that 16 years after the study by Foster *et al.* (1978) the Afon Goch was still supplying copper to Dulas Bay and that *E. modestus* living there could be exposed to approximately 50 ppb.

Copper toxicity tests carried out, using identical methods, on the nauplii of *E. modestus* from two different natural populations at Dulas Bay and Menai Bridge showed differences in mortality. Naupliar origin as a parameter influencing the outcome of toxicity tests has already been analysed in detail earlier in this study (see Chapter Three: Factors affecting copper toxicity to *Elminius modestus* nauplii). In this case, however, the populations differed mainly in terms of pollution of the surrounding medium. Nauplii produced by the population at Menai Bridge, a relatively unpolluted location (see Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994) where copper concentrations are of the order of 1 ppb (see Chapter Three). were not as copper tolerant as the ones produced by the population from Dulas Bay, a highly copper contaminated site, which showed comparatively higher LD_{50}s.

It appears that copper tolerance was inherent in the larvae from Dulas Bay adults and could either be genetic or possibly a physiological adaptation to the prevailing copper

loads.

Assuming that *E. modestus* stored copper in granules, as is the case with *B. (Semibalanus) balanoides* (see Walker, 1977), then animals left in seawater with no added copper for four months could be considered to be fairly free of copper stress (see White & Walker, 1981; AlThaqafi & White, 1991). Thus, according to Walker & Foster (1979) after 3 months in 'clean' water, half of the total copper load of *B. (Semibalanus) balanoides*, originally from Dulas Bay, had been lost; and increasing the time to 6 months did not significantly increase the amount of lost copper (Walker & Foster, 1979). Copper levels in FUVSW are below 1 ppb (see Chapter Three) and would not have imposed any copper stress on the *E. modestus* barnacles. In addition, the 4 months would have guaranteed that neither the ovary nor the eggs of these barnacles would have been affected by copper, since the larvae used in the tests would have been fertilised from eggs produced by ovaries developed within one-two months of hatching.

Nauplii produced by the above adults would only show tolerance to copper if such a characteristic had been inherited from their parents. Failure to resist copper levels above those tolerated by Menai Bridge nauplii (with roughly 1 ppb environmental copper) would mean that tolerance was simply a result of developmental acclimation in a copper-contaminated environment.

Nauplii produced by Dulas Bay laboratory-acclimated *E. modestus* adults were in fact copper tolerant, since their

modestus adults were in fact copper tolerant, since their LD_{50}s were even higher than those for natural Dulas Bay nauplii. This therefore indicated that copper tolerances shown by these Dulas Bay nauplii were genetically controlled, and were not the expression of a differential egg mortality. Bryan & Hummerstone (1971) observed an equivalent phenomenon when studying the copper adaptation of the polychaete *Nereis diversicolor* from a range of estuaries in the South West of England; these included Restronguet Creek, a highly metal contaminated site, which had been receiving mining wastes for 200 years. Worms from that location were copper tolerant, probably as a result of a genetically controlled difference between resistant and non-resistant populations, since transfer of such high-copper animals to low-copper content sediments belonging to the 'cleanest' estuary did not affect their LT_{50}. Similarly, the barnacle population at Dulas Bay would have a copper resistant genotype in order to guarantee its success in such a contaminated environment.

The morphology of barnacle opercular plates has been shown to be dependent on certain environmental parameters such as temperature (Barnes & Healy, 1965, 1969, 1971) and salinity (Furman 1989, 1990). In addition, it can be influenced by pollution, which was first suggested by Furman (1989) when studying morphometric characters of *B. improvisus* from Gdynia, a highly polluted location in the Baltic sea. The variations in *E. modestus* opercular plate dimensions over a 200 mile range along the west coast of Britain were

positively correlated with potential pollution (see Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994). Furthermore, a link was established between copper concentration and the morphology of *E. modestus* opercular plates in laboratory reared populations (Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994).

Dulas Bay is within the 200 mile range mentioned above, so, its average annual temperature should be comparable to that of those sites. Its salinity regime, however, should only be comparable to that of Aberystwyth, Birkenhead and Connah's Quay. Furman (1989, 1990) showed that *B. improvisus* from low salinity locations tended to have smaller opercular plates, but this was not the case for *E. modestus* specimens from Birkenhead and Connah's Quay (see Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994). Salinity differences are therefore unlikely to be directly implicated in the variation in plate dimensions seen here, which resulted, most likely, from the high metal levels at Dulas Bay.

Birkenhead and Connah's Quay waters are generally considered polluted as they receive effluents from the heavily industrialised Merseyside and Wirral areas. As an example, metal concentration in water samples from those sites were indicative of high anthropogenic input in the form of copper, lead and mercury when compared with other sites such as Menai Bridge (Royo-Gelabert, 1991 unpublished; Royo-Gelabert & Yule, 1994).

Dulas Bay is contaminated with metals, to such an extent

that only seven species are present in significant quantitics within the bay (Foster *et al.*, 1978; Royo-Gelabert, 1993, pers. obs.), two of these being barnacles viz. *B balanoides* and *E. modestus*. Copper levels in the bay ranged from 1.78 ppb to 47 ppb at high water during spring tides to 48 ppb to 61 ppb during the ebbing in neap tides. The other metals present in high concentrations in Dulas Bay are iron, manganese and zinc (Foster *et al.*, 1978).

In contrast with Birkenhead and Connah's Quay, however, Dulas Bay has a single, well-characterised source of contamination (Afon Goch), which is exclusively metallic in nature and where metal ions do not include the highly toxic mercury. Accordingly, barnacles from Dulas Bay occupied an intermediate position between the highly polluted barnacles from the Dee and the Mersey estuaries (Connah's Quay and Birkenhead) and the cleaner location at Menai Bridge. The correspondence of the opercular plate morphology of barnacles from Dulas Bay with those at Aberystwyth and Pembroke Dock highlights the lower level of overall contamination in those areas. Pembroke Dock has little heavy industry nearby, although a major petrochemical plant is located further west near Mildford Haven. The estuary and bay at Aberystwyth exhibits a similar, limited source, input of heavy metal contamination as that shown at Dulas Bay (see Ireland, 1973) which is substantiated by the results of the PCA analysis.

The results of the present work are further evidence of

the usefulness of barnacle opercular plate morphology in identifying chronic, sublethal pollution.

Source of variation	DF	SS	MS	F	P
Time	3	4068.43	1356.14	38.91	0.000
Treatment	1	206.53	206.53	5.93	0.041
TimexTreat	3	74.59	24.86	0.71	0.571
Error	8	278.80	34.85		
Total	15	4628.34			

Table 1 Two-way Analysis of Variance performed on the mean mortality values of *E. modestus* nauplii (stage II) from a natural population, exposed for 48 h (at 22.5°C and 32 ppt) to different copper concentrations and subsequently transferred to unpolluted media where they remained for 24 h. Day 1=values after a lapse of 48 h, Day 2=after a further 24 h

Scutum	A	B	C	D
	0.65814 ±0.06457	0.53666 ±0.04531	0.91490 ±0.08260	0.38426 ±0.03480
Tergum	E	F	G	H
	0.40113 ±0.04004	0.64278 ±0.04294	0.65853 ±0.04910	0.56686 ±0.06702

Table 3 Mean values ± stdev of the standardised dimensions of the opercular plates of *E. modestus* from Dulas Bay

Added copper (ppb)		N DA	DB	MB	LA	LB	No. dead DA	DB	MB	LA	LB
100	a	68	69	45	70	38	1	2	3	2	1
100	b	35	84	67	65	23	1	2	5	1	1
100	c	54	76	61	68	40	6	2	2	2	2
100	d	58	71	49	67	25	2	1	4	4	1
100	e	53	80	46	60	26	3	2	2	2	2
200	a	52	78	63	49	35	8	18	22	3	1
200	b	69	76	69	53	42	22	15	38	2	1
200	c	41	72	57	46	27	18	15	32	2	2
200	d	45	78	84	51	26	8	12	40	2	3
200	e	59	66	60	61	29	13	18	27	3	2
250	a	-	75	-	76	38	-	29	-	6	4
250	b	-	84	-	59	26	-	30	-	9	4
250	c	-	64	-	59	30	-	27	-	2	7
250	d	-	61	-	64	29	-	26	-	11	8
250	e	-	56	-	54	29	-	25	-	13	7
300	a	67	81	52	81	37	57	53	51	31	6
300	b	69	62	43	67	35	65	41	40	21	9
300	c	57	53	43	48	31	56	40	41	18	11
300	d	50	79	48	57	40	41	54	47	25	12
300	e	80	81	37	55	33	66	57	35	21	9
400	a	60	58	45	46	49	59	50	-	36	29
400	b	64	54	36	47	27	63	51	-	34	19
400	c	54	63	49	23	29	53	58	-	18	15
400	d	47	60	52	40	38	-	54	-	31	27
400	e	40	72	51	37	33	39	70	-	29	23

Table 2 Mortality of *E. modestus* nauplii (stage II) from natural adult populations at Dulas Bay (DA, DB) and Menai Bridge (MB), and from one population originally from Dulas Bay but subsequently maintained in clean medium for 4 months (LA, LB), exposed for 48 h (at 22.5°C and 32 ppt) to different copper concentrations, the mortality values shown being the actual figures used in the Probit analysis; a-e=positions in the culture tray

CHAPTER SEVEN

GENERAL DISCUSSION

As stated in the reviews by Holdgate (1979) and Persoone (1988), initially, acute toxicity testing provided valuable information on the effects of anthropogenic inputs in the marine environment. Indeed, the legislative limits to permissible pollution were directly derived from acute toxicity tests. Numerous factors govern the extrapolation of toxicity test results to the natural environment. Such factors range from temperature to life histories (see reviews by Holdagte, 1979; Persoone, 1988, 1989).

Currently, much interest has centred in defining precise protocols for toxicity tests and identifying suitable organisms which can be used to assess the effects in an ecotoxicological context where chronic and physiological effects must also be considered (see Persoone, 1988; Persoone *et al.*, 1989; Persoone & De Paw, 1991; Snell *et al.*, 1991; Ferrando *et al.*, 1993). In their reviews on hazard prediction and pollution both Holdgate (1979) and Persoone (1988) pointed out the need to standardise toxicity tests, which should also include standardisation of the state of the test organism in terms of uniformity of physiological condition, stage of the life cycle and genetics. Snell *et al.* (1991) suggested that rotifers fulfilled many of these criteria. They are easily-maintained, rapidly-reproducing organisms which are small and can be hatched on demand from cysts hence avoiding acclimation.

The present study has provided ample evidence on the suitability of the acorn barnacle *Elminius modestus* (Darwin) for the assessment of the possible effects of copper pollution and contamination in the sea. Thus, copper exposure both under laboratory conditions and in the natural environment, gave rise to a range of detrimental effects on *E. modestus* larval and adult forms, which could be categorised as acute, chronic and physiological (see review by Persoone, 1988).

E. modestus nauplii fulfil the requirements of acute toxicity testing pointed out by Holdgate (1979) and Persoone (1988). Thus, they are small and can be obtained from field parents whenever necessary, avoiding the culture of adults in the laboratory, acclimation, etc. and guaranteeing, upon adequate manipulation (larvae from a single egg mass can be used), genetic and physiological uniformity. In addition, barnacle nauplii are highly representative of crustacean zooplankton populations, often forming the bulk of zooplankton biomass in certain inshore locations (Barlow & Monteiro, 1979). Such representativeness is important when trying to extrapolate laboratory results to the natural environment (see review by Persoone, 1988).

Acute toxicity effects in the present study were determined solely in the nauplius larvae of *E. modestus* and clearly showed the difficulties in standardising such procedures. Median lethal doses (LD_{50}s) were used as a measure of copper toxicity and early attempts provided highly

variable LD_{50}s but identified many sources of variability in the test procedure.

Test standardisation was largely achieved through tight control of the number and stage of nauplii per test, range of copper concentration (100 ppb-500 ppb), volume and preparation of test media (serial dilution) and realistic criteria to assess mortality.

The development of a standard protocol allowed the investigation of a number of parameters which affected measured LD_{50}s in *E. modestus* nauplii. Within the optimal range of temperatures for *E. modestus* (Crisp, 1958) increased temperature led to a reduction in LD_{50}. Such correspondence has been widely reported in the literature for several marine species (see reviews by Mance, 1987; Persoone *et al.*, 1989), including barnacle larvae (Lang *et al.*, 1979).

The present study illustrated the importance of natural complexation as an ameliorator of copper toxicity to the marine environment. Thus, copper toxicity to *E. modestus* nauplii followed an inverse relationship with the organic content of the test seawater such that the LD_{50} increased by 45% in raw seawater from the Menai Strait compared to FUVSW. Naturally occurring organic ligands are responsible for most of the copper complexation taking place in estuarine (Mantoura *et al.*, 1978; Engel *et al.*, 1981) and marine waters (Zuehlke & Kester, 1981). Further illustrating the dangers of interpreting LD_{50}s from acute toxicity tests, are the problems experienced with the use of artificial seawater in the current study. Instant Ocean salts and deionised,

distilled water were used in an attempt to eliminate copper and organic material from the test water. In reality, the salts contained significant amounts of both copper and organic matter. Fortuitously or by commercial design the potential toxicity of the copper appeared to be balanced by the complexation ability of the organic matter.

The influence of genetics and condition of the parental population on acute copper toxicity was also clearly demonstrated. Nauplii from the copper polluted reaches of Dulas Bay were considerably more tolerant to copper even when they had been conceived and brooded in uncontaminated laboratory conditions. Similarly, Bryan & Hummerstone (1971) concluded that copper resistance was genetically controlled on observing that the copper tolerance noted in *Nereis diversicolor* inhabiting Restronguet Creek, a highly metal contaminated estuary on the south west of England, did not reduce significantly upon transferal to a low-copper environment.

Furthermore, populations maintained in the laboratory at Menai Bridge, under relatively more favourable conditions, produced larvae that showed higher LD_{50}s than those from natural populations. The difference in sensitivity may derive from poorer egg quality in the natural population (as with bivalve molluscs Holland, 1978) or, since Walker (1992) suggests that barnacle egg quality is invariable (Crisp, 1986, 1987) and the number of eggs laid reduces under unfavourable conditions, the laboratory nauplii showed better

acclimation to laboratory conditions.

Many of these effective parameters would vary spatially and temporarily in the marine environment hence the effects of a pollutant such as copper is likely to vary throughout an organism's life history. Effects on development are not apparent in acute toxicity testing if the organisms require feeding to effect development. Again the complicating effects of attempting acute toxicity testing while feeding organisms is clearly demonstrated in the current study.

Persoone (1988) noted that toxicity tests on early life stages have been mainly carried out on fish. Thus, toxicity studies on larval or early stages of marine invertebrates are few, even when it has been shown that larvae are generally more sensitive to pollutants than adults (Connor, 1972; Ahsanullah & Arnott, 1978; Birge & Black, 1979; Lewis & Cave, 1982). The literature contains some examples of sublethal and lethal copper toxicity to barnacle larvae (Clarke, 1947; Pyefinch & Mott, 1948; Toseland, 1976, Lang *et al.*, 1979, 1980, 1981), although none of them assesses the effects of the metal throughout naupliar development.

During the present work *E. modestus* larvae were reared to the cypris stage at copper concentrations eight to nine times higher that those killing 50% of stage II larvae during acute toxicity tests. Feeding the nauplii with microalgae completely masked the effects of copper, so obvious on unfed larvae. Royo-Gelabert (1991 unpublished), showed similar high survival rates during full development for *Balanus amphitrite* nauplii exposed to high copper concentrations.

The reduction in copper toxicity must, in large part, have been due to complexation. Sunda & Guillard (1978) and Anderson & Morel (1978) have shown that phytoplankton can complex cupric ions, where copper adsorbs to the algal surfaces (Xue & Sigg, 1990) or complexes with algal exudates (Fogg & Westlake, 1955; McKnight & Morel, 1979; Van der Berg *et al.*, 1979, Härdsted-Roméo & Gnassia-Barelli, 1980; Fisher & Fabris, 1982). Manley (1983) showed a reduction in the sensitivity to copper of *Mytilus edulis* pumping rates which was also attributed to algal complexation of ionic copper.

The reduced sensitivity to copper seen during the development of *E. modestus* larvae was, however, not due to complexation alone since the experiments with Algal Culture Medium (ACM) clearly demonstrated the synergy of 'nutritional stress' and copper toxicity. Larvae fed on healthy algal cultures were much less susceptible to copper poisoning than those fed on poorer quality algae. Similarly, Winner *et al.* (1977) showed that *Daphnia magna* fed on a nutritionally-poor diet exhibited less resistance to copper than those fed on a vitamin-enriched diet.

Delayed development or growth rate is one of the most commonly observed detrimental effects of copper on marine organisms (see reviews by Hodson *et al.*, 1979; Lewis & Cave, 1982). However, copper did not affect the time taken for full naupliar development of *E. modestus*. Lang *et al.* (1981). reported that copper either delayed development to stage III or totally suppressed moulting in *B. improvisus* stage II

nauplii. However, these authors only fed their larvae algae at densities of 20 to 40 cells.μl^{-1} considerably below the 100 cell.μl^{-1} routinely used for successful rearing of balanomorph larvae (Yule, 1986; Stone, 1988; Neal & Yule, 1994). Lang *et al.*'s (1981) larvae could well have been exhibiting 'nutritional stress' which increased their sensitivity to copper.

The current experiments therefore provided further evidence on the effects of the physiological state of the test organism on the outcome of toxicity tests. They also showed the inadequacy of extrapolating LD_{50} values from acute toxicity tests to longer temporal scales (see also reviews by Holdgate, 1979; Persoone, 1988; Persoone *et al.*, 1989). *E. modestus* is relatively easy to rear and settle on suitable substratum in the laboratory. It also has a short generation time (Crisp & Davies, 1955) which makes it eminently suitable for investigating the long-term effects of pollutants, such as copper, on adult growth and reproductive capacity. Persoone (1988) pointed out that growth and fecundity were crucial parameters for the prediction of long-term effects of pollutants on aquatic biota

During the current study both growth and reproductive effort were drastically reduced by copper exposure. These animals, however, did produce gametes although fertilisation never took place. Literature contains numerous examples of growth reduction in the presence of copper, e. g. bivalve molluscs, Manley *et al.* (1984), Redpath (1985) and Sanders (1984), and crustaceans Maund *et al.* (1992), see also review

by Jørgensen *et al.*, 1991. Reproductive impairment in the presence of copper has likewise been reported by Biesinger & Christensen (1972) on *Daphnia magna* at a copper concentration of 22 ppb.

The effects detailed above for adult *E. modestus* are largely sublethal but profound in terms of the continued survival of the population. The action of a pollutant may not kill an organism outright, but may affect the manner in which that organism acquires energy and uses that energy for life sustaining processes. The present work with *E. modestus* has shown such effects in response to copper pollution.

The respiration rate of *E. modestus* nauplii varied with copper concentration. At concentrations below 75 ppb oxygen consumption was generally increased. At higher concentrations consumption dropped to just below 'control' levels. Bernard & Lane (1963) interpreted a similar pattern of oxygen consumption with copper concentration in *B. amphitrite* cyprids as the energetic cost of copper regulation at the lower concentrations. At the higher concentrations toxic effects of copper override regulatory mechanisms. Assuming a similar interpretation for *E. modestus* the regulation of copper requires increased energy expenditure which would be at the expense of energy directed towards growth. Organisms under 'nutritional stress' would have much less chance of coping with such an extra demand.

Although not tested in the current study it is likely that the adults would show a similar energetic cost of copper

regulation (see Bowles, 1993 unpublished; Burkitt, 1994 unpublished). One way of redressing such energetic cost would be to increase energy intake. *E. modestus* adults, however, showed significant reductions in ingestion rates at copper concentrations above 10 ppb. The action of copper in reducing ingestion rates has been noted previously in *Brachiouns calyciflorus* (Ferrando *et al.*, 1993) and *Daphnia magna* (Flickinger *et al.*, 1982).

The mechanism by which the alterations in the feeding behaviour occurred remains obscure, although it appears to be a copper-induced behaviour rather than a structural damage resulting from the copper exposure. Thus, barnacles previously exposed to copper reverted to 'normal' (non-contaminated) ingestion rates when they were transferred to clean media.

A large contributor to the lack of somatic and reproductive growth exhibited by adults which were maintained under copper stress must have been their inability to acquire enough energy through ingestion. Indeed the combination of the anticipated energy expenditure on regulation and lower level of ingestion led to such a reduction in 'condition' that several barnacles succumbed to the effects of copper even though they were showing some somatic growth.

In conclusion, the ease with which the various stages of *E. modestus* life cycle can be manipulated, coupled with their general high tolerance to temperature and salinity and low-generation time make it an excellent organism in which to illustrate the effects of pollution in the marine

environment. The current study demonstrates how acute and chronic toxicity can be investigated throughout the barnacles's life history. The changes witnessed in the barnacle's physiology are rapid and sensitive indicators of stress and the long-term pollution problems resultant from reduced survivorship, growth and reproductive effort.

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