EFFECTS OF COPPER ON THE ENERGY BUDGET OF A STREAM DETRITIVORE: VALIDATION AND ECOLOGICAL RELEVANCE.

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ABSTRACT.

It is undisputed that single species toxicity tests are essential for obtaining information on concentrations and durations of exposures to chemicals that result in changes in survival, reproduction, physiology, biochemistry and the behaviour of individuals within a particular species (Cairns, 1983). However, the extent to which single species toxicity tests can be used to predict effects in the natural environment and changes at higher levels of biological organization are largely unknown (Kooijman, 1985). The ultimate aim of laboratory tests is to predict the potential effects of toxicants in natural systems in order to protect the structure and functioning of the ecosystem. Hence, the aim of this study was to investigate the validity (i.e. the extent to which effects observed in the laboratory are mirrored in a more natural environment) and ecological relevance (i.e. the extent to which the tests are indicative of effects at higher levels of organisation which are important for the structure and / or functioning of the ecosystem) of a particular single species laboratory test.

In order to increase its potential ecological relevance, both test species and response criteria for the laboratory test were carefully selected. The effects of copper, a reference toxicant, on energy budget parameters of *Gammarus pulex*, an important stream detritivore, were investigated. The validity and ecological relevance of these energy budget tests, under more natural conditions, were investigated with the use of outdoor artificial streams.

In the laboratory tests, consumption rates were identified as the most sensitive energy budget parameter to copper-induced stress in *G.pulex*. This decrease in consumption rates was due to an increase in the body copper concentration of *G.pulex* via copper uptake from the media, rather than to a rejection of copper contaminated food.

As well as being the most sensitive parameter to copper stress in laboratory tests, consumption rates were identified as the most sensitive energy budget parameter measured in animals deployed in the artificial streams. Further, there was no statistically significant difference between the effects of copper on consumption rates of animals in laboratory tests and in the artificial streams. Therefore tests based on consumption rates as a response criterion were valid in more natural systems. Potential additional effects on the consumption rates of *G.pulex* due to copper-induced reductions in food quality were also indicated.

The ecological relevance of these tests was investigated by trying to predict higher level effects in terms of growth and reproduction of whole organisms in the laboratory and in terms of the structure and functioning of populations and communities in artificial streams. Predictions regarding effects of copper on growth and reproduction of whole organisms were fairly accurate in indicating trends but were unsuccessful in predicting actual growth rates and reproductive output.

Population density and leaf processing were selected as response criteria indicative of effects of copper on the structure and function of the freshwater ecosystem respectively. Effects on these two parameters were observed at the same concentration as adverse effects on consumption rates, and no adverse effects were detected at concentrations below those causing a decrease in consumption rates. Potential consequences, of the observed effects of copper on *G.pulex*, for the structure and functioning of the freshwater community are discussed.

It is concluded that by careful selection of ecologically meaningful response criteria and test species, laboratory tests based on individuals can be representative of potential impact of a toxicant at higher levels of organisation in the natural environment. Consumption rates in *G.pulex* represent a sensitive, valid and ecologically relevant response criterion for the determination of the effects of a pollutant in natural ecosystems.

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CHAPTER 1.

GENERAL INTRODUCTION.

1.1 Introduction.

Chemicals from a variety of human sources are constantly being released into the natural environment. When such chemicals are released at elevated concentrations, or for sufficient periods of time, that they become a threat to the biota, they are considered to be pollutants (Martin, 1976). Many pollutants enter streams and rivers either via direct discharge or indirectly via runoff or deposition. Therefore, it is of necessity to be able to predict the potential ecological impact of pollutants in freshwaters in order to protect the structure and the functioning of the ecosystem.

It has been stated that 'the ultimate objective of ecotoxicological studies is both to predict and diagnose the causes of biological / ecological effects resulting from exposure to chemicals and other stressors in the environment' (Widdows and Donkin, 1991). Hence, many bioassays have been developed which may be concerned with either predicting environmental impacts before they occur and / or with assessing actual damage caused as a result of pollutant release. Cairns (1981) stated that the most important needs in biological monitoring were:

1. 'The development of predictive capability': Reliable and sensitive tests need to be developed which would be indicative of potential deleterious effects to the structure and / or functioning of the freshwater system.

2. 'A means of validating the accuracy of predictions'. Results obtained from standardised laboratory tests may not accurately reflect real effects in more variable, natural conditions.

The most common tests for determining the effects of a pollutant in freshwater ecosystems are single-species laboratory tests (Cairns, 1986). Such tests have many advantages including their high degree of replicability, use in indicating direct cause and effect relationships and their low cost. However, because single-species laboratory tests are highly standardised and controlled, they often have little ecological relevance (Kooijman, 1985; Cairns, 1986).

One of the major problems in ecotoxicology is validation of laboratory tests and extrapolation from laboratory to field situations (Kimball and Levin, 1985). The aim of this study was to investigate the validity and ecological relevance of a specific single-species laboratory test. For a laboratory test to be valid it should display the same pattern of response and sensitivity which is observed under field conditions. Ecological relevance is demonstrated by the ability of a test to indicate effects at higher levels of organization which are important for the structure and / or the functioning of the ecosystem. In order to increase the ecological relevance of laboratory tests, both test species and response criteria were carefully selected. The validation of these laboratory tests and an investigation of their ecological relevance was conducted using more natural test systems i.e. outdoor artificial streams set up to imitate a natural freshwater ecosystem.

1.2 Test species.

The prediction of general environmental impact often involves the use of specific test results from selected test systems or assays (Maltby and Calow, 1989). These tests are usually conducted on a limited range of test species (usually fish or *Daphnia*) which may or may not be useful in indicating effects in all systems. For investigation of the effects of a pollutant in freshwater streams, a test species important in such systems should be selected. Many small freshwater streams are heterotrophic, with the major energy input coming from the terrestrial environment in the form of dead leaves, needles, wood, twigs, flowers and buds. In an analysis of gut contents and determination of the principle pathways of energy flow in streams, Minshall (1967) showed that matter imported from

the terrestrial environment provided the main energy source for primary consumers and indirectly, for the whole benthic community. Allochthonous detritus has been found to contribute 50 to 99 percent to the total energy budget of upland-stream communities (Nelson and Scott, 1962; Cummins et al, 1966; Fisher and Likens 1973). Hence an appropriate test organism for determining the effects of a toxicant in freshwater lotic environments would be one selected from members of the freshwater detritivorous food web.

Detritus entering streams may be classified on the basis of particle size (Cummins, 1974): coarse particulate organic matter (CPOM, > 1mm), fine particulate organic matter (FPOM, > 0.45μm < 1mm), dissolved organic matter (DOM, < 0.45 μm). Much of the organic matter entering streams is initially in the CPOM size range and a major input is in the form of dead leaves (Fig. 1). The direct nutritional value of this plant litter for detritivorous invertebrates is usually poor but after leaching, leaves undergo 'conditioning' whereby the leaf material is colonised by microorganisms increasing the palatability of the leaf material to invertebrates (Cummins, 1974). Initially, fungi are more active than bacteria in the conditioning process (Barlocher and Kendrick 1973 a, b; MacKay and Kalff 1973; Kostalos and Seymour, 1976; Suberkropp and Klug, 1976; Pattee et al, 1986). The hyphae of fungi allow deep penetration of the leaf matrix while bacteria tend to be more restricted to surfaces (Harley, 1971).

Freshwater detritivores have been categorised into 'functional feeding groups' on the basis of the type of food they utilise and their mechanisms of feeding (Cummins, 1973; Cummins et al, 1973; Merrit and Cummins, 1978 in Anderson and Sedell, 1979; Anderson and Cummins, 1979; Cummins and Klug, 1979).

'Shredders' (e.g. *Gammarus sp*) comminute CPOM into FPOM and DOM (Petersen and Cummins, 1974; Sedell et al, 1975; Cummins et al, 1980; Webster and Benfield, 1986) which is then utilized as a food source by 'collectors' which may either filter the FPOM

out of the water column (e.g. *Simulium sp*) or gather it up off the stream bed (e.g. *Baetis sp*). Both shredder and collector invertebrates are preyed upon by predatory invertebrates and fish (Fig. 1).

The ability of shredders to process CPOM, coupled with their low assimilation efficiencies (i.e. approximately 30 %: Nilsson, 1974; Barlocher and Kendrick, 1975), suggests they can potentially generate large amounts of FPOM and DOM (Grafius and Anderson, 1980) and therefore greatly influence the food availability for collector invertebrates. More than 30% of the processing of CPOM to FPOM in freshwater streams has been attributed to the action of shredder invertebrates (Petersen and Cummins, 1974; Cuffney et al, 1990). Loss of CPOM and gain of FPOM in laboratory streams was found to be directly related to density of leaf shredding snails (Mulholland, 1985). Hence shredder invertebrates are key in the processing of leaf litter and the transfer of energy to other trophic groups.

Because of the important role of shredders in detritivorous systems, any effects of toxicants on these species would not only affect the shredder species itself but could have considerable consequences for the rest of the freshwater community (Fig. 1). The leaf shredding amphipod, *Gammarus pulex* (L), was selected as the test species for this study due to its wide distribution throughout Europe and its importance in detritivorous food webs. Adverse effects of a pollutant on this shredder species would result in a decrease in the processing of CPOM and hence a reduction in the amount of FPOM and DOM entering the system. Reductions in the amount of FPOM entering the system could lead to a limitation in the food supply for collector invertebrates. Direct and indirect effects of pollutants on shredder and collector population densities respectively, could result in a limitation in the food supply for predatory invertebrates and fish.

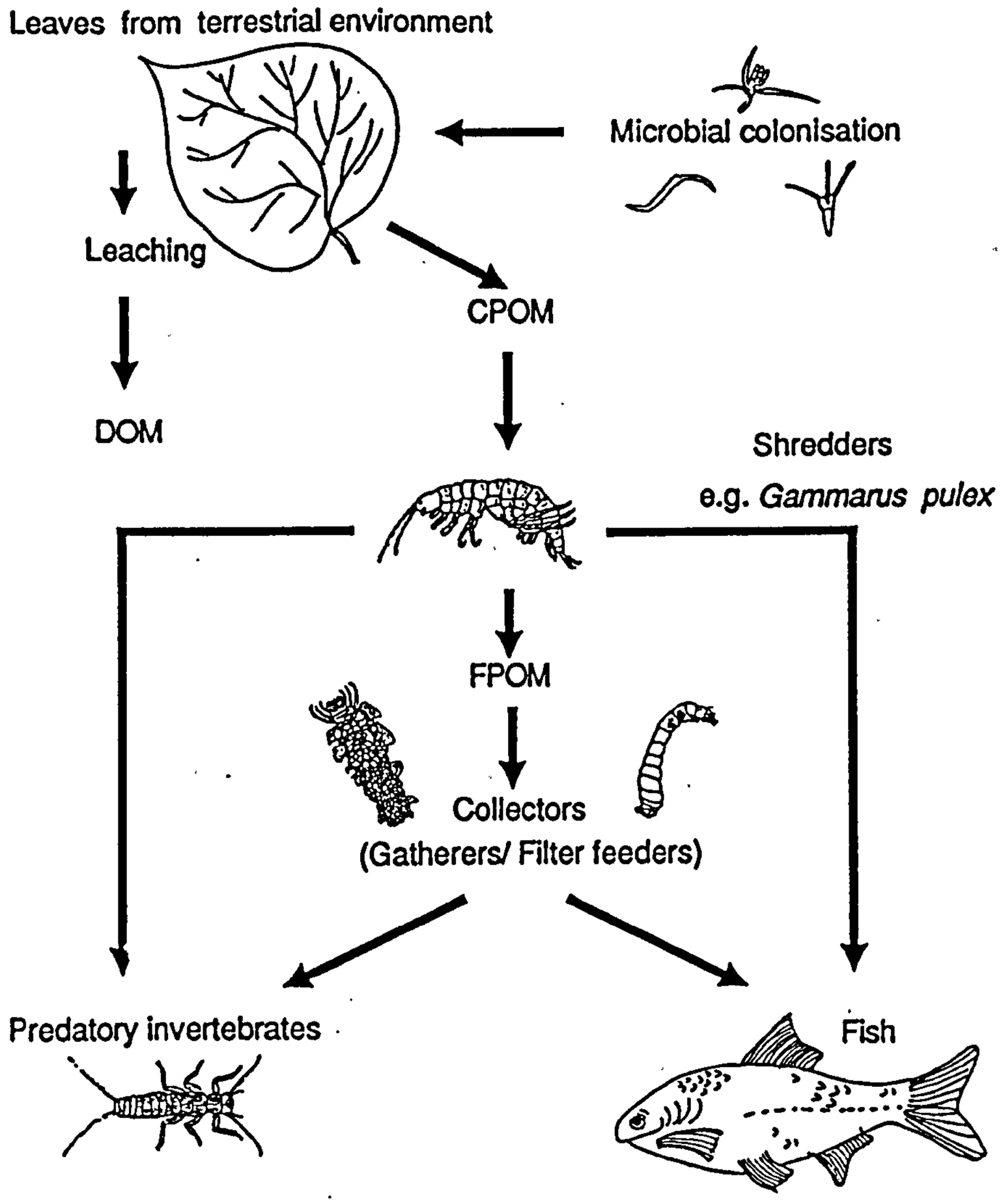


Fig. 1. A simplified detritivorous food web.

1.3. Response criteria.

The ecological relevance of many response criteria used in tests predicting general environmental impact of a pollutant can also be questioned. In papers reviewed by Maltby and Calow (1989), acute, lethal toxicity tests accounted for more than 80 percent of the studies published upto 1987. However, these acute tests have been found to often under-estimate the potential effect of a pollutant in natural systems and many species that

appear tolerant in acute tests may be absent or have restricted distributions in the field (Howarth, 1989).

The measurement of changes in the physiological responses of individual organisms offers many advantages over measurements made at other levels of biological organisation (e.g. biochemical or molecular levels). Physiological responses are capable of quantifying the organism's condition i.e. the performance and efficiency with which it functions (Widdows 1985). They may also be assessed relatively easily and quickly and can be related to population and ecosystem effects (Calow, 1991). The use of physiological energetics could prove useful for determining the effect of a pollutant in freshwater ecosystems; energy is a parameter that can be used throughout many levels of organisation, (individual to ecosystem), and from one level to another. Hence a study of the energy budget of an individual would allow extrapolation for the prediction of effects at higher levels of organisation necessary for the demonstration of ecological relevance.

The energy budget for an individual animal can be summarised using Winberg's (1960) energy budget equation:

$$C - F = A = R + U + P \qquad \text{eqn 1.1}$$

Where C is the energy ingested, F energy lost as faeces, A energy absorbed, R respiration, U energy lost as excretion and P energy accumulated for production (growth and reproduction).

Effects of a pollutant on an individual's energy budget may be integrated by determining the effects on 'scope for growth' (SfG). Warren and Davis (1967) defined SfG as the difference between energy intake and total metabolic losses and hence approximates the production (P) term in the above energy budget equation. Growth and reproduction are fundamental components of fitness and are linked in a direct manner to population

dynamics. However, these parameters are often problematic and time consuming to measure in the laboratory and therefore their use may be restricted in routine tests. A consideration of the parameters of Winberg's (1960) energy budget equation however allows the estimation of scope for growth (Warren and Davis, 1967) which is directly linked to growth and reproductive rates and to population processes. When SfG is positive growth and reproduction may proceed, however when it is zero, or negative, there can be no growth or reproduction and if negative energy balance is maintained the animal will die (Calow and Sibly, 1990).

Measurements of SfG may be quickly, easily and routinely performed under laboratory conditions. A measurement of SfG provides an immediate assessment of the energy status of the animal as well as an insight into the individual components which effect the changes in reproduction and growth rate (Widdows, 1985).

A procedure for measuring SfG in *G.pulex* has been developed (Naylor et al, 1989), and it has since been demonstrated that SfG is a sensitive indicator of stress caused by pH, oxygen, 3,4-dichoroaniline and zinc in laboratory studies (Naylor et al, 1989; Maltby et al, 1990a). The laboratory SfG assay has also been successfully modified for field deployment and proved sensitive to stress caused by chlorinated ethers, sewage and coal mine effluents, (Maltby et al, 1990b; Maltby, 1992).

1.4. Reference toxicant.

Copper was used as a reference toxicant for the purposes of this study. It is an essential element which has widespread industrial use. In members of the Crustacea, copper is necessary for the functioning of various enzymes, particularly the mono-oxygenases (Walsh, 1979). In addition, haemocyanin, the oxygen carrying pigment of crustaceans including *G.pulex*, has a non-haem oxygen binding centre based on cupric ions (Ghiretti, 1962; Van Bruggen, 1980).

As well as releases of copper from mining (Atkinson, 1987), copper is used in the manufacture of various alloys with zinc, nickel and tin, in metal plating and the production of copper wire and piping (Mance, Brown and Yates, 1984). Copper is used in the textile industry, in glass and ceramic manufacture, as a catalyst in the production of vinyl chloride, in the manufacture of wood preserving agents, rayon and paint pigments and various copper compounds are used as fungicides, algicides and molluscicides (Mance, Brown and Yates, 1984).

Copper in the waste water of these industries may exist as the monovalent ion Cu^{+} or more commonly as the divalent ion Cu^{2+}. It may exist in the aqueous environment as; the divalent cupric ion, complexed with inorganic anions or organic ligands such as carbonates, chlorides, humic and fulvic acids, as hydroxide or sulphide precipitates, or adsorbed to particulate matter (Jenne, 1968; Florence, 1977; Sylva, 1976; Vecta and Morgan, 1978; Wilson, 1978; Moore and Ramamoorthy, 1984; Tessier and Campbell, 1987). It is generally believed that the free cupric ion is most toxic to aquatic organisms and inorganic complexes are more toxic than organic complexes (Gatcher et al, 1973).

The complex speciation of copper in aquatic environments renders it a challenging chemical for use in ecotoxicological tests. When copper enters natural waters it partitions into various compartments; a portion enters the particulate compartment, a portion forms colloidal complexes and the remainder enters the soluble compartment (including organic and inorganic complexes). The partitioning of copper depends on many physicochemical characteristics of water and is especially dependent on pH, alkalinity and water hardness (Jenne, 1968; Florence, 1977; Sylva, 1976; Vecta and Morgan, 1978; Wilson, 1978; Moore and Ramamoorthy, 1984; Tessier and Campbell, 1987); toxicity generally increasing with increased pH (Stumm and Morgan, 1981) and decreased alkalinity and water hardness (Stephenson, 1983; Clements et al, 1989).

1.5 Approach.

The primary aim of this study was to determine the validity and ecological relevance of a specific laboratory based test. Laboratory based tests determining the effects of copper on various components of the energy budget of *G.pulex* are described in Chapter 2. Sensitive parameters of the energy budget are identified and the mechanisms underlying these responses determined. The ability of energy budget determinations in predicting longer term effects on the individual in terms of growth and reproduction was also investigated (Chapter 3).

The validity of laboratory tests based on energy budgets was assessed by comparing results obtained in laboratory tests with those observed in artificial streams (Chapter 4). The ecological relevance of laboratory tests was determined by comparing the effects on energy budget parameters of individuals with effects on the structure and functioning of populations and communities in the artificial streams (Chapter 5). Potential indirect effects of copper via toxicant-induced changes in food quality were also investigated and are described in Chapter 5.

Potential consequences of copper pollution for the structure and functioning of the detritivorous community which may be inferred from effects of copper on *G.pulex* are discussed in Chapter 6.

CHAPTER 2

EFFECTS OF COPPER ON THE ENERGY BUDGET OF *GAMMARUS PULEX*.

2.1 Introduction.

In order to prevent damage to a system (as opposed to assessment and recording of damage once it has occurred), there is a need to be able to predict the potential impact of a toxicant in ecological systems at risk. Further, these predictive tests must be able to provide explanations for effects in terms of readily measurable processes, in order to produce models permitting extrapolation to natural systems (Cairns 1981).

The measurement of physiological processes and their integration by means of physiological energetics can provide insight into the growth process and how it might be disrupted by environmental stress and pollution (Bayne and Newell, 1983). Measurement of scope for growth (SfG; Chapter 1, Section 1.3) provides a rapid and quantifiable measure of the energy status of an animal as well as an insight into the individual energy budget components (and mechanisms of toxicity) which result in changes in growth rate (Widdows, 1985).

Most previous work involving the effects of stressors on SfG has been conducted with marine organisms, in particular marine invertebrates. These studies indicated that SfG was sensitive to a diverse range of stressors including temperature and food density (Warren and Davis, 1967; MacDonald and Thompson, 1986), salinity (Shirley and Stickle, 1982a, b), aromatic hydrocarbons (Bayne et al, 1979; Widdows et al, 1987a, b) copper and diesel oil combinations (Widdows and Johnson 1988), and heavy metals (Gilfillan et al 1985).

In freshwater systems, scope for growth has been found to be sensitive to changes in hydrogen ions (Naylor et al 1989), oxygen concentration, zinc, ammonia, 3,4-dichloroaniline (Maltby et al 1990a) and a mixture of chlorinated ethers, (Maltby, 1992).

Effects of a toxicant on the energy budget of an animal could result in either an increase or a reduction in SfG. A reduction in SfG may cause a decrease in the fitness of an animal by adversely affecting growth and / or reproduction. This reduction in SfG may be a consequence of either a decrease in consumption rate and / or an increase in respiration rate.

A decrease in consumption rate may be the result of a behavioural and / or a physiological response. A behavioural response may be elicited if the animal can detect contaminated food and reject it, while a physiological response may be elicited if the toxicant is taken up by the animal, either from the food or the medium in which it lives, and food is rejected due to stress caused by the high toxicant levels within the organism.

Total respiration can be partitioned into three respiratory compartments: standard (R_s), active (scope for activity, R_a) and feeding (R_f / specific dynamic action; SDA). The toxicant could cause the organism to become more active (e.g. by eliciting an escape response) thereby increasing respiratory losses, or less active due to narcotic effects. Feeding has been shown to have an associated metabolic cost (e.g. Bohrer and Lampert, 1988), usually representing 9 to 20% of food energy ingested (Jobling, 1981; Beamish and MacMahon, 1988). This cost is thought to represent the caloric effect of feeding (Garrow, 1974); i.e. the costs of acquiring, handling, ingesting, digesting, absorbing and converting the food into storage products. SDA itself represents the strictly biochemical processes (Carefoot, 1990a) and it has largely been accepted that SDA mainly represents the energetic costs of protein turnover, (Cui and Lui, 1990). In some animals, for example fish, the effects of feeding associated activity and SDA are difficult to separate; in such cases the combined respiratory costs of these two processes are referred to as 'feeding

metabolism' (R_f) (Cui and Lui, 1990). As biochemical processes are difficult to separate from the physical processes of feeding the term 'feeding metabolism' will be used here. A stress induced decrease in consumption rate could result in a decrease in respiration rate due to reduced feeding respiration.

Hence, toxicants may either increase metabolic costs and therefore reduce SfG, or reduce metabolic costs ameliorating effects on SfG. Standard respiration rates may be increased due to increased metabolic demands for repair mechanisms or decreased due to toxicant induced inhibition of enzymes and other metabolic processes.

2.2 Objectives.

The objectives of this part of the study were to investigate the lethal and sublethal effects of copper on *Gammarus pulex* in laboratory tests. Sublethal effects were investigated in terms of the energy budget parameters: consumption (C), faecal production (F) and respiration (R_s, R_a, and R_f). Measurement of these parameters allowed determination of effects on Scope for Growth (SfG) and hence the levels of contamination at which growth and reproduction might be affected.

The effects of copper on the individual parameters of the energy budget equation were assessed in order to address the following:

1. Is SfG in *G.pulex* reduced by exposure to copper?

2. Is a reduction in SfG due to a reduction in energy acquisition or an increase in energy expenditure?

3. Is the response elicited due to behavioural or physiological effects?.

4. Is the major uptake route of copper into the organism via the water or the food?

2.3 Materials and Methods.

2.3. 1. Collection of test animals.

Gammarus pulex were collected from Crags Stream (NGR SK 497745), a small spring fed stream, situated near the village of Clowne, Derbyshire. The collection site was approximately half a kilometre from the spring. This stream was considered relatively clean with a water hardness of 98 mgCa/l, pH 7.8. The stream was narrow with fine substrate (< 0.5 mm) (Graca 1990) and high in macrophyte cover.

Large male animals were collected from the stream using a 2 mm mesh Endcott laboratory test sieve, and were transported to the laboratory in stream water.

2.3. 2. Maintenance of *G.pulex* in the laboratory.

a. Media.

The effect of media on the survival of *G.pulex* was determined. Four different media were assessed: artificial pond water (APW; Naylor et al, 1989), Low chloride artificial pond water (LowClAPW), Media 4 (M4; Elendt and Bias, 1990) and filtered stream water (FSW) (Appendix 1). APW was the simplest of the media, having only four salts added (Ca, Mg, Na and K salts). The low chloride medium contained the same Mg, Na and K salts as APW but much of the calcium salt was added as its sulphate rather than as its chloride. This was in order to reduce the amount of chloride ions in the medium and increase the sulphate ions to levels approximating to those of the natural stream water from which animals were collected. The M4 media contained the same four salts as APW at the same concentration, but also had a complex mixture of micronutrients, buffering nutrients and vitamins added in order to try to supply a full complement of trace

elements. Finally, filtered stream water from the collection site of the animals was used, an analysis of which is given in Appendix 1.

Five replicates of 25 animals per treatment were placed in vessels containing 1.5 litres of media. Animals were fed *Cladosporium* inoculated leaf material, prepared as detailed below (Section 2.3. 2. b), and were maintained at 15^{o}C with a 12 hour alternating light - dark photoperiod. Media were prepared and changed weekly, at which time survival of animals was also recorded.

Results are given in Appendix 2, but in summary, although there was no significant difference in the survival of animals maintained over a period of eight weeks in any of the 4 media (Ancova: $F = 0.055$, $df = 3$, $p > 0.05$), survival of animals maintained in APW was slightly higher than that of animals maintained in the other 3 media (Appendix 2). Therefore APW was selected for maintenance of stocks and all subsequent laboratory tests.

<u>b. Food.</u>

Alder leaves (*Alnus glutinosa*) inoculated with *Cladosporium* fungus had previously been shown to be an optimum diet for *G.pulex* (Nilsson, 1974; Naylor et al, 1989) and were therefore used as the food source in all experiments. Leaves were, collected in the autumn after abscission but just prior to fall, air dried at room temperature and then stored until use. Leaves were then prepared in a standard way by inoculation with *Cladosporium* fungus.

Cultures of *Cladosporium* were maintained on malt extract broth (Oxoid). Cultures were grown up, at room temperature, to evenly fill a petri dish from which discs of fungus could be cut with a sterilized 16mm diameter borer.

16mm diameter leaf discs were cut from rehydrated leaves, avoiding major veins, again using a cork borer. 120 leaf discs were placed in 500 ml conical flasks containing approximately 300 ml of distilled water enriched with salts given in Appendix 3. The flasks were sealed and autoclaved at 121^{o}C, 15 PSI, for 20 minutes. After cooling 6 discs of *Cladosporium*, were added to each flask using sterile techniques. The flask was then resealed and incubated at approximately 20^{o}C, in a shaking water bath, for ten days. After this period the flasks were removed, individual leaf discs laid out on sheets of aluminum-foil and dried in an oven at 60^{o}C for 3 days. Discs were then stored in a desiccator until required. When required discs were weighed on a Mettler ME30 micro-balance and were then rehydrated in test solution for four days before being offered to animals in laboratory tests (Fig. 2).

c. Holding conditions.

Animals were maintained in large 30 litre stock tanks containing 30 litres of APW and were allowed to acclimate to laboratory temperature (15^{o}C), photoperiod (alternating 12 hour light/dark) and food quality (*Cladosporium* inoculated alder leaves) for 10 days prior to use in experiments. Whole leaves, rather than leaf discs, prepared in the same manner as above (Section 2.3. 2. b; Fig. 2), were used for the maintenance of acclimating animals.

d. Test solutions.

All test solutions were prepared by dilution of a 10 mgCu/l stock solution. The stock solution was made by dissolving 19.65 mg of copper sulphate ($CuSO_4(7H_2O)$, BDH; Analar grade) in 500 ml of APW. Stock concentrations were checked by atomic absorption spectrometry (AAS) prior to preparation of test solutions. Both stock and test solutions were prepared and changed daily in all experiments and analysed using AAS or furnace atomic absorption spectrometry (FAAS).

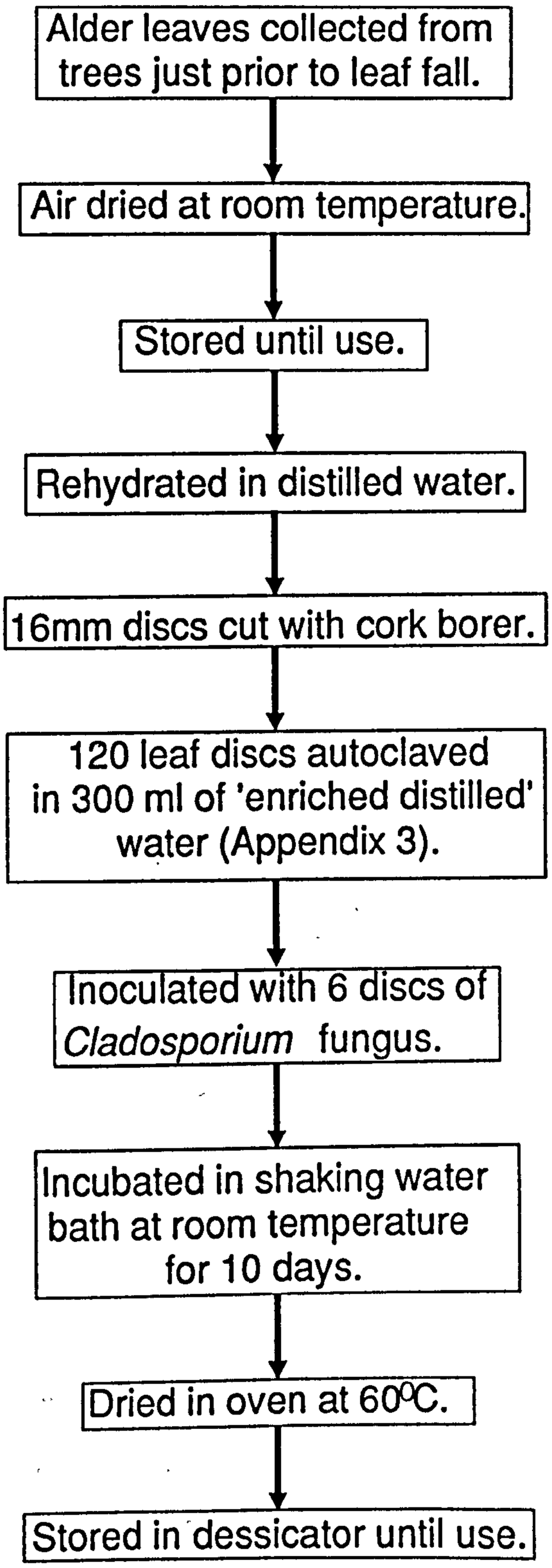


Fig. 2. Preparation of food by inoculation of alder leaf discs with *Cladosporium* fungus.

2.3. 3. Survivorship.

The standard 96 hour LC_{50} was determined in laboratory experiments. Individual, large, male animals were exposed to nominal copper concentrations of 0, 10, 20, 30, 40, 50, 60 µg/l for 96 hours. Three replicates of 10 animals per concentration were maintained individually in small glass tubes (1.5 x 1.5 x 9 cm) with mesh (500 µm) covered ends suspended in a glass tank containing 3 litres of test solution. Animals were maintained at 15°C with an alternating 12 hour photoperiod. No food was offered during the period of the test. Animals were checked daily for moulting or mortality and any moults or dead animals were removed.

To enable comparison of survival with energy budget results, survival of animals, fed alder leaf discs *ad libitum* (prepared as in Section 2.3. 2. b) was also determined over a six day period. 25 animals per concentration were exposed individually to copper (nominally 0, 10, 20, 30, 40, 50, 60 µg/l) in small pots containing approximately 225 ml of test solution. Animals were checked daily for moulting or mortality and any moults or dead animals were removed.

2.3. 4. Energy budget.

a. Consumption and faecal production.

25 animals per concentration were exposed to nominal copper concentrations of 0, 10, 20, 25, 30, 40 µg/l. Individual animals were placed in the upper chamber of small two-chambered pots containing 225 ml of test solution (Fig. 3). Each animal was provided with approximately 5 pre-weighed and pre-soaked leaf discs as food (prepared as in Fig. 2). The bottom chamber of the pot was separated from the top by a 1 mm mesh barrier which allowed faecal pellets to fall through into the chamber below and so prevented reingestion of faeces.

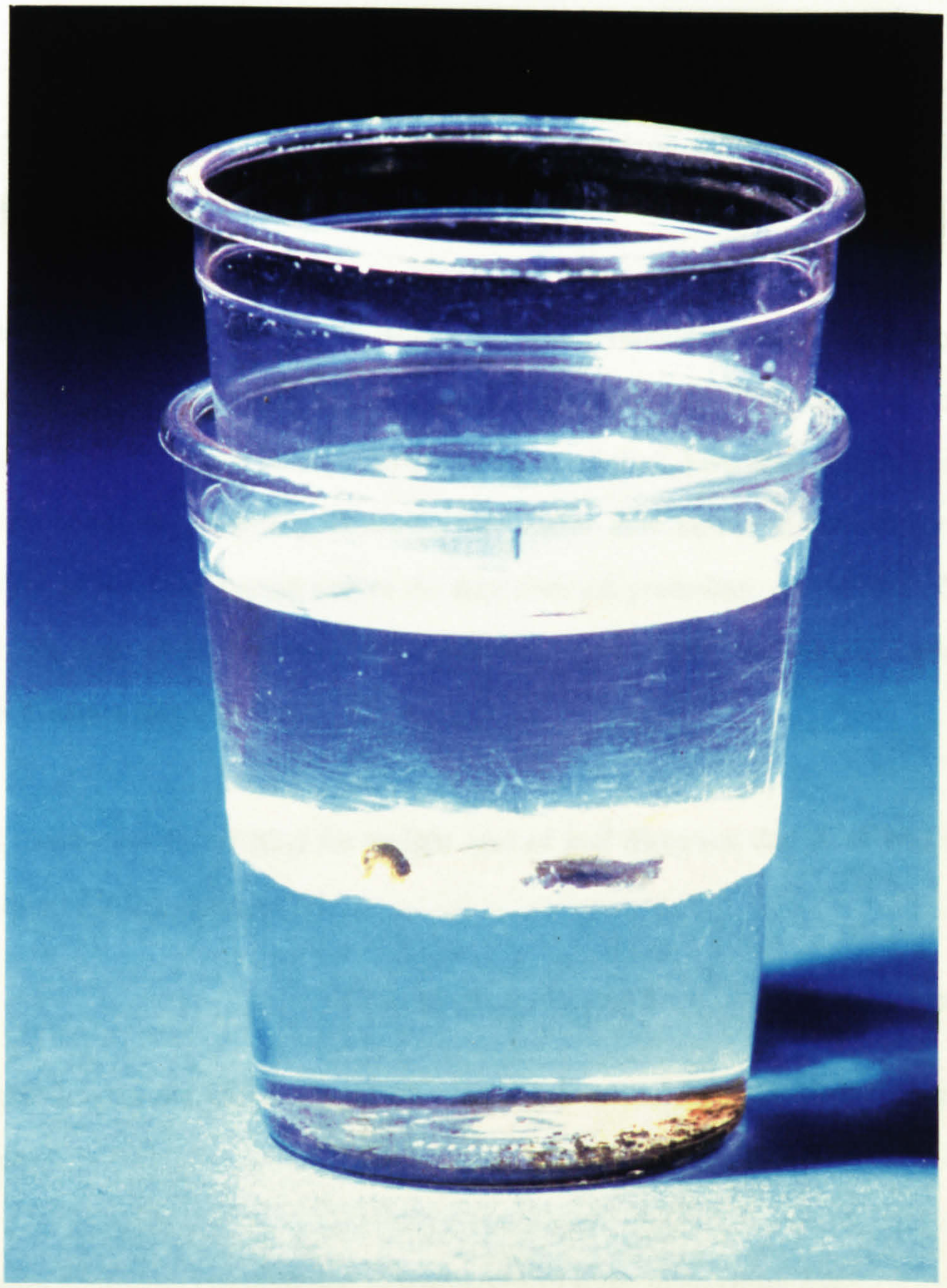


Fig. 3. Two-chamber vessel used for measuring consumption and faecal production in *G.pulex*. Upper chamber containing animal and *Cladosporium* inoculated leaf discs as food separeated from the lower chamber by a mesh divide through which faecal material can fall.

Five pots per treatment containing food but no animal were used to control for weight changes of the food not due to animal feeding.

Animals were exposed for six days after which remaining food was removed. Faeces were filtered onto preweighed filter papers (Whatman No. 1, 5.5cm) and both food and faeces dried for 3 days at 60^{o}C before being reweighed. Five filter papers were processed in the same way, filtering distilled water rather than faeces to control for changes in weight of the filter papers due to the experimental procedure. At the end of the test the animals themselves were removed, dried in an oven at 60^{o}C and weighed using a Mettler ME30 micro-balance.

The correction factor (C_1) for weight loss of leaf discs not due to animal feeding was calculated using equation 2.1:

$$C_1 = (\Sigma\ (L_{c2} / L_{c1})) / 5 \qquad \text{eqn 2.1}$$

Where L_{c1} is the initial weight of control leaves and L_{c2} the final weight of control leaves.

Energy consumed (J/mg/d) was determined according to equation 2.2:

$$C = (((L_1 \times C_1) - L_2) \times E_1)\ .\ (W \times 6) \qquad \text{eqn 2.2}$$

Where L_1 and L_2 denote initial and final weight of leaf material respectively, E_1 is the joule equivalent of food (determined as in Section 2.3. 2. b) and W the dry weight of the animal (mg).

The correction factor (C_f) for change in weight of filters during the experimental procedure was calculated using equation 2.3:

$$C_f = (\Sigma\ (F_{c2} / F_{c1})) / 5 \qquad \text{eqn 2.3}$$

Where F_{c1} and F_{c2} denote the initial and final weight of control filters respectively.

Energy losses due to faecal production (F), in J/mg/day, were determined according to equation 2.4:

$$F = ((F_2 - (F_1 \times C_f)) \times E_f) / (W \times 6) \qquad \text{eqn 2.4}$$

Where F_1 and F_2 represent initial and final weight of the filter papers respectively and E_f is the energy equivalent of faeces (determined as in Section 2.3. 2. b).

<u>b. Joule equivalent of food and faeces.</u>

Joule equivalents of *Cladosporium* inoculated alder leaves (Section 2.3. 2. b) and faecal pellets were determined by bomb calorimetry using a Parr 1425 Semi-micro calorimeter.

Material for bombing was dried in an oven at 60^{o}C, roughly ground with a pestle and mortar and compacted into 5mm diameter pellets using a pellet press. Pellets were dry-weighed using a Mettler ME30 micro-balance and were placed on the sample holder in the bomb. The two ends of a 10 cm length of nichrome alloy fuse wire were bound to the hook terminals and the fuse wire positioned so as to just touch the sample. The bomb was then closed. The bomb was charged with oxygen via the open valve, the valve was closed and the bomb placed into a Dewar flask. The flask contained distilled water at approximately 1^{o}C below room temperature. The ignition wires were then attached to the bomb and the flask was covered with a lid encompassing the stirrer and thermistor.

The drive belt motor was then started and the calorimeter began temperature measurements. When the temperature of the water was stable, a charge was fired by depressing the ignition button. The calorimeter then measured and gave an output of the temperature rise caused by combustion of the sample in excess oxygen. Any burning of

fuse wire was corrected for. This is done by measuring the length of fuse wire remaining and hence determining the length burned. A correction of 2.3 cal/cm (as determined by Parr, the bomb manufacturer) was then applied in all standardization and calorific value determinations.

The bomb was calibrated with a series of benzoic acid samples (of known calorific value i.e. 6318 cal/g). This involved firstly checking whether there was a direct linear dependence between the mass of substance burned and the temperature rise recorded and secondly determining the energy equivalent of the calorimeter (W; cal/^{0}C). The manufacturers of the Parr semi-micro bomb calorimeter recommend sample of masses should be between 25 to 200 mg. However, it was determined that calorific values of samples down to a mass of approximately 10 mg could be accurately determined and there was a linear relationship between the mass of substance burned and the temperature rise recorded ($r^2 = 98\%$, df = 9, $p < 0.001$; Appendix 4, Fig. 67).

As the calorie equivalent of benzoic acid is known, the mass burnt could be converted to calories (Appendix 4, Fig. 68). The energy equivalent (W; cal/^{o}C) is equal to the heat of combustion of the standard benzoic acid sample (Hm; cal/g) (after correction for combustion of the fuse wire (fc; Cal)) divided by the observed temperature rise (T; ^{o}C) eqn 2.5.

$$W = (Hm + fc) / T \qquad \text{eqn 2.5}$$

By a regression of T against corrected Hm, W may be determined as the inverse of the gradient (Appendix 4, Fig. 68).

Once the energy equivalent of the calorimeter (W; 344.477 cal/g) had been determined, the gross heat of combustion (Hc; Calories/g) of food and faeces of known mass (m; g) was then determined from the observed temperature rise (T; ^{o}C) using equation 2.6.

$$Hc = ((W \times T) - fc) / m \qquad \text{eqn 2.6}$$

Hc was then converted to J/mg by multiplying by the joule equivalent of calories (i.e. 4.187) and dividing by 1000.

The energy content of 10 samples of leaf material (prepared as in Section 2.3. 2. b) before being fed upon was assessed in order to determine the energy content of leaf material offered to animals. The energy content of 5 samples of leaf material per concentration remaining after it had been offered to animals exposed to different concentrations of copper (Section 3.3. 4. b) was also determined to check that there was no difference in the energy content of material between treatments (Results are given in Appendix 5). Faecal material gathered during the above experiment (Section 2.3. 4. a) was filtered onto paper filters and therefore could not be used to determine energy values. Hence another set of 10 animals were set up as above, (Section 2.3. 4. a) maintained in APW and were fed *Cladosporium* inoculated alder leaves. Faeces were collected from these animals by pipetting into glass crystallizing dishes. The faeces were then dried in an oven at 60°C and the energy content of 10 samples determined. To ensure copper had no effect on the energy lost as faeces, the energy content of three samples per concentration were also collected, in the same way, from animals exposed to copper concentrations of 0, 20, or 30 µg/l (Results are given in Appendix 5).

<u>c. Standard Respiration.</u>

At the end of the 6 day feeding period, before dry weighing, animals were removed from the two-chambered pots and were placed in 2ml glass syringes. Standard respiration rates (R_s) of animals were then determined by flow-through respirometry (Wrona and Davies, 1984). The aerated test solution held in a reservoir, was pumped at a constant rate (45 rpm), by a Watson Marlow pump (model 202 U/ 1), through the syringes containing the animals.

Each pump served 16 respirometers, 15 of which each contained one animal leaving one without an animal to act as a control. All animals were allowed to acclimate to test conditions (ie. respirometers held at 15^{o}C, with an alternating 12 hour photoperiod) for 15 hours after which approximately 50 µl of water were slowly removed from each syringe using a 50 µl Hamilton micro-syringe and was injected into a Radiometer oxygen electrode (E5046) linked to a Strathkelvin oxygen meter (model 781b). The oxygen concentration across the membrane was allowed to stabilize over a period of 2 minutes at which time the oxygen content (torr) of the injected water was read. The concentration across the membrane was then allowed to return to that of saturated air (i.e. 160 torr) by injection of ambient air. Once the electrode had stabilized at this level the next reading could be taken.

The oxygen content of water leaving the control respirometer was used as a measure of the oxygen content entering the respiration chambers (PEO_2). A sample of water from the control syringe was taken before samples from the experimental respirometer were measured (PLO_2). This was necessary in order to account for any fluctuation in background oxygen levels of the water entering the respiration chambers. Measurements of oxygen content from individual test syringes were replicated twice or, if values varied by more than two torr between replicates, a third replicate was taken.

Flow rates were determined by measuring the time (seconds) to fill 40 µl of the 50 µl micro-syringe used for injection into the electrode. Flow rates were then converted to litres per hour.

After all the oxygen readings had been taken the animals were removed from the syringes and were placed in labelled trays and dried at 60^{o}C for 3 days. They were then weighed using a Mettler ME30 micro balance.

Weight specific oxygen uptake (MO_2: mol/mg/h) was determined using equation 2.7 (Wrona and Davies, 1984):

$$MO_2 = ((PEO_2 - PLO_2) \times AO_2 \times Fl) / W \qquad \text{eqn 2.7}$$

Where PEO_2 and PLE_2 denote the oxygen content (torr) of water entering and leaving the syringe respectively, AO_2 is the solubility coefficient of oxygen in water at 15°C (i.e. 2.01 mol/ l/ torr), Fl is the flow rate of water through the syringes (l/h) and W the dry weight of the animal (mg).

Oxygen uptake (MO_2) was converted to litres per mg per day by multiplying by 22.41 $x10^{-6}$ and 24, and to energy (R: J/mg/d) using an oxyjoule equivalent for carbohydrate metabolism (21×10^3 J/l O_2; Elliott and Davison, 1975).

d. Absorption and scope for growth.

The energy absorbed (A) in J/mg/day was defined as the difference between energy consumed and energy lost as faeces (i.e. A = C - F).

Scope for growth (SfG; J/mg/day) was calculated as the difference between energy gained via consumption (C) and that lost via faeces (F) and standard metabolic processes (Rs) according to equation 2.8:

$$SfG = C - (F + R_s) \qquad \text{eqn 2.8}$$

2.3. 5. Active and feeding respiration rates.

a. Active respiration rate (R_a).

The effect of activity on respiration rate was determined by closed respirometry. Test solutions with copper concentrations (nominally 0, 20, 50 µgCu/l), aerated over-night,

were decanted into three replicates of twenty four, 27ml glass vessels per concentration. The initial oxygen content of the water added to each vessel (PIO_2) was determined by extracting 50 µl of solution from the vessel, prior to addition of the animal and injecting into the oxygen electrode as above (Section 2.3. 4 c). Individual large male *G.pulex* where added and the vessels were sealed air-tight with parafilm covered rubber bungs. Animals could move freely within each vessel. Four vessels per concentration contained no animal and controlled for any changes in the oxygen content of the water not due to respiration of the animals.

Three replicates of ten vessels per concentration were attached to a wooden rod. The rod was attached at each end to a Watson Marlow pump and when the pumps were switched on they caused the rod to rotate (9 rpm). The rotation of the bottles caused a water movement against which the animals would swim. Hence a measure of respiration rate at near maximum activity could be gained. The remaining three replicates of ten vessels remained stationary and respiration rates at normal activity rates could be determined.

All animals where left for 6 hours after which the final oxygen content of the water (PFO_2) was re-assessed as before. Animals where then removed, dried for 3 days at 60°C and weighed.

Weight specific oxygen uptake (MO_2) was determined using equation 2.9:

$$MO_2 = ((PIO_2 - PFO_2) \times V \times AO_2) / (W \times t) \qquad \text{eqn 2.9}$$

Where PIO_2 and PFO_2 denote the initial and final oxygen content (torr) of the vessels respectively, V is the volume of vessel (ml), W the dry weight of the animal (mg), t the time (hours) and AO_2 is the solubility coefficient of oxygen in water at 15°C (2.01 mol/l/torr).

Oxygen uptake (MO_2) was converted to J/mg/day using the method described in Section 2.3. 4. c.

b. Feeding respiration rate (R_f).

Respiration rate of feeding animals was determined by flow through respirometry (see 2.3. 4. c). Three sets of 12 animals were starved for 24 hours prior to the test. Individual *Gammarus* were placed in 36, 2 ml syringes attached to respirometry equipment as described in Section 2.3. 4. c. Animals were allowed to acclimate to test conditions (2ml syringes held at 15^{o}C with an alternating 12 hour photoperiod) overnight for approximately 15 hours. Standard respiration rates of all animals were determined hourly for 2 hours using techniques described in Section 2.3. 4. c. One small preweighed *Cladosporium* inoculated leaf disc presoaked in APW was added to 18 of the respirometers allowing those animals to feed while the remaining 18 animals were unfed. Two control syringes were also used, one containing food but no animal and the other containing neither food nor animal. On adding the food the respiration rate of all animals was determined and the animals then allowed to feed in the chamber for 1 hour after-which the food was removed. Syringes containing non-fed animals were opened and closed as with fed animals to control for any changes in oxygen uptake due to the experimental technique. Respiration rates of all animals were determined again as the food was removed and then hourly for a further 6 hours. Remaining food was dried at 60^{o}C and reweighed to determine quantity of food consumed. Oxygen uptake was determined according to equation 2.7.

2.3. 6. Behavioural detection and selection of food.

Preweighed leaf discs (L_1), prepared as in Figure 2, were soaked for 4 days in nominal copper concentrations of 0, 30, 60, 100, and 200 g/l, solutions of which were changed daily. Hence leaf discs became contaminated with different concentrations of copper. After soaking any surface film was rinsed off by holding the discs between forceps and

gently moving them through APW. Single discs were labelled with colour-coded pins to distinguish between the concentrations they had been soaked in (Fig. 4).

One disc from each concentration was placed in each of 25 pots containing 300ml of APW and an individual animal, hence animals were offered a choice of five discs, one of each concentration (Fig. 4). Five pots containing leaf discs but no animal were used to control for weight losses of the leaves not due to animal feeding. The animals were allowed to feed on the discs for 24 hours during which time they were free to select and feed on any of the discs offered. After 24 hours the remaining discs were removed and dry weighed (L_2) to determine the amount of each disc consumed.

This experiment was repeated with five groups of 10 animals rather than individuals. For this experiment each replicate contained 4 labelled leaf discs per treatment.

The correction factor (C_1) for weight loss of leaf discs not due to animal feeding was calculated according to equation 2.2.

The amount of each offered leaf disc consumed (C_{xi}) was calculated from equation 2.10:

$$C_{xi} = (L_1 \times C_1) - L_2 \qquad \text{eqn 2.10}$$

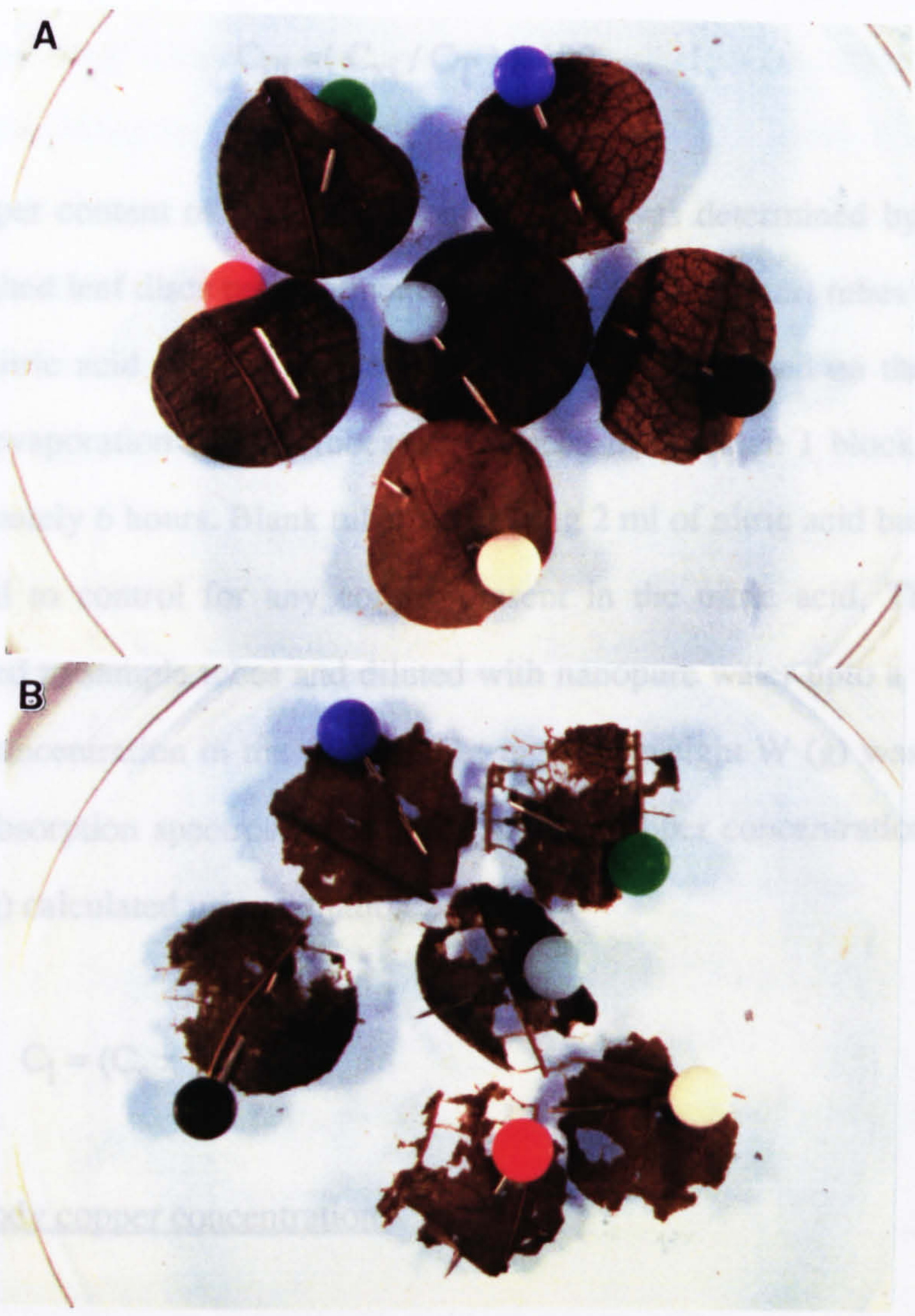


Fig. 4. Design of food choice experiments: A. Leaf discs prior to feeding. Discs of different copper concentrations marked with colour coded pins. B. Leaf discs after feeding period.

The proportion (C_{Pi}) of the total consumption attributable to each disc type was calculated to correct for differing total consumption rates ($C_T = \Sigma C_{xi}$) of individuals and groups and was expressed as a percentage using eqn 2.11.

$$C_{Pi} = (C_{xi} / C_T) \times 100 \qquad \text{eqn 2.11}$$

The copper content of the offered leaf material was determined by acid digestion. Ten, dry weighed leaf discs per treatment were placed in small test tubes to which 2ml of 70% primar nitric acid (Fisons) was added. A marble was placed on the top of each tube to prevent evaporation and the tubes were placed in a Tecam 1 block digester at 90^{o}C for approximately 6 hours. Blank tubes containing 2 ml of nitric acid but no leaf sample were also used to control for any copper present in the nitric acid. The digests were then transferred to sample tubes and diluted with nanopure water upto a volume of 10ml. The copper concentration in the sample (C_S; mg/l) of weight W (g) was then analysed using atomic absorption spectroscopy (AAS) and the copper concentration of the leaf material (C_l; µg/g) calculated using equation 2.12:

$$C_l = (C_s \times 10 / W) \qquad \text{eqn 2.12.}$$

2.3.7. Body copper concentration.

Total copper body concentration of ten individual animals per concentration, used for energy budget determinations, were determined by acid digestion. The same procedure was used as for determining the copper content of the leaf material above (Section 2.3. 6), except that only 1 ml of 70% nitric acid was added to each of the ten replicates per treatment and digestion took about 3 hours. The digests were analysed for copper using furnace atomic absorption spectrometry (FAAS) and the copper concentration of the animals determined using equation 2.12.

2.3. 8. Uptake route of copper.

The relative importance of uptake of copper from food and water was assessed by exposing *G.pulex* to combinations of clean (APW) or contaminated (nominally 30 µg/l) water and food. Contaminated food was either accessible, allowing feeding, or was inaccessible being contained in fine mesh bags, (pore size 1mm), (Table 1).

Table 1. Combinations of contaminated (soaked in 30 µg/l copper) and clean (soaked in APW), food and water used to determine the major uptake route of copper.

Code	Water	Food
a	Control	None
b	Control	Control
c	Control	Contaminated (I/A)
d	Control	Contaminated
e	Contaminated	None
f	Contaminated	Control
g	Contaminated	Contaminated (I/A)
h	Contaminated	Contaminated

(I/A = Inaccessible food)

Contaminated food was prepared by soaking food (prepared as in section 2.3. 2. b) in toxicant for four days prior to the test as in Section 2.3. 6. All test solutions were prepared and changed daily from fresh 10 µg/l stock solutions and were analysed by FAAS.

Individual animals, provided with five leaf discs as food, were placed in the upper chamber of small two-chambered pots containing 225 ml of test solution as in Section 2.3. 4. a (Fig. 3). Animals were exposed to the test combinations for 6 days after which

the animals and remaining food were removed and dried in an oven for three days at 60°C. Animals and food were then weighed using a Mettler ME 30 micro-balance.

The total copper concentration of the animals and the leaves was determined by acid digestion, using the methodology described in sections 2.3. 6. and 2.3. 7.

2.4. Statistical analyses.

The standard 96 hour LC_{50} and the 144 hour LC_{50} of fed animals were determined by probit analysis (Finney, 1971).

All data was tested for normality before applying parametric statistics. After testing for normality using N-Scores, effects of copper on energy budget parameters were determined by one-way analysis of variance and the Tukey multiple range test. Twosample t-tests were then used to determine the significance of differences.

Relationships between consumption rate and faecal production in stressed and unstressed animals were determined using least squares regression techniques and were compared by analysis of covariance. Relationships between feeding respiration rate and consumption were also determined using least squares regression techniques.

The increase in respiration rate due to feeding was determined by integration of the equation describing the relationship between respiration rate and time after the feeding period, such that an equation of the form:

$$y = mx^2 + nx + c$$

was integrated to give an equation of the form:

$$y = 1/3\ mx^3 + 1/2\ nx^2 + cx.$$

The study of Smock (1983) and Rainbow and Moore (1986) indicated a strong exponential relationship between invertebrate dry weight and body concentrations of particular heavy metals. This can be modelled by the power function given below (Rainbow and Moore, 1986):

$$y = ax^b$$

Where y is the body metal concentration (mg/g), x the dry weight of the animal (mg) and a and b are constants.

Hence effects of weight on body copper concentration were determined via linear regression of double log plots of dry weight and body concentration. Relationships between body concentration and copper concentration of the medium, and between body concentration and consumption rates were determined using least squares regression techniques.

Effects of copper on food choice were determined by one-way analysis of variance and the Tukey multiple range test on arcsine transformed data using the twosample t-test to determine where the significance lay.

The major uptake route of copper, via food or water, was determined by one-way analysis of variance followed by the twosample t-test between each combination to determine significant differences.

LC_{50}s were determined using the Pharmacology computer package, and the exponential equation describing the relationship between respiration rate and time after feeding was determined using the Cricket Graph software package. All other tests where performed using the Minitab, Statgraphics, or Fig P software packages.

2.5 RESULTS.

2.5. 1. Survivorship.

Survival of *G.pulex* decreased with increasing copper concentration. The standard 96 hour LC_{50} (µg/l) of *G.pulex* exposed to copper was calculated by probit analysis and is given below:

$$LC_{50} = 64.5\ \mu gCu/l\ \ (95\ \%\ C.I.\ 41.01, 101.37)$$

A 6 day LC_{50} was also determined for animals allowed to feed in order to compare results to energy budget data. The 6 day (144 hour) LC_{50} for fed animals was:

$$LC_{50} = 44.63\ \mu gCu/l\ \ (95\ \%\ C.I.\ 39.64, 49.63)$$

2.5. 2. Energy budget.

a. Consumption and faecal production.

The mass of food consumed and that lost as faeces was converted to energy using joule equivalents for food and faeces given in Section 2.5. 2. b, (Table 2). Copper caused a significant reduction in consumption rates of *G.pulex* relative to the control ($F = 19.033$, $df = 5, 121$, $p < 0.001$). Significant reductions in consumption rates were detected where animals were exposed to a copper concentration of 23 µg/l and over ($t > 2.34$, $df > 34$, $p < 0.05$) (Fig 5). Effects of copper on faecal production of *G.pulex* mirrored those on consumption rates ($F = 12.891$, $df = 5, 121$, $p < 0.001$) with a significant reduction in faecal production detected when animals were exposed to 23 µg/l and over ($t > 2.05$, $df > 34$, $p < 0.05$) (Fig. 5).

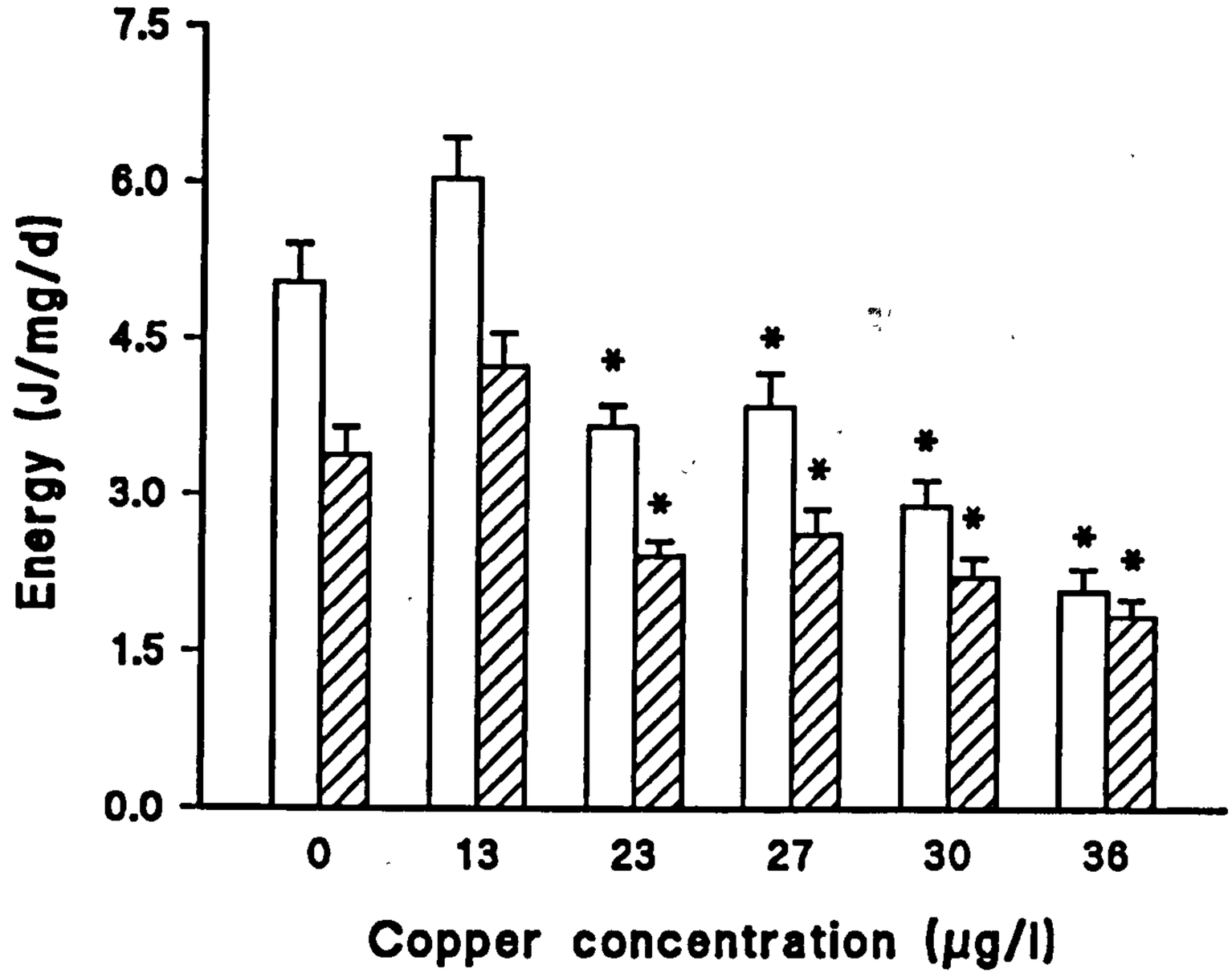


Fig. 5. Energy (J/mg/d) gained via consumption (open bars) and lost as faeces (hatched bars) by *G.pulex* exposed to copper (µg/l) in 6 day laboratory tests. Error bars represent 1 standard error, * data significantly different from control at $p < 0.05$.

There was a linear relationship between consumption rates (C) and faecal production (F) (Fig. 6) which was described by the relationship:

$$F = (0.664 \times C) + 0.151 \qquad \text{eqn 2.14}$$

$$(r^2 = 85.3\%\ df = 118,\ p < 0.001)$$

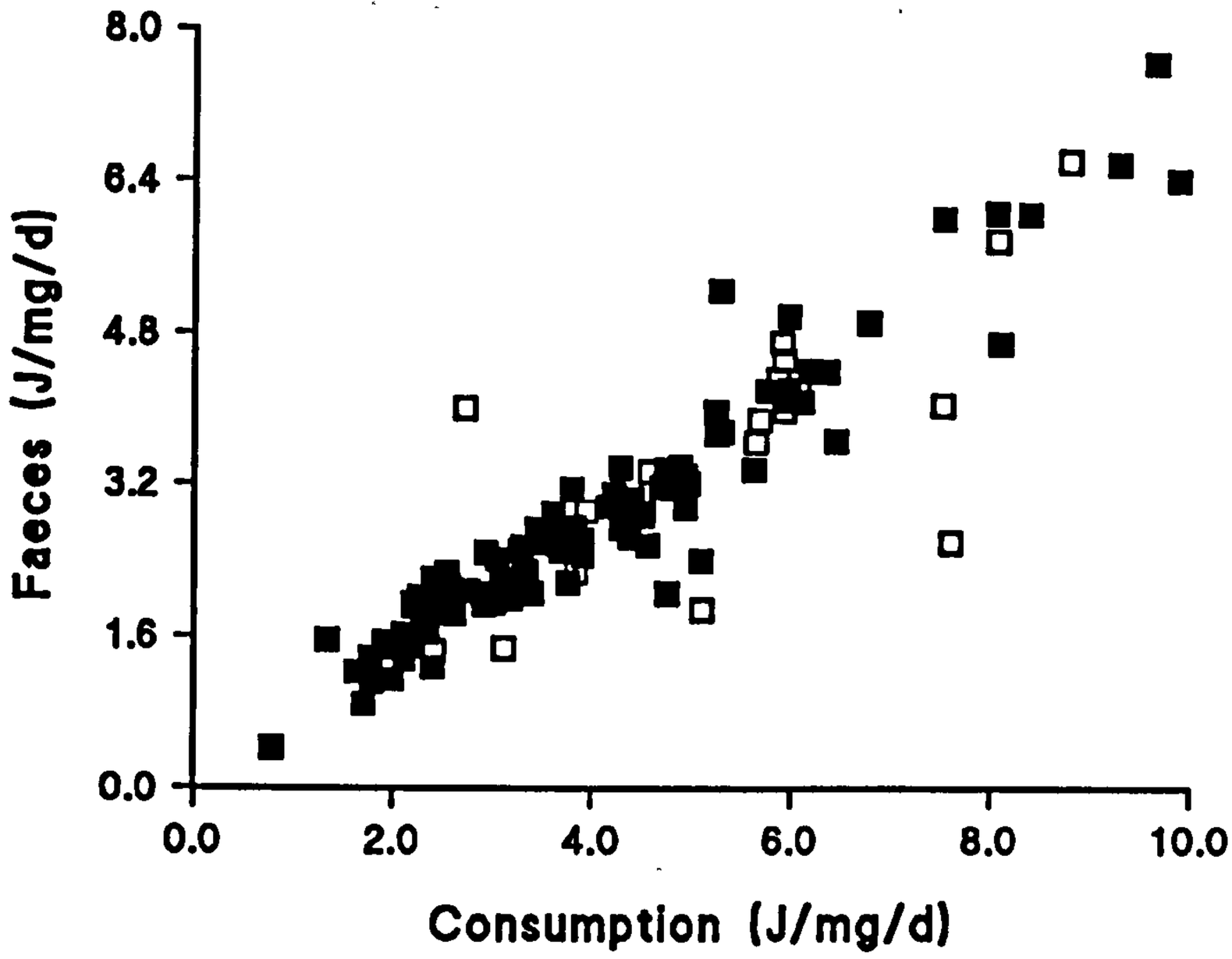


Fig. 6. Relationship between faecal production (J/mg/day) and consumption rates (J/mg/day) of stressed (solid squares) and non-stressed (open squares) *G.pulex* in the laboratory.

Despite generally consuming less energy, there was no significant effect of stressing animals with copper on the relationship (ANCOVA, $F = 2.16$, $df = 1, 121$, $p > 0.05$).

b. Joule equivalent of food and faeces.

The joule equivalents of food and faeces, determined by bomb calorimetry, are given in Table 2.

No effect of copper on the joule equivalent of food ($F = 2.053$, $df = 5, 28$, $p > 0.052$) or faeces ($F = 0.687$, $df = 2, 7$, $p > 0.05$) was found, (Appendix 5), hence the values given in Table 2 were applied to all calculations.

Table 2: Joule equivalents (J/mg) of food and faecal material.

Sample	Joule equivalent	SE	n
Food	22.146	0.6906	10
Faeces	19.944	0.1828	10

c. Standard respiration rates (R_S).

There was no significant effect of copper on standard respiration rates (R_S) at any of the concentrations tested ($F = 0.871$, $df = 5, 68$, $p > 0.5$), (Fig. 7).

d. Absorption and scope for growth.

Despite the linear relationship between consumption and faecal production a significant effect of copper on absorption was detected at 30 µg/l rather than 23 µg/l as expected from consumption data ($F = 11.438$, $df = 5, 121$, $p < 0.001$) (Fig. 7). The lack of a significant effect of copper on absorption at lower concentrations was likely to be a result of the slightly greater effect of copper on consumption rates compared to faecal production. Although concentrations of 23 µg/l and over significantly reduced both C and F, C was effected to a greater extent. This had little effect on the C:F regression as the downward trend was still strong, however, the slightly greater effect of copper on consumption rates than on faecal production caused effects on absorption to be detected only at greater concentrations (i.e. 30 µgCu/l and over).

Due to the linear relationship between consumption rates and faecal production, absorption efficiency (i.e. A / C) should be fixed. However, analysis of variance indicated that copper did cause a decrease in absorption efficiency ($F = 6.405$, $df = 5, 121$, $p < 0.001$) with a significant decrease at concentrations of 30 µgCu/l and over ($t > 4.27$, $df > 26$, $p < 0.001$). Above 30 µg/l the mean absorption efficiency was 35.142 percent (SE =

0.983) while at concentrations of 30 µgCu/l and over it was 24.943 percent (SE = 1.482). The above arguments for the cause of the lower sensitivity of absorption rates, despite the linear relationship between consumption and faecal production, also apply for absorption efficiencies.

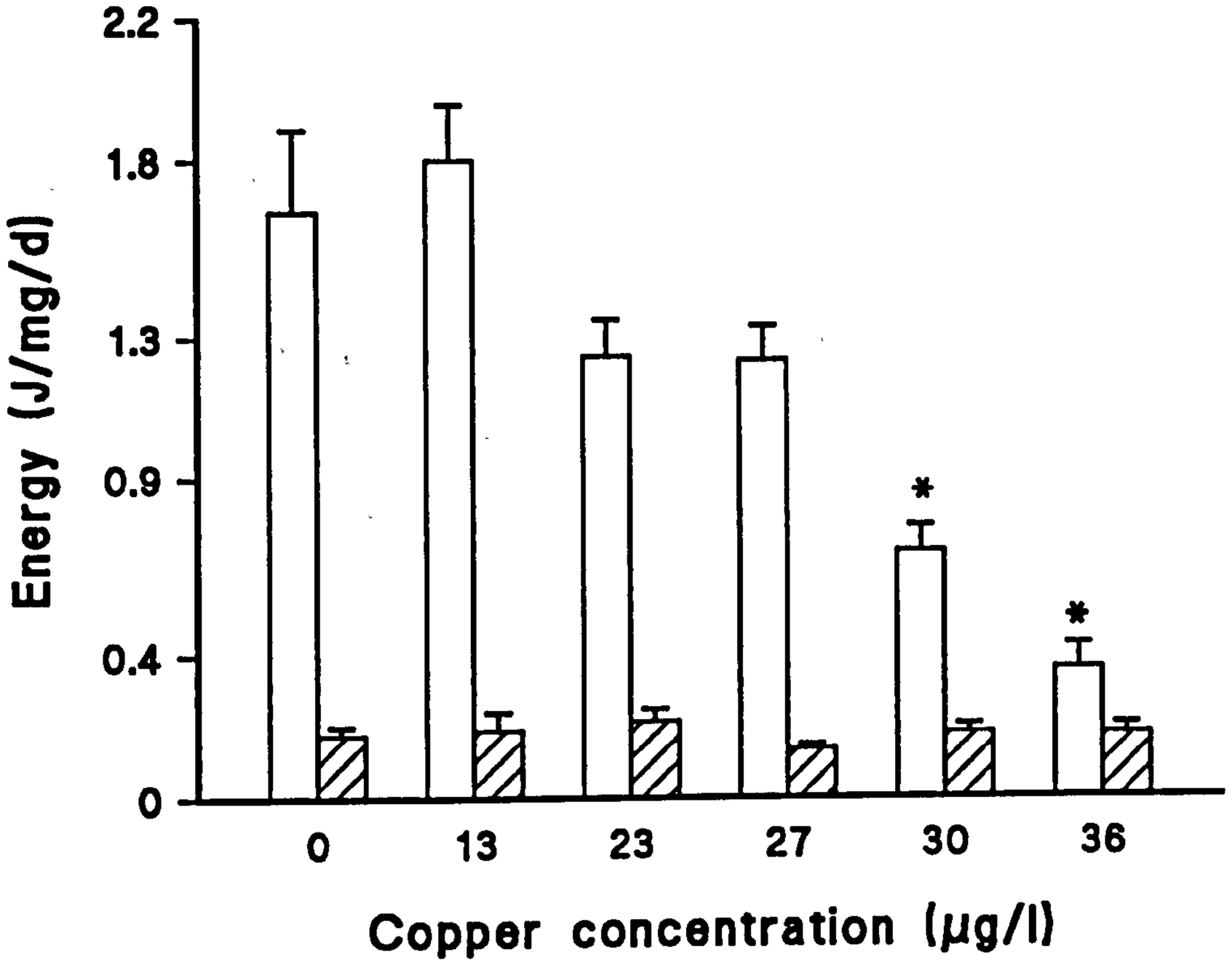


Fig. 7. Energy (J/mg/d) absorbed (open bars) and lost via standard respiration (hatched bars) by *G.pulex* in 6 day laboratory tests. Error bars represent 1 standard error, * data significantly different from control at $p < 0.05$.

SfG is the difference between energy absorbed (A; open bars) and that lost during respiration (R; hatched bars). Hence, Figure 7 indicates that SfG remained positive at all concentrations tested. As respiration rates remained constant while absorption rates were significantly reduced at concentrations of 30 µgCu/l and over, a significant reduction in SfG was detected at concentrations 30 µg/l and over ($F = 13.759$, $df = 5, 68$, $p < 0.001$).

The energy budget parameter most sensitive to copper contamination, causing a significant reduction in SfG was consumption rate. Most energy consumed was lost via faeces (approximately 70 percent) (Fig. 5), while respiration only accounted for a relatively small proportion of the energy consumed (approximately 10 percent) (Fig. 7).

2.5. 3. Active and feeding Respiration rates.

a. Active respiration (R_a).

The standard respiration rate of *G.pulex* did not vary with copper concentration, therefore the standard respiration of the whole data set (line a, Fig. 8) was compared to the respiration rates of active animals. There was a significant elevation in respiration rates of *G.pulex* due to activity ($t > 6.35$, $df > 64$, $p < 0.001$). Maximum activity caused a 2.96 fold increase in respiration rates compared to standard levels (Fig. 8).

Under control conditions and when exposed to 21 µgCu/l there was a significant difference between animals at maximum activity and those at normal activity levels ($t > 2.37$, $df > 45$, $p < 0.05$), However the respiration rate of animals at maximum activity and those at normal activity when exposed to 52 µgCu/l were not significantly different ($t < 1.07$, $df = 36$, $p > 0.05$) (Fig. 8).

Although there was no significant relationship between copper concentration and respiration rates at maximum activity ($r^2 = 0.6\%$, $df = 86$, $p > 0.05$), the relationship between copper concentration and respiration rates of animals at normal activity levels was statistically significant, although variability was high ($r^2 = 4.01\%$, $df = 97$, $p < 0.05$). Hence *G.pulex* appeared to respond to copper by increasing its activity rate and in so doing incurred extra respiratory cost (Fig. 8).

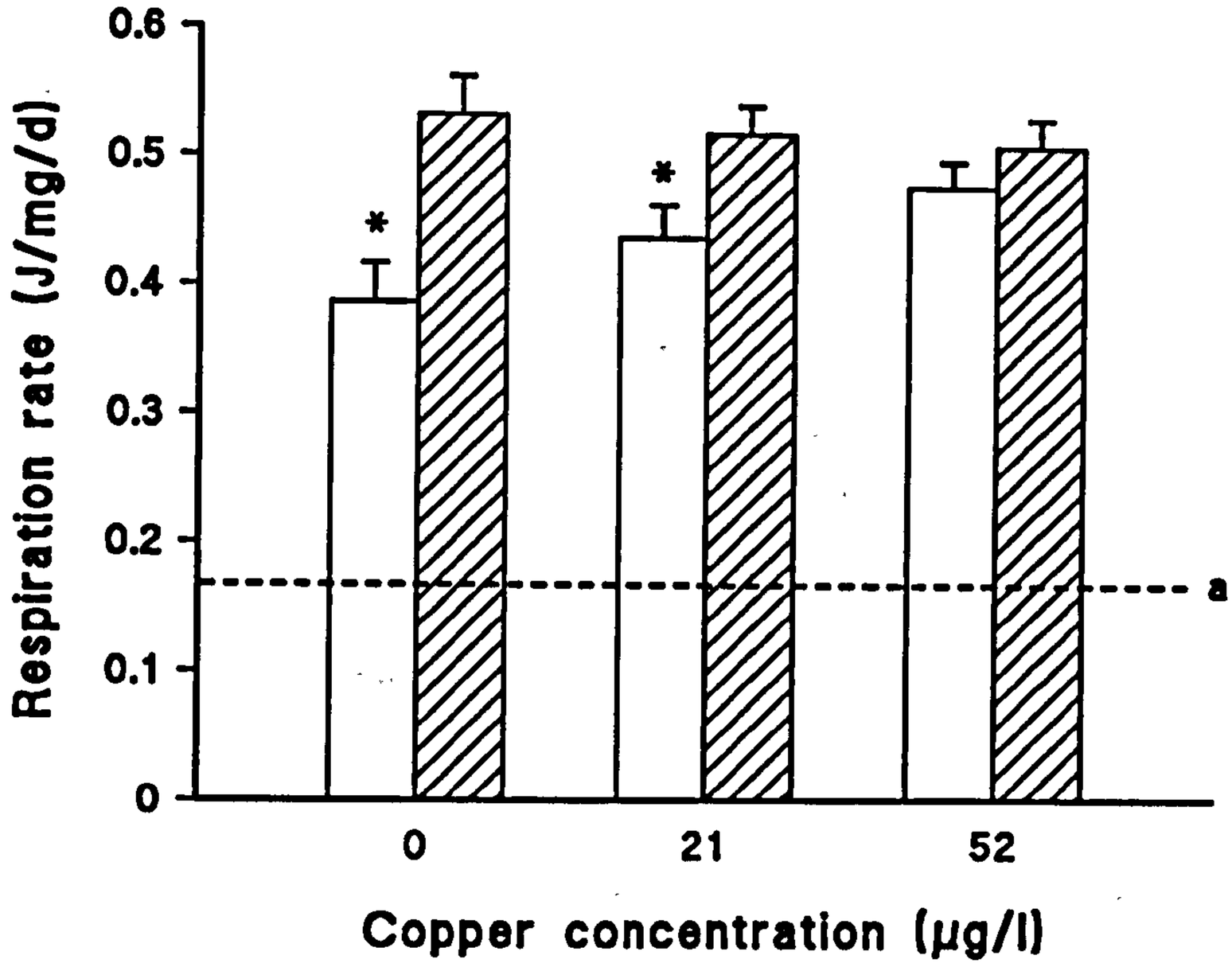


Fig 8. Effects of copper on respiration rates of *G.pulex* at normal (open bars) and maximum (hatched bars) activity. line a = Standard respiration rate (i.e. J/mg/d). Error bars represent 1 standard error, * data significantly different from respiration rate at maximum activity ($p < 0.05$).

b. Feeding respiration rate (R_f).

There was no significant difference between the oxygen uptake of nonfed animals and fed animals during the three base line measurements prior to feeding ($F = 0.778$, $df = 5, 34$, $p > 0.05$) (Fig. 9). The oxygen uptake of nonfed animals did not significantly change at any point over the period of the test ($F = 1.557$, $df = 8, 134$, $p < 0.05$); hence the experimental technique (opening and closing syringes etc) caused no change in oxygen uptake (Fig. 9).

In contrast there was a significant increase in oxygen uptake when animals were fed (($F = 8.914$, $df = 8, 134$, $p < 0.001$) (Fig. 9). Oxygen uptake increased dramatically during the 1 hour feeding period and then declined exponentially after removal of the food.

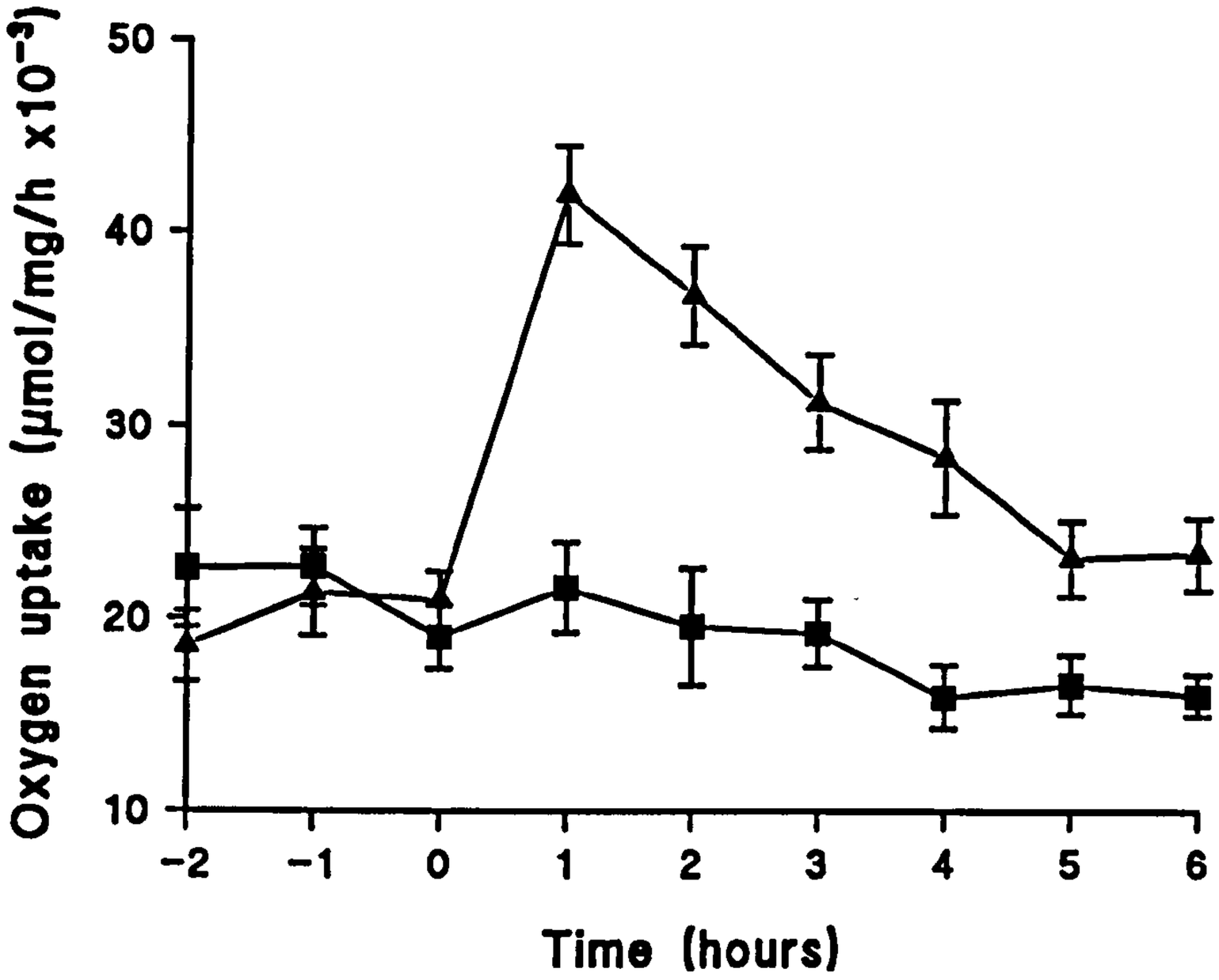


Fig. 9: Effects of feeding on oxygen uptake of *G.pulex*. Non-fed animals (solid squares) animals allowed to feed (solid triangles) (food added at time 0 and removed after 1 hour) Error bars represent 1 standard error.

By extrapolation of a log transformation of the exponential part of this curve (i.e. time +1 to +6) oxygen uptake of fed animals returns to prefeeding levels 5.61 hours after food was removed. Hence feeding respiration was represented by the elevation in oxygen uptake above prefeeding levels between hours +1 and +6.61 (Fig. 10).

The elevation in respiration rate caused by feeding respiration was calculated by integration of equation 2.15.

$$y = 47.867 - 6.318\,x + 0.349\,x^2 \qquad \text{eqn 2.15}$$

$$(r^2 = 93.4\%,\ df = 6,\ p < 0.01)$$

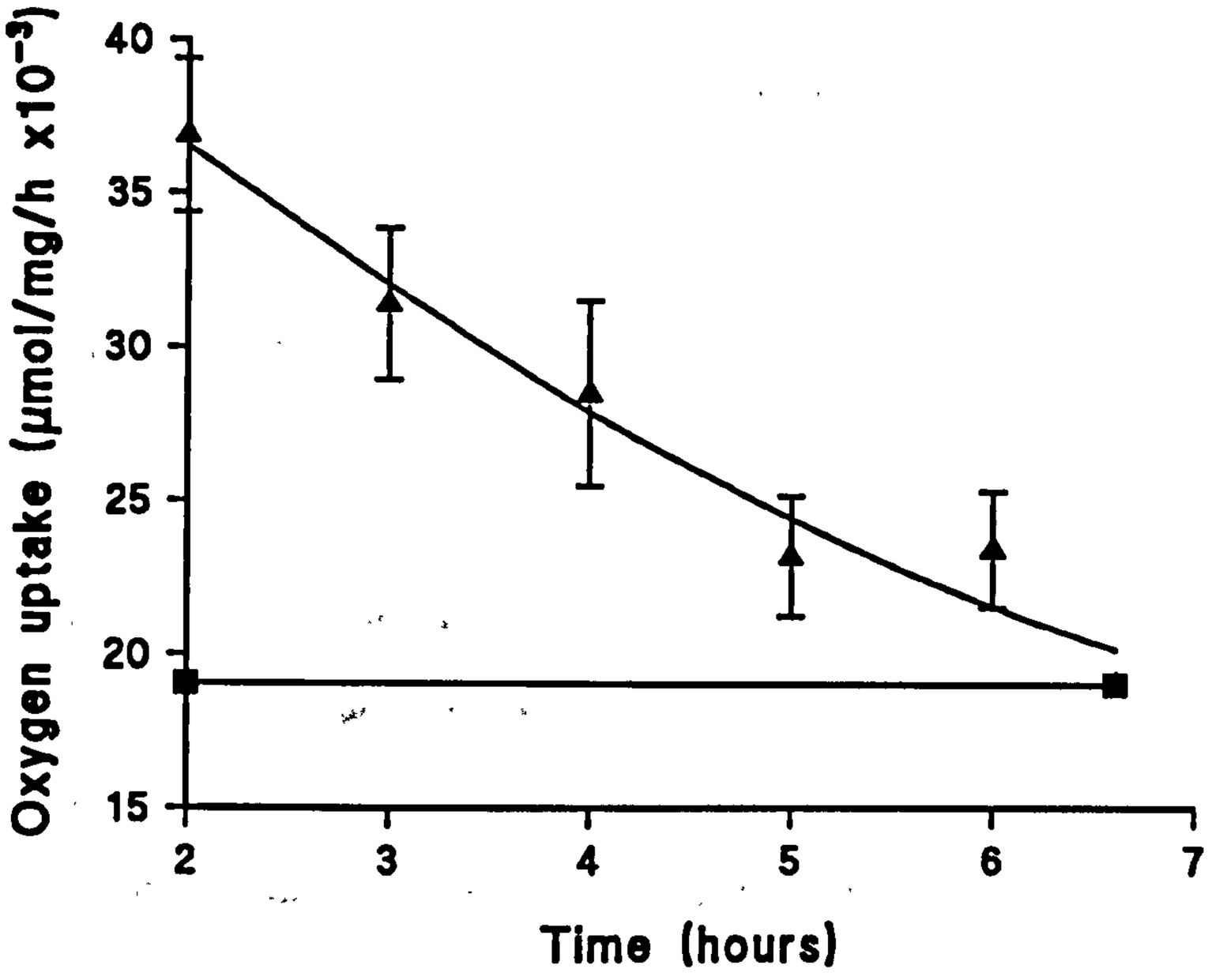


Fig. 10: Elevation of oxygen uptake due to feeding (solid triangles) over the mean prefeeding level (solid squares) Error bars represent 1 standard error.

Hence the elevation in oxygen uptake associated with feeding respiration was equal to 0.281 µmol/mg. This was converted to J/mg by multiplying by 22.41×10^{-6} and 21×10^{3} (see Section 2.3. 4. c) to give a value of 0.132 J/mg.

Although feeding induced an elevation in oxygen uptake, there was no significant relationship between the amount of food ingested and the increase in oxygen consumption ($r^2 = 0.01\%$, df = 15, $p > 0.05$). Hence R_f could not be quantified as a proportion of ingested or assimilated energy but represented 3.6 times the standard respiration rate during the 5.6 hours in which respiration rate was increased.

The increase in respiration rate during the hour in which animals were allowed to feed (hour 0 to 1) was likely to be largely due to the mechanical costs of handling and ingesting the food. This cost represented 1.5 times standard levels.

2.5. 4. Overall energy budget.

Overall energy budgets for this population of *G.pulex* indicated that the greatest energy loss, (approximately 70 percent), was via the production of faeces (Fig. 11). Respiratory losses were comparatively low with standard metabolism representing only 3 to 8 percent of ingested energy. Although active and feeding respiration rates were not measured at each copper concentration these respiratory costs were found to represent constant multiples of standard respiration rates and therefore for the purposes of this study they were calculated as such; maximum active respiration represented an additional 6 to 16 percent and feeding respiration represented 8 to 20 percent of ingested energy (Fig. 11). The contribution of feeding respiration to the energy budget is likely to be an overestimate. As no relationship was found between respiration rates and the amount consumed, an estimate for feeding metabolism was taken from the elevation in respiration rates over standard levels. This elevation was only apparent for approximately 6 hours after feeding and hence the contribution of feeding metabolism to the overall energy budget is likely to be much less than predicted.

At concentrations of 27 µg/l and below, the energy remaining for production is positive and growth and reproduction can occur. At concentrations of 30 µgCu/l and over, the energy required for maintenance exceeds that available, hence the animals are in negative energy balance. At concentrations above 30 µgCu/l animals must use their own body reserves for metabolism. One parameter not measured in this study is the energy loss due to excretion. Excretion has been found to account for 10 to 30 percent of total metabolic energy in a number of amphipod species (Dresel and Moyle, 1950) and represents approximately 3 to 10 percent of absorbed energy in *G.pulex* (L. Maltby, pers comm). If

this estimate of energy losses due to excretion were incorporated into the budget, P would become negative at 23 µg/l although not at 27 µg/l.

On the basis of this energy budget it would be predicted that exposure to copper concentrations between 23 to 30 µg/l and over, would result in a reduction of growth and reproduction. An investigation of this is the subject of chapter 3.

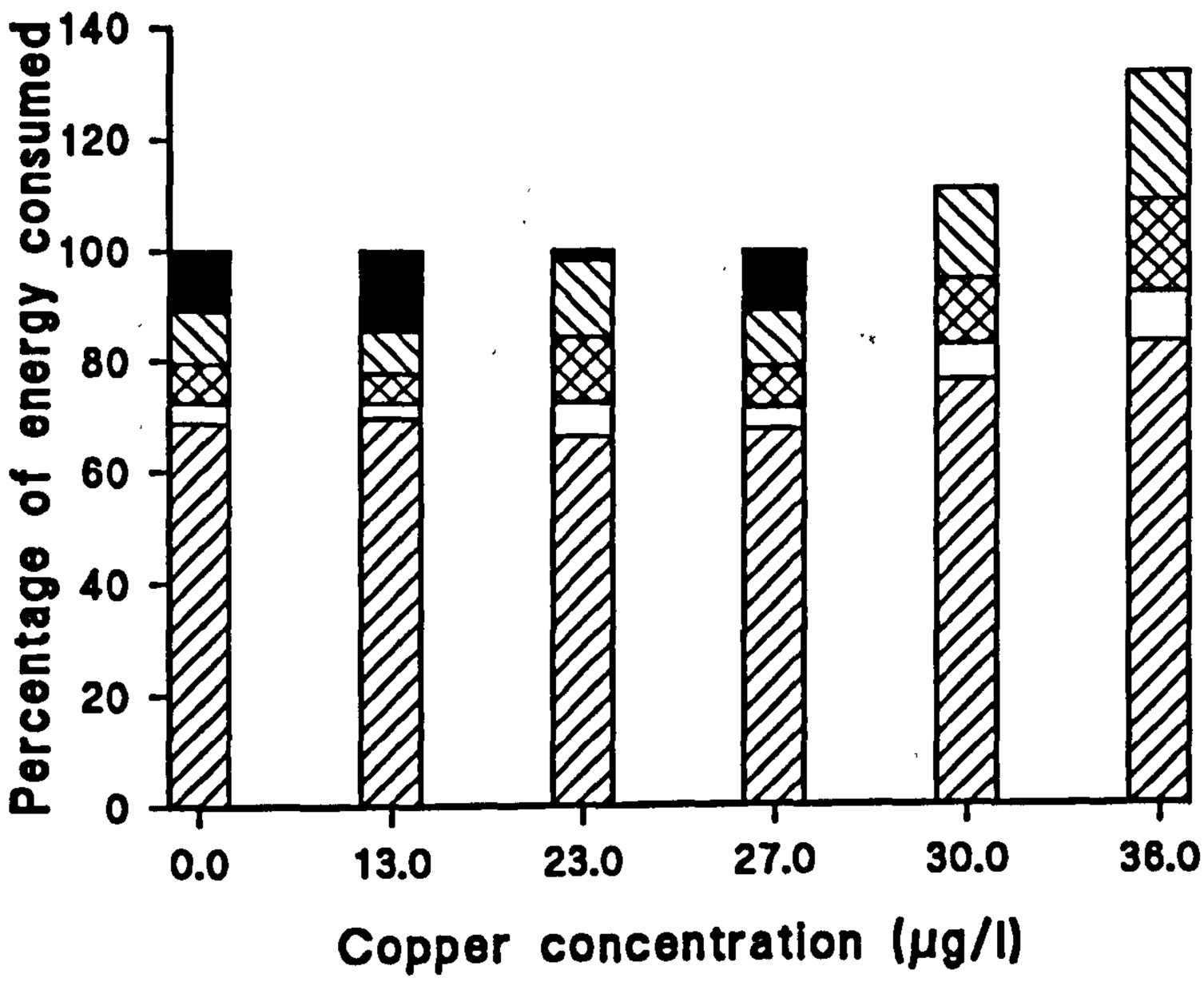


Fig. 11: Overall energy budget for *G.pulex* exposed to copper. Bars represent percentage of ingested energy lost via faecal production (hatched bars), standard respiration (open bars), active respiration (cross-hatched bars), feeding respiration (back-hatched bars) and that remaining for production (solid bars).

2.5. 5. Behavioural detection and selection of food.

The concentration of copper in the leaf material offered to individual *G.pulex* ranged from 21.7 µg/g to 287 µg/g with the average weight of a leaf disc being approximately

3mg. Percentage consumption of the leaves was highly variable between treatments and there was no significant difference in the percentage of each leaf type consumed by the animals when offered the choice between leaves of different copper contents ($F = 3.828$, $df = 4, 124$, $p > 0.05$) (Fig 12).

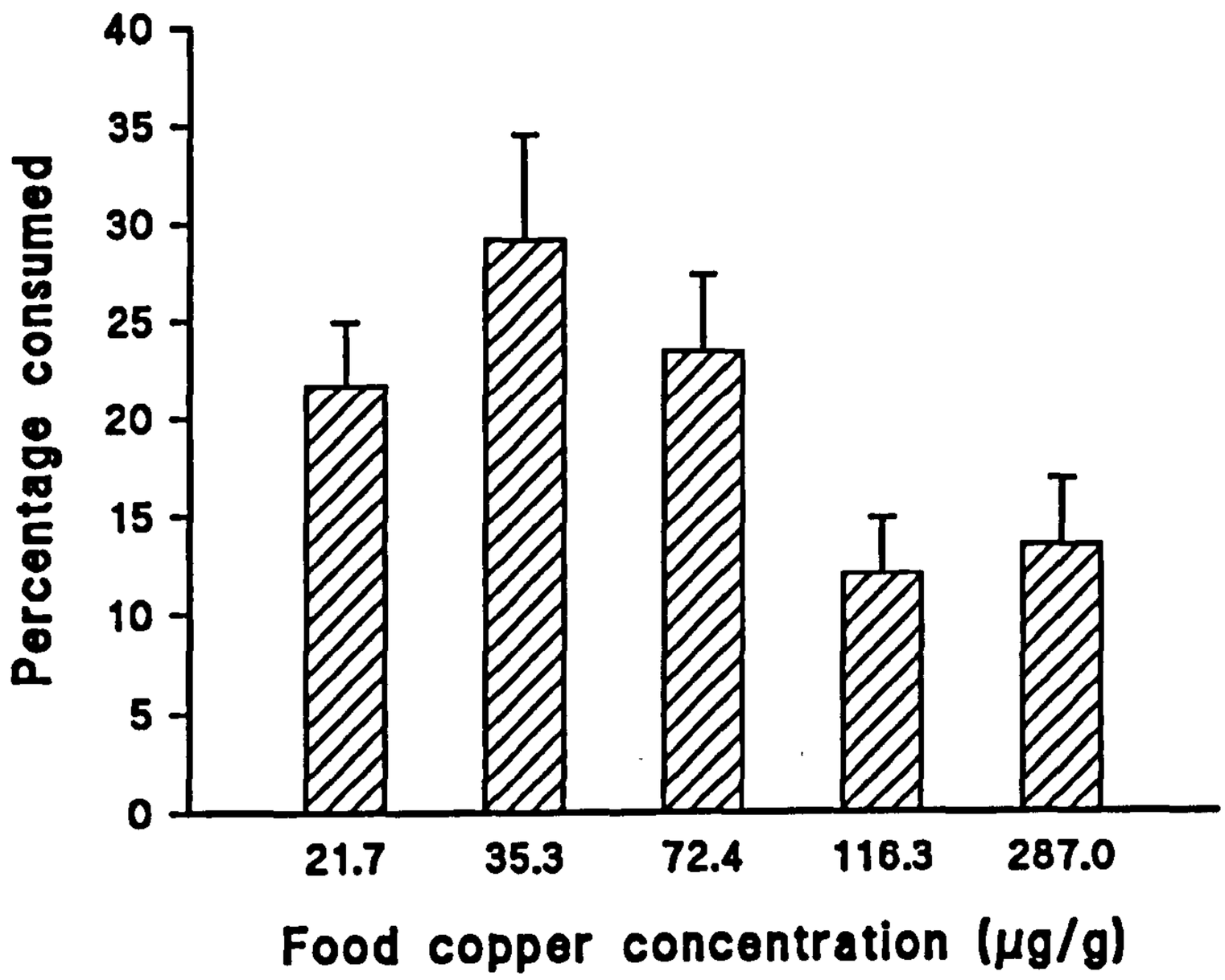


Fig. 12. Food preferences of individual animals offered food of different copper concentration (µg/g). Error bars represent 1 standard error.

The use of groups masked some of the individual variability in the quantity of each leaf type eaten (Fig. 13). The copper concentrations of leaf material offered to groups of animals ranged from 19.6 to 364.5 µgCu/g. There was a significant increase in the consumption of copper contaminated leaves ($F = 4.846$, $df = 4, 23$, $p < 0.001$) with the exception of those with copper concentration of 69.5 µgCu/l. Hence animals appeared to consume a greater proportion of contaminated leaves over the proportion of control leaves consumed (although the increase was less than 10 percent), therefore the observed

decrease in consumption rates due to exposure to copper were unlikely to be due to a behavioural rejection of contaminated food.

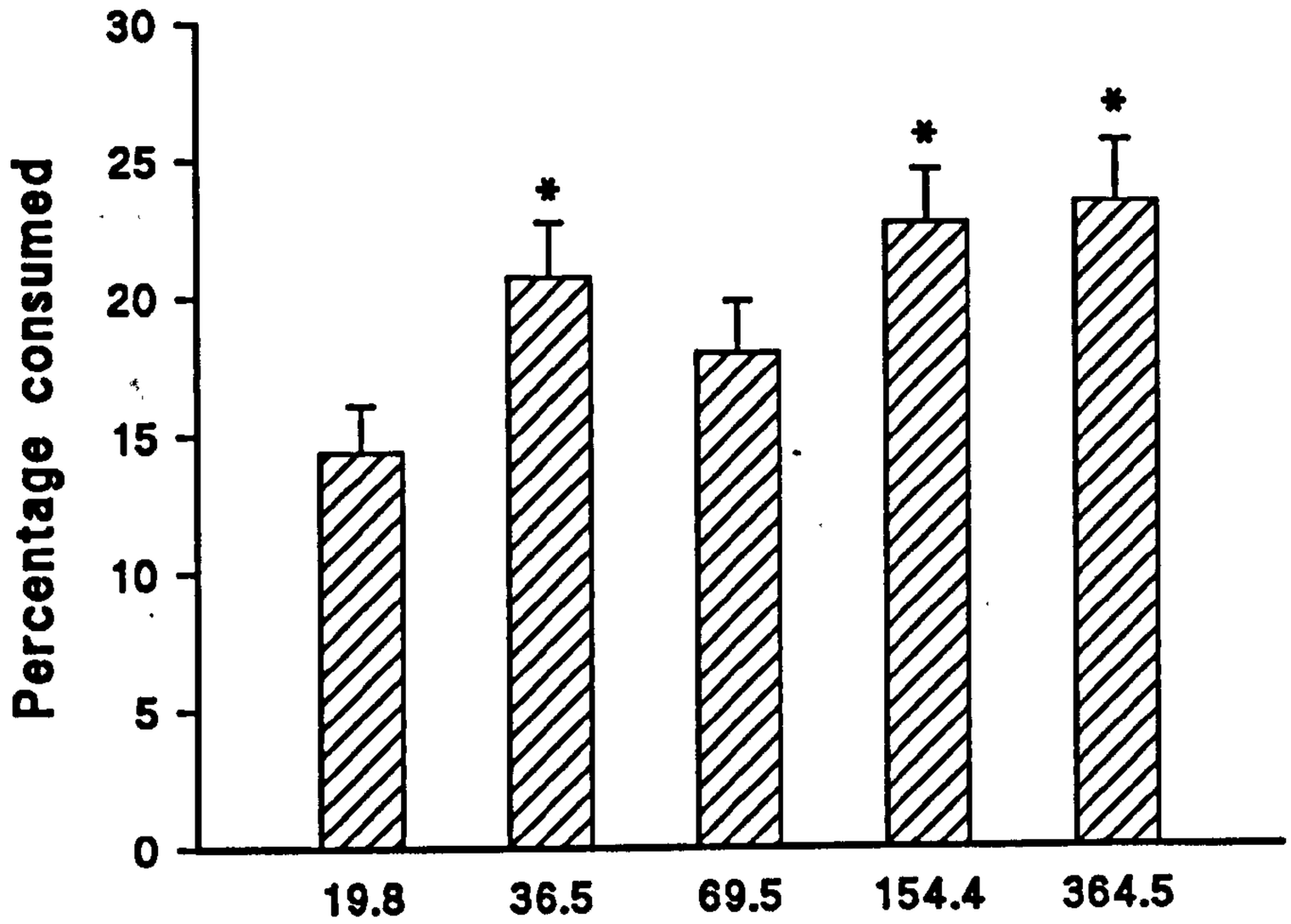


Fig. 13. Food preferences of groups of 10 animals offered food of different copper concentrations (µg/g). Error bars represent 1 standard error, * data significantly different from control at $p < 0.05$.

2.5. 6. Body copper concentration.

Contrary to previous work (Rainbow and Moore, 1986) no significant relationship between dry weight and body loading was found ($r^2 < 39.1\%$, df = 8, $p > 0.05$). This was probably due to the fact that only large animals were used (mean weight = 10.5 mg; SE = 1.8), whereas Rainbow and Moore (1986) studied animals over a weight range of near 0 (birth) to 5 mg. As there was no residual effect of dry weight on body concentrations, absolute values of body loading could justifiably be compared in this study.

Control animals had a mean body copper concentration of 33.62 µg/g. Copper concentrations of animals exposed to copper increased linearly, resulting in a significant correlation with external copper concentration of the media (r^2 = 41%, df = 57, p < 0.001) (Fig. 14).

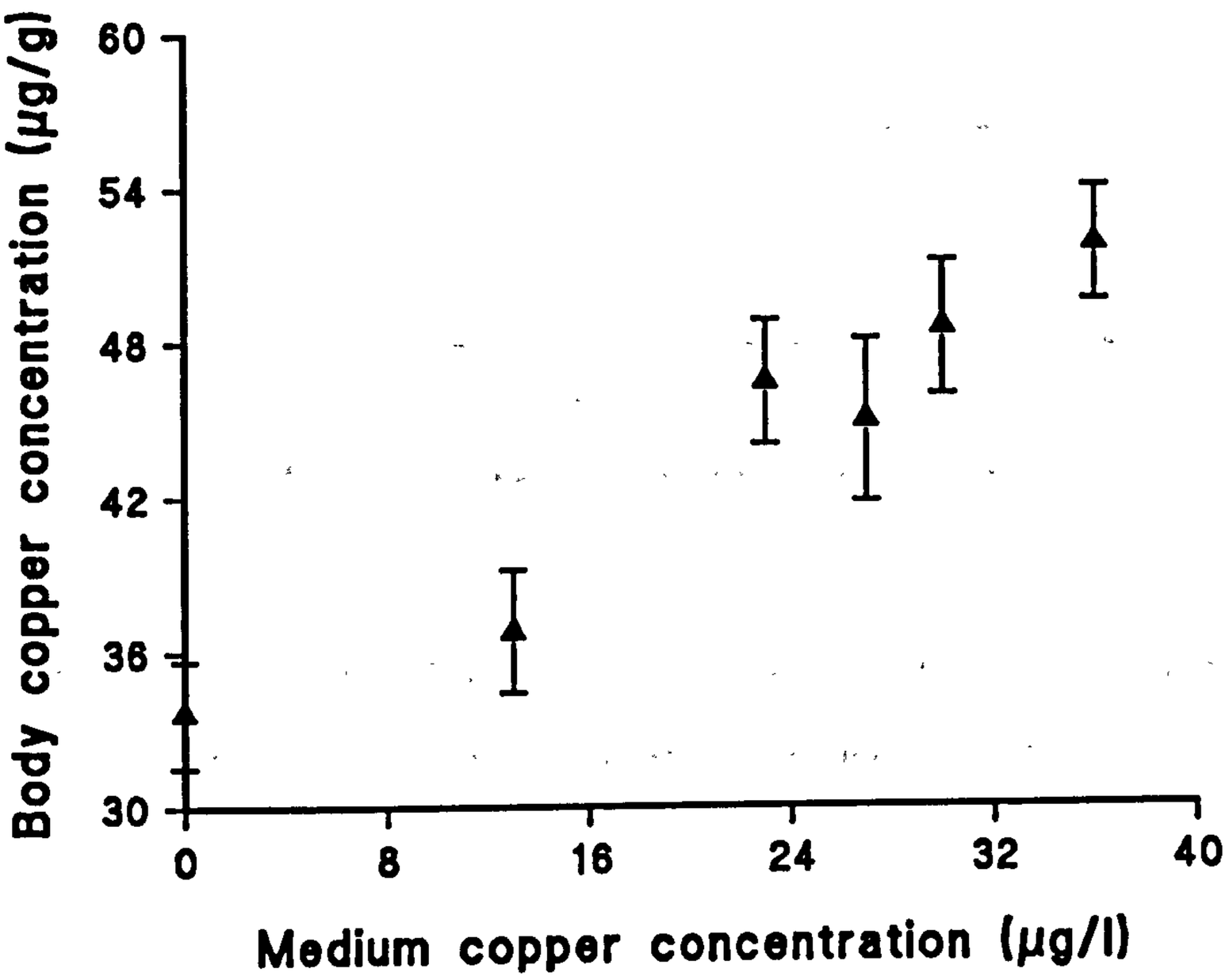


Fig. 14. Body copper concentration of animals (µg/g) exposed to different copper concentrations in the external media(µg/l). Error bars represent 1 standard error.

There was also a significant negative correlation between consumption rates and animal body concentrations (r^2 = 16.9%, df = 56, p < 0.001) (Fig 15). Therefore it is likely that the decrease in consumption rates observed in response to exposure to copper is due to the gain of an excessive body copper concentration.

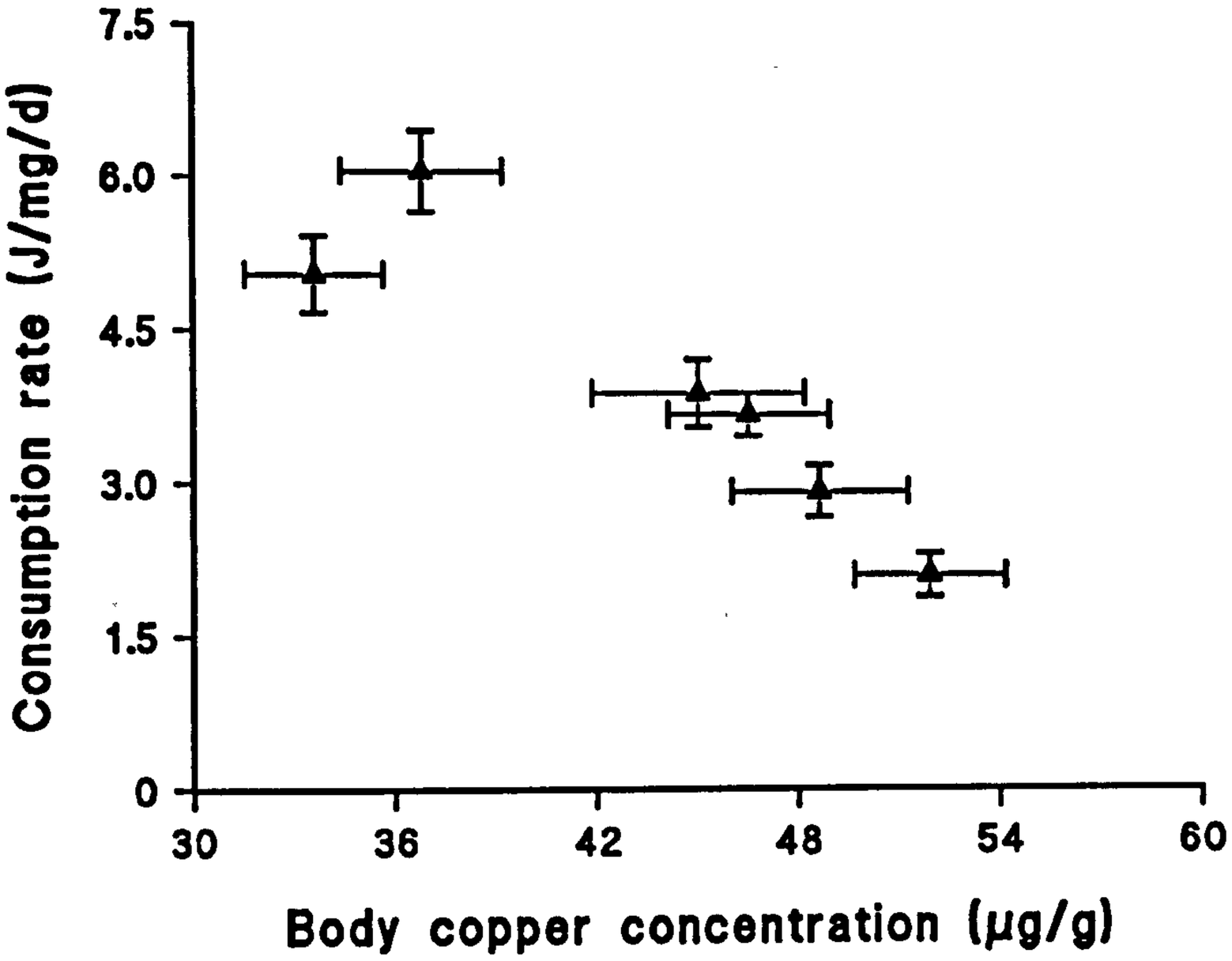


Fig 15. Relationship between body concentrations (µg/g) and consumption rates (J/mg/d) of animals exposed to copper. Error bars represent 1 standard error.

2.5. 7. Uptake route of copper.

In order to examine the uptake of copper from the media, *G.pulex* was exposed to nominal copper concentrations of 0 or 30 µgCu/l actual initial concentrations were 0 µg/l (clean) or 10.855 µgCu/l (SE = 0.148) (contaminated) (Table 3). Animals were provided with approximately 12mg of leaf material when fed with the initial copper content of the food being 68.4 µg/g (SE = 12.8) and 240.28 µg/l (SE = 83.57) for clean and contaminated food respectively.

Total copper concentrations of food increased when subsequently placed in contaminated water whether or not it had been presoaked in toxicant solutions (Table 3). Hence the leaf material appeared to adsorb copper out of the media. There was no significant difference between the copper content of clean and inaccessible or accessible contaminated leaf

material offered to *G.pulex* in contaminated water ($t > 1.4$, $df = 17$, $p > 0.05$). There was however a significant difference between inaccessible and accessible contaminated food in contaminated water ($t > 3.84$, $df = 11$, $p < 0.01$) (Table 3).

In contrast, the copper concentration of contaminated leaves decreased when subsequently placed in clean water and the copper content of all food subsequently placed in clean water was significantly lower than that of food in contaminated water ($t > 8.2$, $df = 15$, $p < 0.001$) (Table 3). There was no significant difference between the copper content of clean and accessible contaminated leaves in clean water ($t < 0.21$, $df = 13$, $p > 0.05$) or between clean, accessible contaminated leaves or inaccessible leaves ($t < -2.5$, $df < 17$, $p < 0.01$) (Table 3).

Table 3. Concentrations of copper in the food (μg/g) and water (μg/l) of different combinations studied: a to d represent clean and e to f contaminated water; a and e were offered no food, b and f clean food, c and g contaminated inaccessible food and d and h contaminated food (see Table 1).

code	Media Copper Concentration	Food Copper Concentration
a	0.000	/
b	0.000	29
c	0.000	65(I/A)
d	0.000	39
e	10.855	/
f	10.855	325
g	10.855	229(I/A)
h	10.885	409

((I/A) = Inaccessible food, i.e. contained in a fine mesh bag).

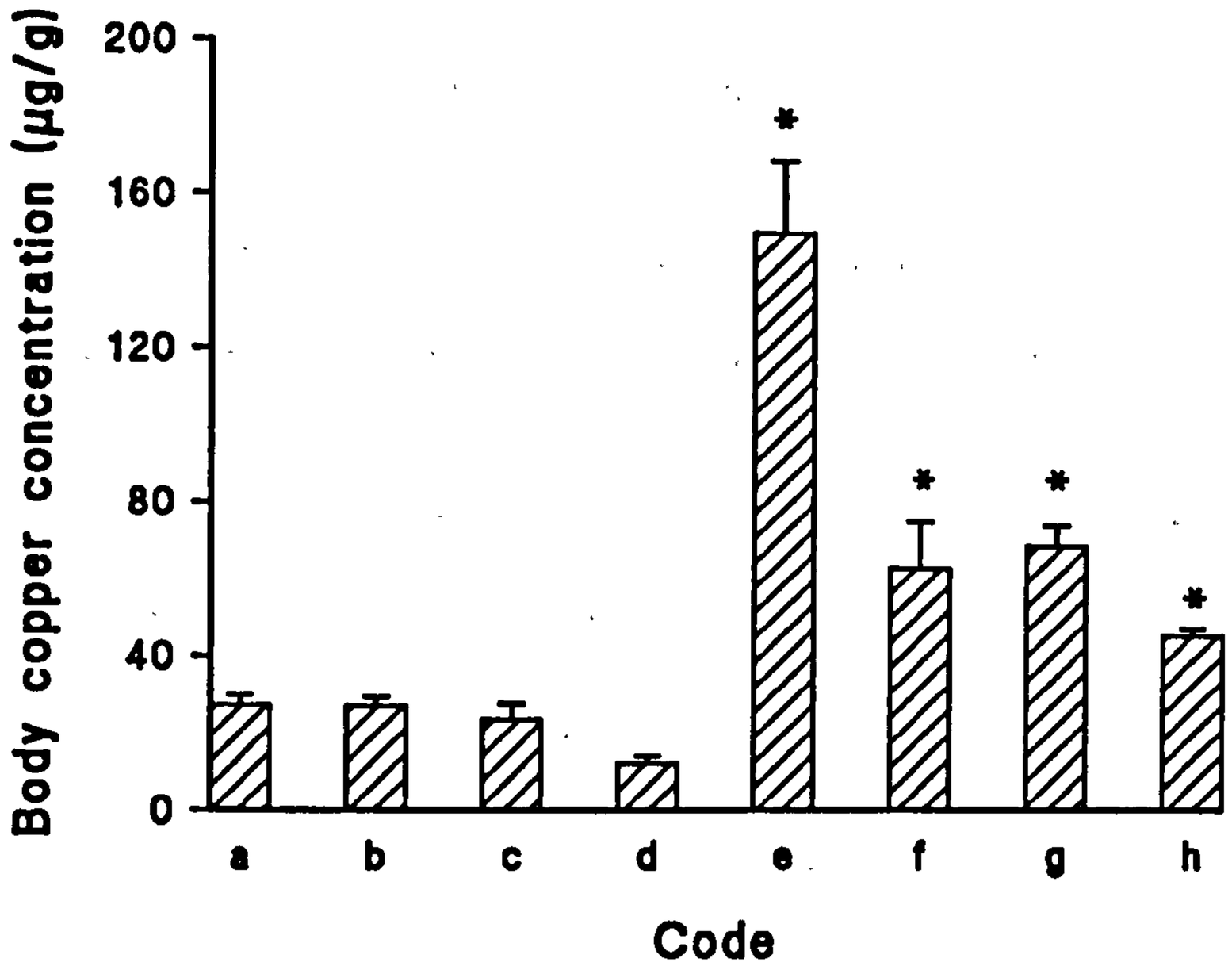


Fig 16. Body concentration (µg/g) of animals exposed to different concentrations of copper in food (µg/g) and in the media (µg/l). (see Tables 1 and 3 for definition of codes a - h) Error bars represent 1 standard error, * data significantly different from control (a) at $p < 0.05$.

Body concentrations of animals in contaminated water were significantly greater than control animals exposed only to clean water (a) ($t > 2.92$, $df > 9$, $p < 0.05$) (Fig. 16, Table 4). The greatest body copper concentration (150.48 µg/g) was that of animals exposed to copper contaminated water only (e) (Fig. 16). Body concentrations of these animals were significantly greater than control animals (27.34 µg/g) exposed to clean water only (a) and animals exposed to all other food and water combinations ($t > 5.89$, $df > 13$, $p < 0.001$).

Body copper concentration of animals exposed to contaminated water but with food present were significantly lower than those in contaminated water with no food ($t > 3.91$, $p < 0.01$, $df = 13$; Fig. 16, Table 4). This was probably due to copper being adsorbed to

the food and hence removed from the water. This is supported by the greater copper content of leaves in f and h (although not g) over initial contents (Table 3).

Table 4. Significant differences in body copper concentrations of animals exposed to different combinations of contaminated or clean food and water: a to d represent clean (i.e 0 μgCu/l) and e to f contaminated (i.e 10.86 μgCu/l) water; a and e were offered no food, b and f clean food, c and g contaminated inaccessible food and d and h contaminated food (see Tables 1 and 3).

code	a	b	c	d	e	f	g	h
a	0	NS	NS	*	*	*	*	*
b		0	NS	*	*	*	*	*
c			0	*	*	*	*	*
d				0	*	*	*	*
e					0	*	*	*
f						0	NS	NS
g							0	*
h								0

* Significantly different ($t > 2.52$, $df > 8$, $p < 0.05$)

NS no significant difference ($t < 1.45$, $df > 9$ $p > 0.05$)

When exposed to copper in the medium, there was no significant uptake of copper from food (i.e. there was no significant difference between body copper concentrations of animals exposed to contaminated water and offered either contaminated food (g and h) or non-contaminated food (f) ($t < 1.45$, $df > 9$, $p > 0.05$)) (Fig. 16; Table 4). Further, the body copper concentration of animals that were unable to eat the contaminated food (g), were significantly greater than those which were (h) ($t > 4.17$, $df > 11$, $p < 0.001$) (Fig.

16, Table 4). This again indicates that the major uptake route of copper is via the water and not the food.

Further evidence supporting this was indicated by there being no significant difference between body copper concentrations of animals exposed to clean water and offered clean food or inaccessible contaminated food. There was in fact a significant reduction in the body copper concentrations of animals in clean water offered contaminated food ($t > 5.02$, $df > 15$, $p < 0.001$; Fig. 16, Table 4).

2.6. Discussion.

The standard 96 hour copper-LC_{50} for *G.pulex* in this study was 64.5 µg/l (95 % C.I. 41.01 - 101.37). This compares with lower estimates of other workers although differences in water hardness between studies makes direct comparison difficult (Table 5).

Stephenson (1983) showed that copper was 4 to 6 times more toxic in soft water than in hard water. The lower LC_{50} values recorded in the study of Taylor et al (1991) are likely to be due to the softness of the water. Despite differences in water hardness, animals in this study appear to be slightly more sensitive than those in the study of Stephenson possibly due to inter-population differences or differences in experimental procedures.

Table 5: LC_{50} values of *G.pulex* exposed to copper in laboratory tests.

Test	Water hardness (mg/l $CaCO_3$)	LC_{50} µg/l	Ref
96 h LC_{50} (Not fed)	104	21	1
96 h LC_{50} (Not fed)	249	109	1
48 h LC_{50} (Fed)	85	47	2
96 h LC_{50} (Fed)	85	37	2
240 h LC_{50} (Fed)	85	33	2
96 h LC_{50} (Not fed)	200	65	3
144 h LC_{50} (Fed)	200	45	3

1. Stephenson (1983), 2. Taylor et al (1991), 3. This study.

Effects of copper on energy budget parameters were greater than those observed for survivorship. There was a two fold difference between the 6 day LC_{50} (45 µgCu/l) and the copper concention causing a significant decrease in consumption rates of *G.pulex* (i.e. 23 µgCu/l). Consumption rates and faecal production were the most sensitive of the response criteria measured. The linear relationship between faecal production and consumption rates of animals in this study gave a regression equation (i.e. eqn 2.14; F = 0.664 C + 0.151) comparing favourably with those of Maltby and Naylor (1988) viz:

$$F = 0.74\ C - 0.303$$

(r^2 =91.8%, n = 226)

and Crane and Maltby, (1991)

$$F = 0.5811\ C + 0.545$$

$$(r^2=64.4\%)$$

$$F = 0.6169\ C + 0.623$$

$$(r^2=60.3\%)$$

for two populations, the first from Crags stream and the second from Haseley brook.

The intercept of the line is closest to approaching the origin in this study (eqn 2.14), while the gradient of the line is comparable to the other studies. Hence faecal production is predictable from a knowledge of consumption rates.

However, despite the linear relationship between consumption rates and faecal production, significant effects of copper toxicity on absorption were not detected at 23 µgCu/l but at 30 µgCu/l. The lack of a significant effect of copper on absorption at lower concentrations was likely to be a result of the slightly greater effect of copper on consumption rates compared to faecal production as mentioned previously (Section 2.5. 2. c).

In most studies of the effects of toxicants on scope for growth, standard metabolism is used as the measure of energy loss via metabolism as differences in standard metabolic rates have long been recognised as having the greatest influence in energy expenditure (Vansteenbergen, 1987; Waterlow, 1988). In this study there was no effect of copper on standard respiration rates at any of the copper concentrations investigated (although the relative cost of respiration does increase). Hence, a significant effect of copper on SfG was apparent at 30 µgCu/l.

Consumption rates were therefore the most sensitive of the energy budget parameters measured and were the major parameter causing a decrease in SfG. The increased sensitivity of energy supply over energy expenditure has been noted in other studies, for example Widdows (1985) working with *Mytilus edulis* stated that consumption rates were the energy budget component which was typically the most responsive to pollution. This has been found to be the case for a diverse range of species and chemicals; the mussel *Mytilus edulis* in response to oil pollution, diesel oil and copper (Widdows et al, 1987a, b; Widdows and Johnson, 1988) *Arca zebra* in response to a complex mixture including lead, aromatic hydrocarbons, tributyl tin and polycyclic biphenols (Widdows et al, 1990) the marine snail *Thais lima* in response to oil (Stickle et al, 1984) and *G.pulex* in response to zinc, 3,4-dichloroaniline, oxygen, pH, sewage and a sewage/ coal mine effluent mixture (Maltby et al, 1990a, b).

Despite the evidence from such studies, one must be cautious about ignoring respiratory costs since measurement of standard respiration rate is likely to under-estimate energy losses via metabolic processes. Animals require metabolic energy in natural situations for a diverse range of additional functions. The two most important of these are the metabolic demands for activity and for feeding.

Active and standard metabolism combined, in this study, represent about 10 percent of ingested energy compared to about 22 percent in fish (Cui and Lui, 1990). Results given in Section 2.5. 5. a, indicate that active metabolism is 2.96 times basal metabolism. This compares well with other studies; active metabolism was two to three times basal in *G.oceanicus* (Bulnheim, 1972: In Maltby, 1991), two to four times in *G.minus* (Culver and Poulson, 1971), and approximately three times in *G.pulex* (Maltby 1991).

Copper appears to affect energy losses due to active metabolism by causing a change in the behaviour of *G.pulex* such that it increases its activity. This increase in activity has an associated cost causing an increase in respiration rate. Changes in the activity of *G.pulex*

when faced with concentrations of toxic chemicals in the media was first noted by Jones (1937) who stated that, 'When placed in toxic salt solutions, *Gammarus* swims continually for the greater part of the survival time...'. This avoidance response was well illustrated by Costa (1966) for *G.pulex* exposed to copper in apparatus whereby half the chamber contained clean media and the other half contaminated media. The animals displayed a prompt and well developed negative reaction on entering the contaminated zone, swimming vigorously back into the clean zone.

Swimming activity is energetically costly and there is a positive linear relationship, between activity and oxygen uptake (Halcrow and Boyd, 1967). Therefore exposing animals to copper would result in elevated respiration rates due to an avoidance reaction resulting in increased activity. This increase in activity and consequent elevation in respiration rate was observed in this study on exposure of animals to copper for a six hour period.

There must, however, be a limit to the period that animals exposed to contaminated water will display this avoidance response when there is no refuge from it. Hence, this increased activity and consequent increased respiration rate may be transitory. In fact the activity of animals exposed to copper during the 6 day measurement of other energy budget parameters did not appear, by observation, to be any different from control animals exposed to clean water.

Effects of elevation in respiration rate due to feeding have been demonstrated by many other workers for various aquatic organisms, (e.g. Warren and Davis, 1967; Nelson et al, 1977; Jobling and Davis, 1980; Cui and Wooton, 1988; Carefoot, 1990 a, b, c; Cui and Lui 1990). Most studies have been conducted on fish and SDA was found to generally account for 9 to 20 percent of ingested energy. However, in fish it is rather difficult to separate SDA and active metabolic rates. Measurements of SDA in *Daphnia* accounts for 11 to 13 percent of ingested energy (Bohrer and Lampert, 1988). SDA of *Ligia* varied

according to the chemical composition of the diet being 2.7 percent of ingested energy when fed a complete chemical diet as opposed to representing 12 to 17 percent of consumption when fed *Ulva lactura* or *Nereocystis luetkeana* (Carefoot, 1990a). In general the higher the protein content of the diet the greater is the associated cost due to SDA.

Although some workers have found a significant positive correlation between the elevation in respiration rate and the quantity of food ingested (e.g. Beamish, 1974; Bohrer and Lampert, 1988; Carefoot, 1990a, b, c), others, including this study, have failed to do so (e.g. Nelson, 1977; Barber et al, 1990). This lack of correlation may have been due to the short duration of feeding in which there was little variation in the quantity of food consumed, or may be a real 'all or nothing' response of respiration rate upon feeding.

Nelson et al (1977) found an increase in oxygen consumption of 7 to 40 percent above standard metabolism depending on diet. In this study feeding respiration represented 3.6 times the standard respiration rate or approximately 8 to 20 percent of the energy consumed in SfG tests. However, this is likely to be an overestimate due to the short duration of these tests.

The common use of standard respiration rates in determination of effects of a toxicant on SfG is therefore thought to be most useful, and determination of active and feeding respiration rates in routine SfG tests, unnecessary. Effects of activity on respiration rate are likely to be transitory. The measurement of feeding respiration rates is problematic and since it represents a constant increase over standard levels, not being related to the quantity of food consumed, the relative contribution of feeding metabolism to the total energy budget is constant across concentrations. However, experiments where animals are allowed to feed for different periods of time and consume more variable amounts of food are necessary to confirm this.

Overall energy budgets for this population of *G.pulex* indicated that the greatest energy loss, (approximately 70 percent), was via the production of faeces. Respiratory losses were comparatively low with standard metabolism representing only 3 to 8 percent of ingested energy and maximum active respiration an additional 6 to 16 percent. Feeding respiration represented 8 to 20 percent of ingested energy although this was measured over a short time period and overall costs were likely to be much lower. Low energy requirements for maintenance should relate to lower whole-body protein turnover, (Hawkins, 1991). A consequence of this could be greater stability in the face of toxicants due to a greater amount of metabolizable energy available for other processes such as production. However, if high protein turnover is required for protective mechanisms or repair in the face of toxic stress, then fitness would be increased by greater protein turnover and hence higher respiration rates.

SfG, ($A - R_S$), was significantly reduced at copper concentrations of 30 µg/l and over and became negative at a copper concentration of 36 µg/l. Hence, if SfG is a reliable indicator of an individual's energy balance, growth and reproduction should be reduced at concentrations above 30 µg/l. When in negative energy balance, animals must use their body reserves for metabolism, and death may ensue.

The observed decrease in SfG on exposure to copper was primarily due to a decrease in consumption rates. This decrease in consumption rates could have been due to either detection of copper in the food and behavioural rejection, or the gain of a body concentration of copper and subsequent cessation of feeding.

Although invertebrates such as *G.pulex* have the ability to distinguish between different leaf types, preferred fungal species and optimally conditioned food (e.g. Barlocher and Kendrick, 1973a; Arsuffi and Suberkropp, 1984 and 1985), they did not discriminate between non-contaminated and copper contaminated food (Section 2.5. 5) It therefore seems unlikely that copper-induced reductions in consumption rate were due to a

behavioural response. There was, however, a significant negative relationship between body copper concentration and consumption rates, (Section 2.5. 6.), suggesting that the cause of decreased consumption rates was an increase in body copper concentration.

Copper may enter *G.pulex* either via the food or water. Previous studies on uptake routes have shown the potential of both water and food as sources of metals. Tessier et al (1984) suggest the main route of accumulation of copper (and lead and zinc) by the freshwater mollusc, *Elliptio complanata*, was by uptake via the gills and mantle and not by the ingestion of particulate material. Brown (1977) reported that *Asellus meridianus* accumulated copper and lead from solution while tolerant animals also accumulated both metals from metal enriched food. Abel and Barlocher (1988) found that *G.fossarum*, eating leaves contaminated with cadmium, suffered increased mortality and accumulated cadmium in their tissues. However, in soft water the direct effects of cadmium uptake via the water were much more severe than effects suffered through ingestion of contaminated food.

In an investigation of the influence of feeding habits on whole body metal concentrations in aquatic insects, Smock (1983), indicated that the location at which the organism feeds (e.g. sediment, water column etc) and mechanism of feeding (e.g. shredding, filter feeding) are of great importance in determining the body metal load. Availability of metals from food depends on their chemical form and very stable compounds may not be broken down by digestion (Bryan 1976).

The uptake route experiment performed as part of this study indicated that the medium was the main source of copper to *G.pulex* (Section 2.5. 7.). The greatest body concentration was gained when animals where placed in copper contaminated media with no food present. The body concentration gained by animals exposed to contaminated media but with food present was lower than when food was not supplied probably due to a decrease in copper availability caused by adsorption or complexation of copper with

leaf material. Because control leaf material accumulated copper when placed in contaminated media and contaminated leaf material lost copper when placed in control media, it was difficult to distinguish between body concentration gained via food or media. One way to overcome this problem is to provide animals with food which they are unable to consume. When animals where exposed to contaminated media and contaminated food that was accessible, there was no significant difference in the body concentrations of animals in treatments where contaminated food was or was not accessible. This clearly shows that the major route of uptake of copper was via the water and uptake of copper from the food was not important.

Copper is an essential metal and is required by *G.pulex* for the functioning of a variety of enzymes and in the oxygen binding centre of the oxygen carrying pigment, haemocyanin. White and Rainbow (1985) estimated theoretical minimum metabolic requirements of copper in crustaceans to be 26.3 µg/g dry weight for enzyme production and 65.5 µg/g dry weight for haemocyanin production, giving a total requirement of 83.7 µg/g.

This theoretical value was considerably higher than the mean measured values in *G.pulex* in this study (33.6 µg/g; SE = 2.06). The estimates of White and Rainbow (1985) related to decapod crustaceans, it may be that *G.pulex* contains less haemocyanin than a decapod crustacean and would therefore be expected to have a lower metabolic requirement. However, even when copper concentrations of *G.pulex* measured here are compared to those reported in the literature for other *Gammarus* species, they still appear unusually low (Table 6).

Table 6. Body copper concentration (μg/g) of *Gammarus* sp. reported in the literature.

Species	Body concentration	SE	Notes	Reference
G.pulex	75	/	/	1
G.pulex	82.6	34	metaliferous site	2
G.pulex	104	22	clean site	2
G.pulex	33.6	2.06	clean site	3

1. Bourne, 1985. 2. Rainbow and Moore, 1986 3. This study.

Although decapod crustaceans can regulate the body concentration of essential metals such as copper, malacostracea, such as *Gammarus*, do not and body concentrations are positively correlated with external concentrations (Fig. 14). Previous results have apparently suggested that amphipods can regulate metals (e.g. Rainbow and Moore, 1986; Johnson and Jones, 1989; Xu, 1990), however this apparent ability to regulate was in fact due to low net metal accumulation in these larger animals compared to body growth rate in a short time period (Rainbow and Moore, 1986; Weeks and Rainbow, 1991).

Hence it has been determined that SfG (A - Rs) in *G.pulex* is reduced by exposure to copper. This reduction in SfG was primarilly a result of a reduction in the amount of energy acquired. Consumption rates of *G.pulex* were reduced due to an increase in body copper concentration due to uptake of copper from the medium.

2.7. Summary.

1. The standard 96 hour copper LC_{50} for *G.pulex* was found to be 64 µgCu/l. This was lower than previously reported values suggesting that animals in this study may be slightly more sensitive to copper than those used by other workers.

2. Copper caused a significant reduction in consumption rates at a concentration of 23 µg/l, while basal metabolism was unaffected at all concentrations studied. Despite a linear relationship between consumption rates and faecal production, a significant reduction in absorption and SfG was detected at 30 µg/l rather than 23 µg/l. Effects of copper on consumption rates of *G.pulex* was the major determinant causing this decrease in SfG.

3. Copper contamination caused an increase in the activity of *G.pulex* and a subsequent elevation in respiration rate. This increased activity represented a 2.9 fold increase over the standard respiration rate. Feeding caused a significant increase in the respiration rate of *G.pulex* although this was not related to the quantity of food consumed. The elevation in respiration rate due to feeding represented a 3.6 fold increase over standard metabolism. The measurement of standard metabolic rate is considered adequate for routine use of the SfG test as Ra and Rf represent a multiple of Rs and therefore do not increase the sensitivity of the SfG test.

4. Overall energy available for production (P) became negative at copper concentrations of 30 µg/l and greater. Faecal production represented the major energy loss accounting for approximately 70 percent of ingested energy. Standard respiration represented 3 to 8 percent of ingested energy while active and feeding respiratory costs accounted for 6 to 16 percent and up to 8 to 20 percent of ingested energy respectively.

5. The observed copper-induced decrease in consumption rates was unlikely to be a result of behavioural rejection of contaminated food but the negative correlation between body loading and consumption rates indicates that a physiological response may be more likely. The major source of this copper burden was from the water rather than the food. Animals gained an increased body concentration with increasing copper concentration of the media, with no evidence for the ability to regulate copper over external copper concentration range of 0 to 36 μg/l.

CHAPTER 3.

EFFECTS OF COPPER ON GROWTH AND REPRODUCTION OF *GAMMARUS PULEX*.

3.1. Introduction.

a. Growth.

Growth in *Gammarus pulex* proceeds by a series of moults with approximately 10 moults between birth and first reproduction (Sutcliffe et al, 1981). Time between moults is dependent on temperature, instar number and food level (Nilsson, 1974, 1977; Willoughby and Sutcliffe, 1976; Welton and Clarke, 1980), with the average time between moults at 15^{o}C being 10 days (Sutcliffe et al, 1981). Growth rates of *G.pulex* at 15^{o}C were found to be maximal in 6 to 9 mg wet weight animals (Sutcliffe et al, 1981).

Growth rates of *G.pulex*, from birth to death, have been shown to follow a sigmoid curve. Growth rates may be described by a logarithmic linear equation (Sutcliffe et al, 1981; McCahon and Pascoe, 1988b):

$$\log_e Y = \log_e A + bt. \qquad \text{eqn 3.1}$$

where Y denotes the size of animals at time t, A denotes the size of newborn individuals and b is the weight specific growth rate (Gs). The weight specific growth rate is often multiplied by one hundred and expressed as percent per day.

Because of the exponential nature of growth rates in *G.pulex* (eqn 3.1) a regression of $\log_e$ wet weight of animals against time would yield a linear relationship with the gradient of the line denoting the specific growth rate (Gs).

Growth rate of *G.pulex* may be measured in a number of ways; (i) wet weight, (ii) dry weight, (iii) length, or (iv) number of antennal segments. Each of these approaches have limitations for use in growth studies.

(i). Measurement of wet weight may incur error in the drying process and hence animals should be blotted dry for a standard length of time. Wet weighing requires a high degree of handling and may stress animals during the process.

(ii). Dry weighing animals is destructive and hence the growth of individual animals may not be followed through time. Animals may be subsampled at periods but this requires the initial weight of animals to be very similar. Alternatively relationship between wet and dry weight may be determined and the wet weight of animals converted to dry weight (e.g. Graca, 1990). However as well as problems mentioned above in measuring wet weight of animals additional error may be incurred in wet to dry weight conversion.

(iii). As *G.pulex* has a naturally curved body form measurement of body length is not easily performed. The use of a digitiser may aid the measurement of length but animals must first be anaesthetised before they may be measured. The stress caused by anaesthetization, like handling, may injure, kill or alter the moulting pattern of animals.

(iv). The number of antennal segments on the first and second antenna are quantitatively related to body size (Sutcliffe and Carrick, 1981; Gee, 1988; Pokl, 1992). Amphipods are usually born with a fixed number of antennal segments (five in *G.pulex*) and the number increases throughout the life of the animal (Sexton, 1928; Nilsson, 1977; Sutcliffe and Carrick, 1981; Sutcliffe et al, 1981). A linear relationship has been found between the number of antennal segments (x) and the total body length (y) in *G.pulex* of the form $y = a + bx$, (where a and b are constants), (Sutcliffe and Carrick, 1981; Gee, 1988).

However, Antenna may often be broken during the moult (Sutcliffe and Carrick, 1981; Pokl, 1992) and examination under the microscope again requires anaethetization. One solution to the problem of stress is to count the number of segments of the moult (Sutcliffe and Carrick, 1981). However this requires animals to be maintained individually in order to collect the moult and moults not being eaten before collection. Further, this would give a measure of the size of animals at the previous moult and not at the current time.

The most useful measure of growth in *G.pulex* is likely to be the one which causes least stress to the animal during its measurement.

b. Reproduction.

Breeding in *G.pulex* generally occurs during the summer months (Maitland 1966). Theoretically a female *G.pulex* may produce up to 10 broods in her life time, although the maximum observed in the laboratory is 5 to 7 (Nilsson, 1977; Welton and Clarke, 1980). The reproductive cycle of *Gammarus* has been described in detail by many workers (Hynes, 1955; Zerib 1980; McCahon, 1987; McCahon and Pascoe, 1988c) and has recently been reviewed by Sutcliffe (1992).

(i). Precopular pair formation.

The male can fertilise the oocytes only at the time of the female moult and for this reason guards the female in precopular for several days prior to copulation.

During precopula the male grasps the female lengthways, with his first pair of gnathopods (Ward, 1985; Sutcliffe, 1992). Copulation occurs as soon as the female moults: The male turns the female ventral side up and sperm are transferred close to the openings of the females oviducts (Ward 1985). The precopular pair then separate and pairs of oocytes (one from each oviduct) are released and become distributed along the length of the brood

pouch (Sheader and Chia, 1970). The brood pouch is formed from four pairs of oostegites or brood plates and is the site for external fertilization (Hynes 1955).

(ii). Factors affecting mate choice:

Mating in *G.pulex* is positively size assortive (Greenwood and Adams, 1984; Ward, 1984), this has been hypothesized to be due to male - male competition (Birkhead and Clarkson, 1980), or mechanical constraints (Greenwood and Adams, 1984).

Whilst a male should endeavour to pair with the largest female possible as she carries the most eggs (Birkhead and Clarkson, 1980; Greenwood and Adams, 1984), tests in artificial streams with varying current, found that the upper limit was set by an interaction between mechanical constraints rather than male - male competition (Greenwood and Adams, 1984). Birkhead and Clarkson, (1980), and Dick and Elwood, (1989), however, found that *G.pulex* was able to mechanically manipulate and copulate with all size classes of female.

Male *G.pulex* appear to have developed a capacity to discriminate between females according to their size and stage of their moult cycle (Ward, 1984; Dick and Elwood, 1989). The female herself has also been found to exercise some choice over her potential mate by straightening and flexing her abdomen very vigorously in order to break away from the male grasp and escape (Ward, 1984).

(iii). Female reproductive cycle:

The reproductive cycle of *G.pulex* may be divided into three distinct phases each lasting the duration of one inter-moult period (Zerib, 1980) (Table 7):

During oogenesis a single strand of oocytes develop sequentially from the oogonia in each of the two ovaries (Sutcliffe, 1992). Oogenesis itself occurs during one intermoult period while oocytes are provisioned during the second (Hartnoll and Smith 1978, cited

in Sheader 1983; Steele and Steele 1969). Therefore, there are 2 batches of oocytes in the ovaries of mature females at any one time.

Table 7: Sequence and site of reproductive events spanning three intermoult periods of female *G. pulex*.

Intermoult period	event	site
1	Oogenesis	ovary
2	Provisioning	ovary
3	Embryogenesis	brood pouch

Provisioning involves the major investment in terms of energy of the female to reproduction (Bradley et al 1991). This is the vitellogenic stage in which yolk is formed and the oocytes grow rapidly to their full size (Sutcliffe 1992). Bradley et al (1991) postulated two alternative models for egg provisioning in *Daphnia*:

1. parallel provisioning, i.e. all oocytes are provisioned at the same time;

2. serial provisioning, i.e. oocytes are provisioned one at a time in sequence.

These are also possible mechanisms for *Gammarus*.

The provisioned oocytes may only leave the ovary when the female moults and the oviduct wall becomes flexible and is able to stretch, allowing the large oocytes to pass into the ventral marsupium or brood pouch (Sutcliffe 1992).

During a third intermoult period the brood is incubated within the brood pouch where embryogenesis occurs (Sexton 1928). Embryogenesis may be observed to pass through

several stages (Hynes, 1955; Steele and Steele, 1969; Sheader and Chia, 1970; Zerib, 1980; McCahon, 1987; McCahon and Pascoe, 1988c). The most detailed account, given in McCahon and Pascoe (1988c), describes six developmental stages prior to hatching.

Hence female *Gammarus* reproduce by producing a succession of broods: females with late stage embryos or hatched young in their brood pouch (current brood) have large oogonia in their ovaries (subsequent brood) which will be deposited soon after the previous batch of young is released from the brood pouch. At the same time, oocytes are being produced to become provisioned as soon as the previous batch is released from the ovaries and deposited in the brood pouch (Hynes 1955; Steele and Steele, 1969 and 1975).

(iv). Duration of brooding.

The time period required for embryogenesis is dependent on temperature such that as temperature increases the duration is reduced in a curvilinear fashion (Kinne, 1960; Steele and Steele, 1973; Nilsson, 1977; Welton and Clarke, 1980). At 15^{o}C embryogenesis of *G.pulex* has been found to vary between 20.6 - 30 days (Nilsson, 1977; Pinkster et al, 1977; Welton and Clarke, 1980; Maltby, 1991).

(v). Egg loss.

During embryogenesis there may be substantial loss of developing eggs from the brood pouch (Steele and Steele, 1969; Sheader and Chia, 1970; Fish and Mills, 1979; Welton and Clarke, 1980; Sheader, 1983). This may be due to egg disease or mortality, accidental loss, or consumption of eggs by the female if she is stressed (Sheader and Chia, 1970; Sheader, 1983).

(vi). Hatching of young.

Embryogenesis ends with the hatching of young by rupture of the chorionic membrane after which the young may be released immediately or may remain in the brood pouch for

several days post-hatch (Sheader and Chia, 1970; Fish and Mills, 1979; Sutcliffe, 1992). Release of all young from the brood pouch may take several days, offspring of some *Gammarus* spp have been observed to return to the brood pouch after release (Sheader and Chia, 1970; Fish and Mills, 1979). There is also evidence for the feeding of young *G.pulex* whilst still in the brood pouch (Welton et al, 1983). Once offspring are released from the brood pouch they may be cannibalised by adults, including their mother (Sutcliffe, 1992).

(vii). Number of offspring per brood.

In a study of reproduction in Gammaridae, Hynes (1955) found the average number of eggs per brood in *G.pulex* was 16, however the range of variation was great (1 to 43). Brood size has been found to be dependent on season (Hynes, 1955; Steele and Steele, 1969; Sheader and Chia, 1970; Sheader, 1983) and size of female, with the relationship between fecundity and female size being either linear or curvilinear, (Sexton, 1928; Cheng, 1942; Hynes, 1955; Kinne, 1960; Steele and Steele, 1969, 1975; Sheader and Chia, 1970; Fish and Mills, 1979; Sheader, 1983; Ward, 1986; Graca, 1990).

(viii). Offspring size

The size of *G.pulex* at birth was found to be approximately 1.6 to 1.8 mm (McCahon, 1987). Thompson (1986) found evidence for the heritability of body size in *Asellus aquaticus* as well as non-random mating. However although non-random mating also occurs in *Gammarus* (Ward, 1984) there has been no evidence for the heritability of size (Sheader, 1983).

Toxic compounds may affect the reproductive performance of females in several ways including:

1. a direct lethal effect on the current brood resulting in a decrease in the number of viable offspring produced (Maltby and Naylor, 1990);

2. a reduction in the amount of energy available for the provisioning of eggs, if provisioned in parallel, would result in offspring from the subsequent brood being smaller or, if provisioned in series the result would be, a reduced number of offspring of normal body size (Maltby and Naylor, 1990; Bradley et al, 1991);

3. a lethal effect on the eggs whilst they are being provisioned resulting in offspring being reduced in number but of normal body size (Maltby and Naylor, 1990);

4. reduction in number of offspring from current and/ or subsequent brood due to female aborting and consuming eggs when stressed.

3.2. Objectives.

The objectives of the work described in this chapter were to:

1. investigate the effects of copper on the growth and reproduction of *G.pulex*;

2. investigate whether information from energy budgets could be used to predict observed effects on growth and reproduction.

3.3. Materials and Methods.

3.3. 1. Growth.

a. Parameter measured.

As discussed above the method chosen for determining growth rates should be that which causes least stress to the animal. Measurement of animal length or number of antennal segments requires anaesthetization of the animals whilst wet weight measurements

require standard blotting of animals before weight determinations. Therefore effects of periodic anaesthetisation or wet weighing on survival were determined.

Two replicates of ten juvenile *G.pulex* per treatment were placed in 2 litres of APW maintained for six weeks at 15°C with a 12 hour light-dark photoperiod. Animals were fed *Cladosporium* inoculated alder leaves (prepared as in Chapter 2, Section 2.3. 2. b) cut into fine pieces. At 14 day intervals for the first 4 weeks and weekly there after, animals were removed and subjected either to anaesthetisation or wet weighing. Survival of animals was recorded just prior to each treatment.

In order to anaesthetize animals they were placed in small pots containing 20 ml of APW. Soda water was then added to the water so that the carbon dioxide concentration of the water increased and the animals ceased to move. Upon transfer to aerated APW, normal activity was resumed.

In order to wet weigh animals, excess water was removed by blotting animals with paper tissue. Animals were then placed in foil weighing boats and weighed on a Mettler ME30 micro-balance.

Results presented in Appendix 6 show survivorship of animals from the different treatments was significantly different (ANCOVA: $F = 4.7$, $df = 1$, $p < 0.05$). Wet weighing of animals was slightly less stressful than anaesthetisation and therefore increase in wet weight was selected as the parameter by which to determine growth rates as this experimental procedure caused the animals least stress.

b. Effect of copper on juvenile growth rates.

Small juvenile *Gammarus* (approximately 9 mg wet weight) were collected from Crags stream (Section 2.3. 1, Chapter 2). All animals were maintained in artificial pond water (APW) and were exposed to nominal copper concentrations of 0, 15 and 30 µg/l via a

flow-through apparatus. Aerated test solution held in a large 30 litre reservoir was pumped continuously, via a Watson Marlow peristaltic pump, through small 0.5 litre containers. Copper solutions were made from a 10 µg/l stock solution prepared with copper sulphate as in Chapter 2 (section 2.3. 2. d). All stocks and test solutions were prepared and checked daily by FAAS.

Test solutions were pumped through the flow-through system for 1 week prior to exposure of the animals in order to reduce loss of copper from solution by adsorption to the vessel and tubing walls. Fresh stock and test solutions in the holding reservoir were prepared and replenished daily. Two experiments were conducted, one using animals held individually and the other using animals maintained in groups. Four replicates of five individual *G.pulex* per concentration were exposed individually in 5 x 5 x 5 cm cylindrical cages with 1 mm mesh covered ends. These cages were deployed in the 0.5 litre vessels. Four replicates of 14 animals per concentration were exposed as groups. All animals were acclimated to laboratory conditions (15^{o}C, alternating 12 hour light to dark photoperiod, leaves conditioned with a mixed fungal flora) for 10 days prior to use in laboratory tests.

Animals were fed *ad libitum* with finely chopped alder leaves inoculated with a mixed fungal flora derived from the river Don (NGR SK 216209) near Penistone, Yorkshire (See Appendix 7 for physicochemical data). Vessels containing the groups of animals and individual caged animals were emptied and replenished with food weekly to remove any accumulation of faecal waste. Survival of animals during the test was noted and animals were wet weighed on day 0, and then at fortnightly intervals for a further 6 weeks. The individual wet weight of all animals was determined, after blotting dry with tissue for 10 seconds, using a Mettler ME30 micro-balance accurate to 0.001 mg and hence effects of copper on growth rates was determined.

c. Prediction of juvenile growth rates.

As outlined in Chapter 1, scope for growth gives an estimate of the amount of energy available for growth and reproduction. Therefore, effects of copper on growth and reproduction should be predictable from a knowledge of the effects of copper on scope for growth. Scope for growth has been defined here as the difference between the amount of energy absorbed and the amount of energy lost via standard respiration (eqn 2.8). However there will be additional energy losses due to respiratory costs of activity and feeding and excretion, for example. Energy remaining for growth and reproduction after these additional factors are accounted for will be termed 'production' (P; J/mg/d) for the purposes of this study.

If it is assumed that all the energy available for production is invested solely in growth (i.e. assuming no other energy demands, e.g. reproduction or storage), then investment in growth (determined as a measure of growth rate (Gs; % dry weight/day) may be calculated.

A measure of the net amount of energy accumulated during an instar, (Pg; J/mg), may be gained by subtracting the energy required for moulting, (C_m), from the total amount of energy accumulated within an instar (E_i).

In order to obtain a value for E_i one needs to multiply a measure of the energy available for production (P, J/mg/d) by the average time over which this energy was acquired (Tm). Information on consumption rates, faecal production, standard, active and feeding metabolism were taken from Chapter 2 (section 2.5. 4). Excretion has been shown to account for 10 to 30 percent of absorbed energy in several species of amphipods, (Dresel and Moyle, 1950), while in *G.pulex* excretory losses are likely to be at the lower end of this scale, representing 3 to 10 percent of absorbed energy (L. Maltby, unpublished data) and this lower estimate was used for the purposes of this study. The average time between

moults for juvenile *Gammarus* at 15^{o}C is 10 days (Sutcliffe and Carrick, 1981) but as animals do not feed during or immediately post-moult (Martin, 1965) T_m was assumed to be 9 days and E_i (J/mg) is therefore 9 x P.

In order to obtain a value for energy expenditure during moulting (C_m), increased activity during the moulting process, increased osmotic work due to increased cuticle permeability and subsequent increased fluxes of water and ions and loss of exuviae must be considered. The cost of increased activity and osmotic stress are reflected by changes in respiration rate (R_s; Sutcliffe, 1984) which increases two to four times normal levels during moulting (Bulnheim, 1972; In Maltby, 1991). A median value of three times standard respiratory losses is taken for the purpose of this study. Energy loss via the exuviae may be estimated by multiplying the joule equivalent for exuviae (Jm) by the proportion of body weight lost in the moult (W_m) as in equation 3.2:

$$C_m = (4 \times R_s) + (J_m \times W_m) \qquad \text{eqn 3.2}$$

In order to determine the proportion of body weight lost in the moult (Wm) and the joule equivalent for exuvae (Jm), individual animals were placed in small vessels containing 225 ml of APW and maintained on *Cladosporium* inoculated alder leaves (15^{o}C with a 12 hour light to dark photoperiod) until the animal moulted. Upon moulting both the animal and the moult were removed, dried in an oven at 60^{o}C, and then weighed on a Mettler ME 30 micro-balance. The weight of the moult divided by the weight of the animal gave a measure of the proportion of body weight lost in the moult.

The moults were then compressed into pellets and weighed using a Mettler ME30 micro-balance. The pellet was placed on the sample tray of a micro-bomb calorimeter (see Chapter 2, Section 2.3. 4 a) with fuse wire touching the sample. The energy content of the moult was determined, after ignition of the sample in excess oxygen as described in Chapter 2 (Section 2.3. 4 b), using equation 2.6.

Energy available for growth in each instar (P_g, J/mg dry weight) as defined by the difference between E_i and C_m was converted to an increase in dry weight by dividing by the joule equivalent for juveniles.

$$W_{dwt} = (E_i - C_m) / J_j \qquad \text{eqn 3.3}$$

The joule equivalent for juveniles was determined by micro-bomb calorimetry. Juveniles, newly released from the females brood pouch, were collected and dried in an oven at 60°C. The juveniles were then compressed into a pellet and weighed on a Mettler ME 30 micro-balance. As the juveniles were so small only one pellet was produced consisting of several hundred juveniles. This pellet was placed on the sample tray in a micro-bomb calorimeter with the fuse wire just touching the sample. The sample was ignited in excess oxygen as in Chapter 2 (Section 2.3. 4. b) and the joule equivalent of juveniles determined using equation 2.6.

The increase in dry weight was then converted to an increase in wet weight (W_{wwt}) in order to correspond to the parameter measured in growth experiments. Dry weight was converted to wet weight using equation 3.4 (Graca, 1990) which describes the relationship between wet and dry weight in *G.pulex*.

$$\ln (Wwwt) = (\ln(W_{dwt}) + 1.728) / 1.01 \qquad \text{eqn 3.4}$$

G_s (% wet weight / day) can then be calculated using equation 3.5:

$$G_s = 100 (\ln W_t - \ln W_0) / t \qquad \text{eqn 3.5}$$

Where W_0 represents the initial wet weight of experimental animals W_t is the initial wet weight (W_0) plus the increase in wet weight (W_{wwt}) and t is the duration of the instar.

Hence growth rates (Gs; % wet weight/day), predicted from a measure of production (P; J/mg/d), can be compared to observed growth rates (Gs; % wet weight/day), of animals exposed to copper in laboratory tests (Section 3.3. 1. b). Hence, the accuracy of a measure of production for prediction potential effects of copper on growth rates can be determined.

3.3. 2. Reproduction.

a. Sensitivity of brooding females to copper.

In order to determine the sensitivity of brooding females to copper, consumption rates, faecal production and absorption rates of female *G.pulex* exposed to nominal copper concentrations of 0, 10, 20, 30, and 40 µg/l were determined using the same methodology used for males (Chapter 2, section 2.3. 4 a). Exposure concentrations for experiments on reproduction rates of female *G.pulex* were selected on the basis of these results such that the highest exposure concentration in reproduction tests (nominally 30 µg/l) was slightly greater than that causing a significant reduction in consumption rates (i.e. 26 µg/l, see Fig. 21).

b. Effect of copper on reproduction.

Precopular pairs were collected from Crags stream (see Chapter 2, Section 2.3. 1) in early autumn and were returned to the laboratory where they were maintained in a stock tank containing 20 litres of APW and were fed *Cladosporium* inoculated alder leaves. The tank was checked twice daily for brooding females released from precopula - recognised by a black colouration to the brood pouch. These females were removed from the stock tank and exposed to copper, in groups of up to 100, for one complete instar (approximately three weeks) in three litre glass jars containing three litres of aerated test solution. The nominal copper concentrations used were 0, 10, 20, 25 or 30 µg/l. Fresh 10

mg/l stock solutions and all test solutions were prepared and changed every four days and all solutions analysed using FAAS.

Brooding females were inspected every four days at which time females that had aborted their brood were removed from the test vessels. Towards the end of the instar (day 18 to 28), females were checked every day and when the brood was seen to turn orange, females were removed and placed individually in two-chambered pots (Fig. 3, Chapter 2) containing 225 ml of APW. Males, gently separated from precopular pairs, were added to the pots to see if they would pair with the female. When the brood was released (i.e. current brood - see section 3.1. b. (c)), juveniles were able to pass through the mesh to the lower chamber of the pot where they would not be ingested by the adults.

Juveniles were immediately removed with a fine pipette and were counted. A subset of 5 juveniles per brood were removed and anaesthetised with soda water before measuring their length (mm) using a Kontron Electronic (UK) videoplan and Reichert Jung Polyvar widefield microscope. Length was measured as the distance from the anterior edge of the head to the posterior edge of the telson. As fecundity increases with increasing size of female, (Sheader and Chia, 1970; Steele and Steele, 1969, 1975; Hynes, 1955; Ward, 1986; Kinne, 1960; Sexton, 1928; Fish and Mills, 1979; Sheader, 1983), effect of female size on the number of offspring was controlled for by expressing all results as number of offspring per unit weight of female. Given that the brood may not all be released at once (Section 3.1. b (f)), females were left three to four days from the release of the first juveniles in order to release the whole brood.

No females remated during this experiment so females were dried at 60^{o}C and weighed on a Mettler ME30 microbalance. Lack of remating may have been attributable to a number of reasons including season and experimental conditions.

Seasonal effects may have been a factor in the lack of remating of *G.pulex* after the release of the current brood as this experiment was conducted in the late autumn. Breeding in *G.pulex* generally occurs in the summer months (Maitland, 1966) and is greatly reduced over the cold winter period. Hence at the time of this experiment reproduction of animals in the field was very much reduced. The experiment was therefore repeated in the spring. However, the time of year alone could not have been the reason for the lack of remating of animals in the autumn experiment as there was also a lack of remating in the spring.

The lack of remating was therefore more likely to be due to experimental conditions and the experiment was repeated the same spring with a slightly different experimental design. Four groups of approximately 25 brooding females per concentration were exposed to copper in one litre glass jars. Just prior to the release of the current brood, females were removed from the exposure jars and placed in groups in large containers of APW with several males which had been freshly separated from precopular pairs. The current brood of these females could not be collected but in this way a few females did remate and hence effects of copper on the subsequent brood were investigated. Information was therefore gained on the effects of copper on both the current and the subsequent broods, although not from the same animals.

<u>c. Brood Cannibalism.</u>

In order to determine if animals which aborted their broods subsequently consumed the eggs, individual gravid females were placed in small pots and exposed to 30 µg/l copper for one complete instar or until the female aborted her brood or died. 20 females were placed in pots with a mesh divide through which aborted eggs could fall preventing the female from consuming them (Chapter 2, Fig. 3). A further 20 females were placed in pots without a mesh divide so aborted eggs could be consumed after they were released.

Solutions were changed every two days and copper concentrations determined using FAAS. The pots were inspected daily and the fate of any aborted broods determined.

d. Prediction of reproductive output.

In Section 3.3. 1. c, it was shown that, assuming all energy available for production is invested in growth, growth rates (Gs; % dry weigh/day) may be calculated. Similarly, if it is assumed that all the energy available for production is diverted into reproduction it should be possible to calculate the reproductive investment of a female. By comparison of this estimate with observed effects of copper on reproduction (Section 3.3. 2. b) the ability of production estimates to predict potential longer term effects on reproduction can be determined.

In a review of the costs of reproduction, Calow (1979), listed several traditional ways of estimating reproductive cost (C). He stated that the best method is an estimate of the amount of energy invested in reproduction (Rep) as a proportion of the amount of energy taken in (I) and energy available for metabolic purposes other than reproduction (Rest) during the reproductive period should be compared with energy 'needed' for metabolic purposes by a healthy parent such that:

$$C = \frac{I - (I - Rep)}{(Rest)}$$

However, in this study, not all the parameters necessary for a measure of energy used for all metabolic purposes other than reproduction (Rest) during the reproductive period were measured. Therefore, reproductive investment in this study was estimated as a measure of reproductive output (RO; mg/mg) for one reproductive event. This method assumes (not necessarily correctly) that there is a direct relationship between metabolic economy and the size of the parent such that:

$$RO = \frac{\text{biomass of gametes}}{\text{biomass of parent}}$$

or:

$$RO = \frac{\text{Number of offspring x mass of offspring}}{\text{mass of female}} \qquad \text{eqn 3.6}$$

A similar procedure to that used in section 3.3. 1. c to predict growth rates, may be used to predict reproductive output (RO; mg/mg) from a measure of production (P; J/mg/d).

In order to predict female energy expenditure in offspring (Pr; J/mg) from energy budget determinations (Chapter 2), the energy required for moulting (Cm; J/mg, calculated as in Section 3.3. 1. c, eqn 3.2) is subtracted from the net amount of energy accumulated in an instar (Ei; J/mg). A value for Ei is gained by multiplying the amount of energy available for production (P; J/mg/d, as defined in Section 3.3. 1. c) by the average time over which this energy is accumulated (Tm; days).

Brood development time in *G.pulex* at 15^{o}C has been found to range between 20.6 to 30 days (Nilsson, 1977; Maltby, 1991; Pinkster et al 1977, Welton and Clarke, 1980). Brood development time could not be determined precisely in this study as experiments were performed on groups of animals but was estimated to be approximately 25 days. However females do not feed during or immediately post moulting (Martin 1965) and feeding rates of animals in precopula are reduced (Welton et al 1983). Welton et al (1983) found that 69 percent of animals not in precopula had full guts compared to only 28 percent with full guts in precopular animals. Therefore it may be assumed that an average of 58 percent of the females time is spent feeding giving a T_m of 14.92 days. This figure was derived as follows (Table 8):

Table 8: Average amount of time spent feeding by females during one complete instar.

Stage	no. days	% time feeding	no. days feeding
Postmoult	1	0	0.00
Non-precopular	20	69	13.8
Precopular	4	28	1.12
Total			14.92

Hence, E_i will be equal to 14.92 x P.

The predicted energy expenditure in offspring (Pr; J/mg i.e. Ei - Cm) can then be converted to a measure of reproductive output (RO; mg/mg) by dividing by the joule equivalent for eggs (Je; J/mg) (eqn 3.7).

$$RO = Pr / Je \qquad \text{eqn 3.7}$$

The joule equivalent for eggs was determined by microbomb calorimetry. Eggs were gently removed from the brood pouch of gravid females with a fine needle. The eggs were then freeze dried before compacting into pellets. The pellets were weighed on a Mettler ME30 micro-balance and placed on the sample tray in the micro-bomb. The pellet was ignited, in excess oxygen, by a piece of fuse wire just touching the sample (see Chapter 2, Section 2.3. 4. b) and the joule equivalent for eggs (Je; J/mg) determined using equation 2.6. Hence a measure of predicted reproductive output is gained for comparisson with that observed in terms of biomass of brood produced per milligram of female

The predicted measure of reproductive output obtained could be compared to that observed once the observed length of offspring (L), was converted from mm to mg dry weight (DW). This was done using equation 3.8 relating length to dry weight in *G.pulex* (Maltby, 1991):

$$\ln (DW) = 3.0 \ln(L) - 5.643. \qquad \text{eqn 3.8}$$

Hence, observed reproductive output was calculated according to equation 3.6 and compared to that predicted from energy budget determinations according to equation 3.7.

3.4. Statistical analyses.

Percentage survival of individuals and groups of *G.pulex* during growth experiments were normalized by arcsine transformation. Survival of animals exposed to different copper concentrations over the period of the test was compared by analysis of covariance after ensuring regression lines of survival against time were significant.

Log_e wet weight of animals against time were plotted and where significant, effects of copper on growth rates were investigated by analysis of covariance, otherwise effects of copper on changes in weight over time were analysed using 2-way analysis of variance.

All data were checked for normality using n-scores techniques before applying parametric statistics. Effects of copper on the size-specific number of offspring and the size of offspring from the 'current' brood was investigated using one-way analysis of variance and and the Tukey multiple range test. Determination of significant differences between groups was obtained using the twosample t-test. Due to non-normality, the Kruskall-Wallis test was employed to determine effects of copper on the 'subsequent' brood and the Mann-Whitney U test employed to determine where significance lay.

Effects of copper on the abortion rates of females was investigated by one-way analysis of variance on arcsine transformed data.

All statistical analyses were performed using Minitab, Statgraphics or Fig P computer software packages.

3.5. Results.

3.5. 1. Growth.

a. Survival of individuals.

Animals were exposed to actual copper concentrations of 0, 19.44 or 32.56 µgCu/l during these tests. Regressions of arcsine transformed survival against time were significant at all concentrations ($r^2 > 66.2\%$, $df = 14$, $p < 0.01$; Fig. 17). When compared by analysis of covariance there was no difference between the survival of animals exposed to 19.44 µgCu/l and that of the controls (ANCOVA: $F = 0.14$, $df = 1, 128$, $p > 0.05$). However survival of animals exposed to 32.56 µgCu/l was significantly reduced compared to the control ($F = 7.26$, $df = 1, 118$, $p < 0.05$; Fig. 17) and mortality of animals at 6 weeks was so low growth rates of animals from this treatment could only be determined over a 4-week period.

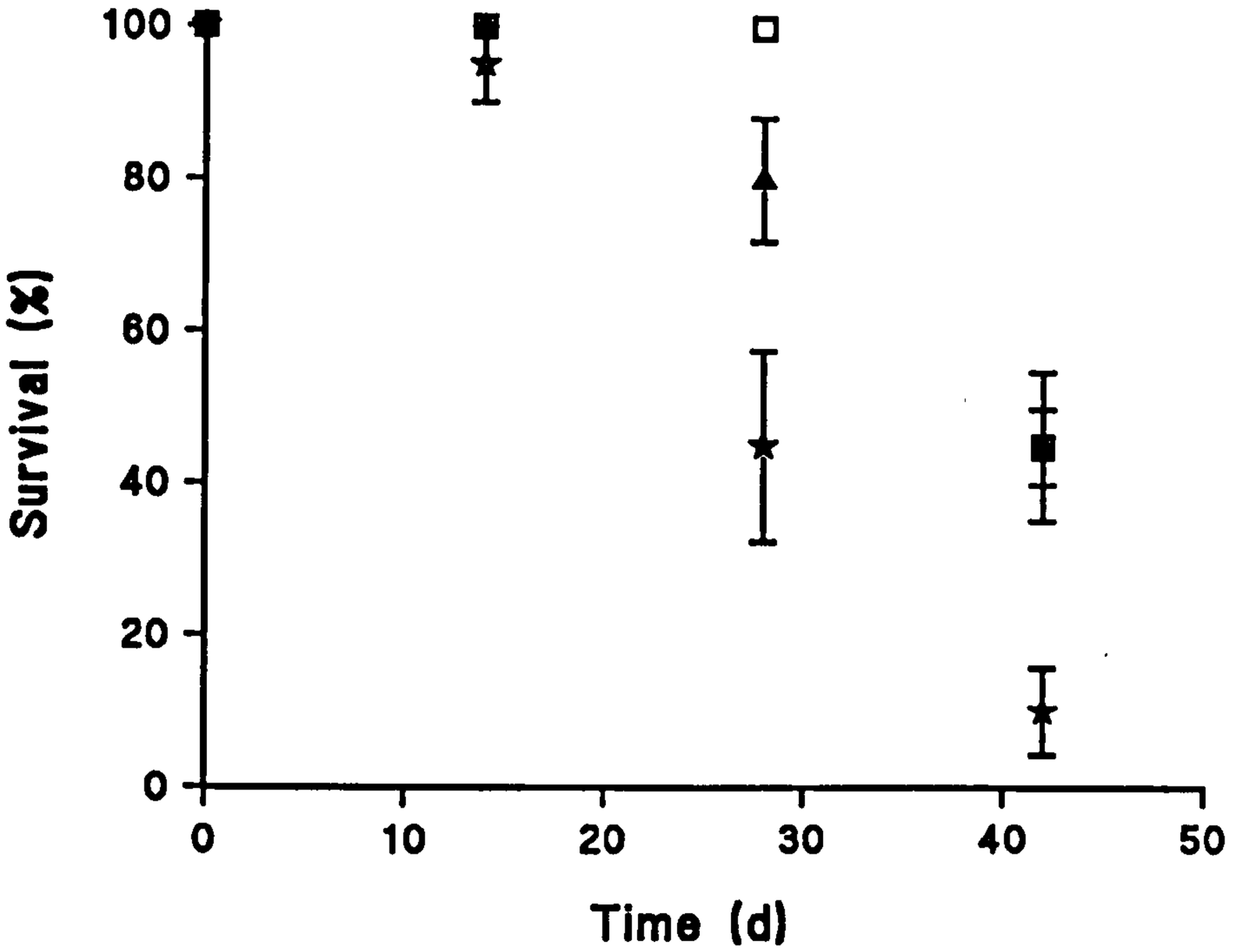


Fig. 17. Survival of individual animals exposed to 0 (open squares), 19.44 (solid triangles), or 32.56 (solid stars) μgCu/l during growth rate experiments. Error bars represent one standard error.

b. Individual growth rates.

There was no significant difference ($F = 0.394$, $df = 2, 58$, $p > 0.05$) between the initial weights of individual animals exposed to 0, 19.44 or 32.56 μg/l copper which were 8.998 (SE = 0.263), 8.694 (SE = 0.269) and 8.667 (SE = 0.328) respectively. Weight specific growth rates declined with increasing copper concentration (Table 9).

Table 9. Average growth rates (Gs) of individual animals exposed to copper.

Copper concentration (µg/l)		
Nominal	Measured	Gs x 100 (%mg/d)
0.00	0.00	0.41
20.00	19.44	0.37
30.00	*32.56	0.13

* Wet weights of animals exposed to 32.56 µg/l copper were only determined over a four week period due to high mortality (See Section 3.5. 1. a).

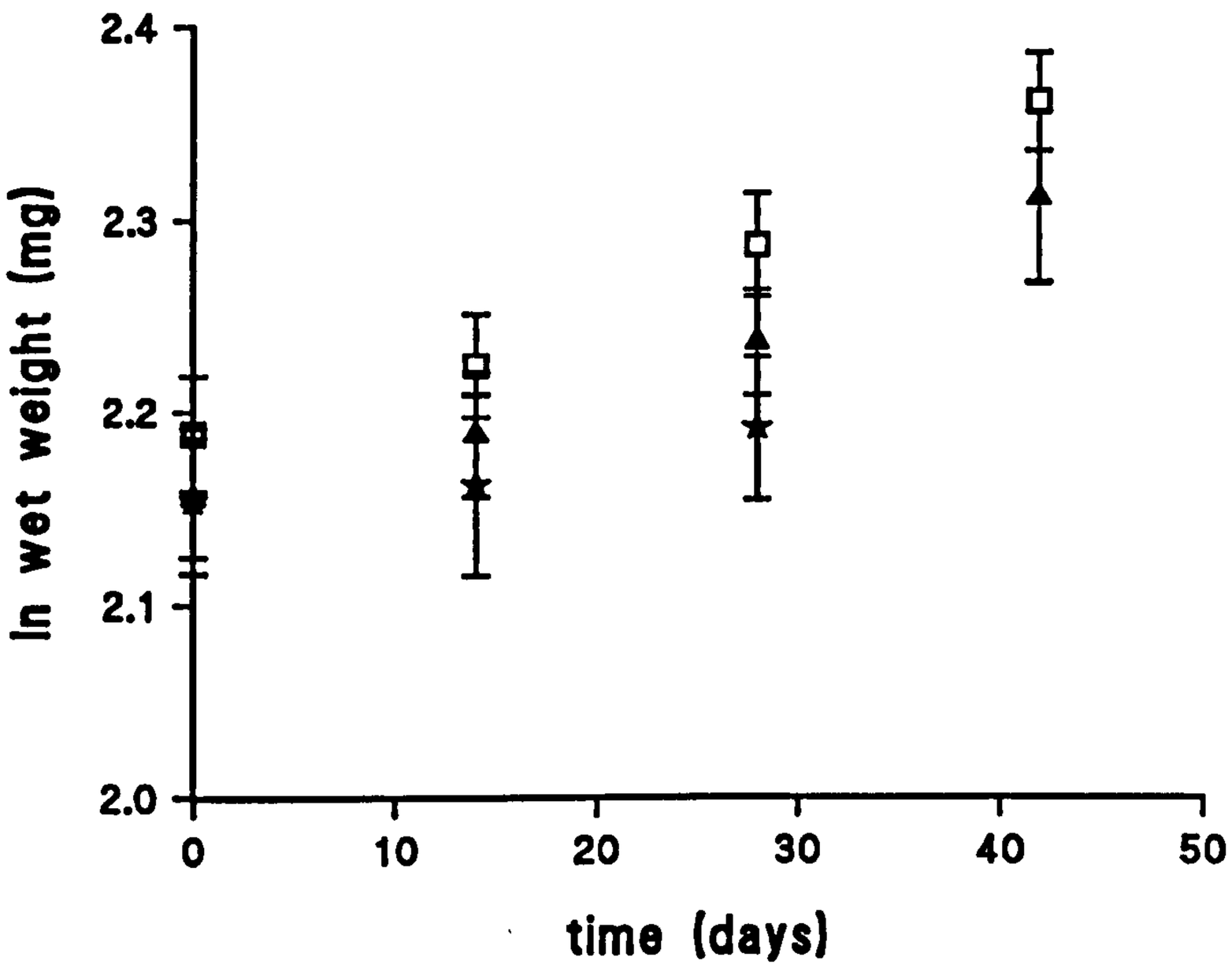


Fig. 18: Effects of copper on the growth rates of animals exposed individually to copper concentrations of 0, (open squares), 19.44 (solid triangles), or 32.56 (solid stars) µg/l. Error bars represent 1 standard error.

Although specific growth rates were reduced by exposure to copper, two-way analysis of variance showed neither exposure to 19.44 µg/l ($F = 0.04$, $df = 3$, $p > 0.05$) nor to 32.56 µg/l copper ($F = 0.37$, $df = 2$, $p > 0.05$) caused a statistically significant reduction in growth rates of individual animals (Fig. 18, Table 9).

c. Survival of grouped animals.

Regressions of arcsine transformed survival against time were significant at all concentrations ($r^2 > 50.4\%$, $df = 14$, $p < 0.05$) (Fig. 19). Survival of grouped animals exposed to 19.44 µgCu/l was not significantly different from that of the controls (ANCOVA: $F = 0.36$, $df = 1, 15$, $p > 0.05$). However survival of animals exposed to 32.56 µgCu/l was significantly reduced compared to the control (ANCOVA: $F = 36.24$, $df = 1, 7$, $p < 0.05$; Fig. 19). There was 100 percent mortality of animals exposed to 32.56 µgCu/l after six weeks so growth rates of animals from this treatment could only be determined over a four-week period.

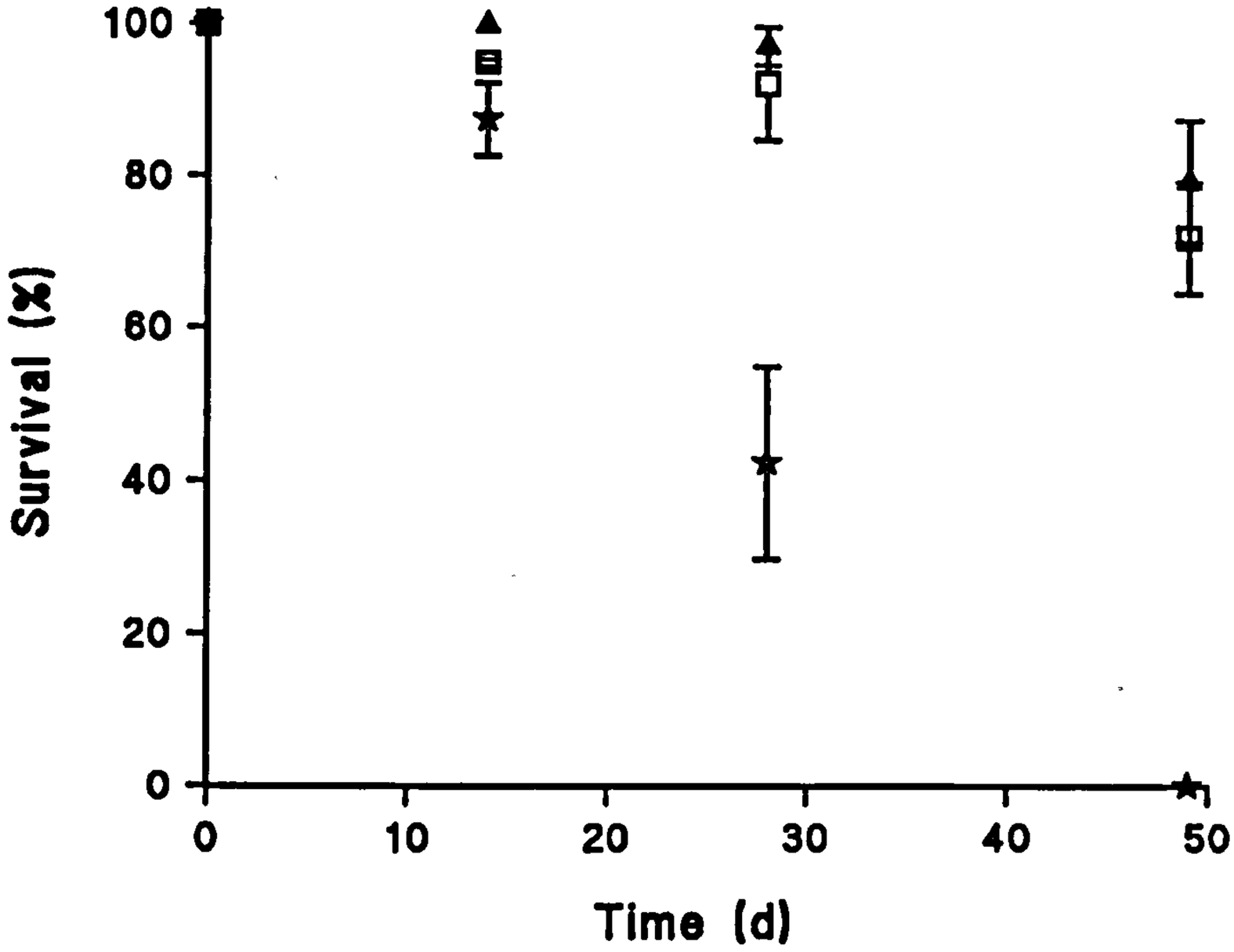


Fig. 19. Survival of groups of animals exposed to 0 (open squares), 19.44 (solid triangles), of 32.56 (solid stars) μgCu/l during growth experiments. Error bars represent one standard error.

d. Growth rates of grouped animals.

There was no significant difference ($F < 2.124$, $df = 3, 3$, $p > 0.05$) between weights of animals in any of the 4 replicates and hence replicates could be combined for analysis. There was no significant difference between the initial weight of control animals and either of the two sets of treated animals although the initial weights of animals exposed to 19.44 μgCu/l were significantly higher than those of animals exposed to 32.56 μgCu/l copper ($F = 7.173$, $df = 2, 6$, $p = 0.001$). Specific growth rates of animals held in groups declined with increasing copper concentration (Table 10).

Table 10. Average growth rates (Gs) of groups of animals exposed to copper.

Copper concentration (µg/l)		Gs x 100 (%mg/d)
Nominal	Measured	
0.00	0.00	1.03
20.00	19.44	0.77
30.00	*32.56	0.76

* Wet weights of animals exposed to 32.56 µg/l copper were only determined over a four week period due to high mortality.

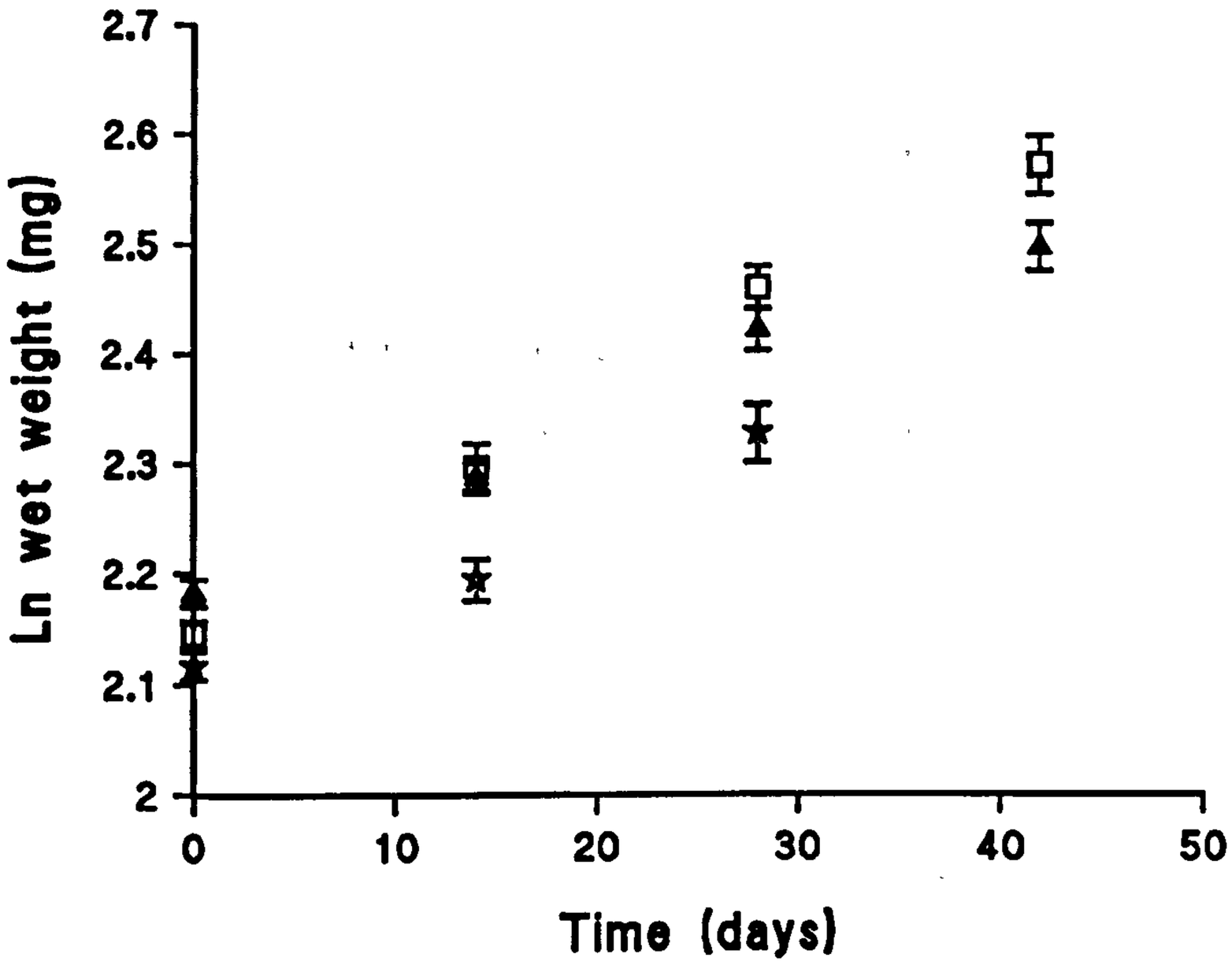


Fig. 20: Effect of copper on the growth rates of animals exposed in groups to copper concentrations of 0 (open squares), 19.44 (solid triangles), or 32.56 (solid stars) µg/l. Error bars represent 1 standard error.

Analysis of covariance indicated that there was a significant effect of copper on the growth of grouped animals with a reduction in growth rate both at 19.44 µg/l ($F = 10.75$, $df = 1, 30$, $p < 0.001$) and at 32.56 µg/l copper ($F = 19.52$, $df = 1, 26$, $p < 0.001$) (Fig. 20).

Growth rates of animals held in groups were greater than of those held in individual cages (Tables 9 and 10). Initial sizes of animals held individually or in groups were very similar hence this could not have been the cause for the difference in growth rates of the two sets of animals. The reduced growth rates of individual animals may have been due to additional experimental stresses of caging, for example, reduced flow rates or restricted movement. However mortality rates of animals held in groups were slightly higher than for individually caged animals suggesting that stresses of caging were not entirely responsible for the lower growth rates of individual animals. The greater growth rates of animals held in groups may be an artifact of differential mortality rates. If smaller animals are less tolerant than larger ones (McCahon and Pascoe, 1988b) then these animals are more likely to die as a result of copper-induced stress. Hence upon reweighing the group of animals the mean weight will be higher, thus distorting true growth rates. In contrast when growth rates of individual animals are measured no such bias would be introduced.

e. Prediction of juvenile growth rates.

Growth rates (Gs; % wet weight/day) of animals exposed to copper were calculated from production data obtained in Chapter 2 (Section 2.5. 6). The total amount of energy accumulated during an instar (i.e. nine days) and the energy required for moulting were calculated to give a measure of the net amount of energy accumulated during an instar (Pg: Table 11). Total amount of energy accumulated in an instar (Ei; J/mg) was equal to 9 x P. The energy required for moulting was calculated using equation 3.2 where a measure of Rs was taken from Chapter 2, (Section 2.5. 2 c) and the proportion of body weight lost

in the moult and its joule equivalent were 33.9 percent (SE = 1.24, n = 46) and 11.459 J/mg (SE = 0.456, n = 5) respectively.

Table 11 . Effect of copper (Cu; µg/l) on the amount of energy available for production (P; J/mg/d) and lost via standard respiration (R; J/mg/d) and hence the total amount of energy accumulated in one complete instar (Ei; J/mg), energy required for moulting (Cm; J/mg) and net amount of energy accumulated (Pg; J/mg).

Cu	P	R	Ei	Cm	Pg
0.0	0.498	0.177	4.482	4.416	0.066
13.0	0.833	0.191	7.496	4.458	3.038
23.0	0.022	0.217	0.194	4.536	-4.342
27.0	0.386	0.139	3.478	4.302	-0.827
30.0	-0.347	0.184	-3.119	4.437	-7.556
36.0	-0.686	0.179	-6.177	4.422	-10.599

The joule equivalent of juveniles was determined as 17.337 J/mg (n = 1). Hence the change in dry weight (Wdwt) was calculated by dividing Pg by Jj according to equation 3.3. This was then converted to a change in wet weight (Wwwt) using equation 3.4 and finally to growth rate (Gs) using equation 3.5. Results are presented in Table 12.

The predicted growth rates (Gs) were then compared to those observed, results are given in Table 13.

Table 12. Effect of copper (Cu; µg/l) on the change in dry weight (Wdwt; mg/d), wet weight (Wwwt; mg/d) and growth rates (Gs; % wet wt/d) of *G.pulex*. (/ denotes negative values which could not be log-transformed).

Cu	Wdwt	Wwwt	Gs
0.0	0.004	0.022	0.026
13.0	0.175	0.987	1.092
23.0	-0.240	/	/
27.0	-0.048	/	/
30.0	-0.407	/	/
36.0	-0.611	/	/

Table 13. Comparison of observed growth rates (Gs: mg/d x 100) compared to those predicted (Gs: mg/d x 100) from a measure of the energy acquired within an instar (E_i: J/mg) and the energy lost during the moult (C_m: J/mg).

Copper concentration (µg/l)	Predicted G_s	Observed G_s	
		Ind	Group
0.00	0.026	0.41	1.03
13.00	1.092	-	-
19.44	-	0.37	0.79
23.0	-	-	-
27.0	-	-	-
30.0	-	-	-
32.56	-	0.13	0.78

While predicted growth rates for animals exposed to 13 µgCu/l compare favourably with those observed for control animals held in groups, predicted growth rates of controls seriously under-estimate those observed. Growth rates may have been under-estimated due to an under-estimate of production (e.g. over-estimate of feeding respiration) or an over-estimate of the energy required for moulting (C_m).

At copper concentrations of 23 µg/l and above, energy required is predicted to exceed that available, hence animals would fall into negative energy balance and death is predicted to ensue (Table 13). However, although observed growth rates are reduced they do not become negative at any concentration up to 32.56 µgCu/l.

3.5. 2. Reproduction.

a. Sensitivity of brooding females to copper.

Actual copper concentrations in this test were 0, 17.2, 25.6, 37.0 and 47.6 µgCu/l. Copper caused a significant reduction in consumption rates ($F = 22.903$, $df = 4, 91$, $p < 0.001$), being significantly reduced at concentrations of 25.8 µg/l and over ($t > 4.04$, $df > 27$, $p < 0.001$). Likewise faecal production was reduced by exposure to copper ($F = 6.184$, $df = 4, 91$, $p < 0.001$) being significantly reduced at concentrations of 25.8 µg/l and over ($t > 2.68$, $df > 27$, $p < 0.05$) (Fig. 21). Absorption was similarly reduced by exposure of females to copper ($F = 47.767$, $df = 4, 91$, $p < 0.001$) with a significant reduction at a copper concentration of 25.8 µg/l ($t > 5.2$, $df > 27$, $p < 0.001$). The sensitivity of female *G.pulex* to copper contamination was comparable to that of males, where copper concentrations of 23.0 µg/l caused significant decreases in consumption and faecal production and 30 µg/l caused a significant reduction in absorption rates, (Chapter 2, Section 2.5. 2. d). The average absorption efficiency of females was 33.67 percent and

was only significantly different ($F = 22.268$, $df = 4, 91$, $p < 0.001$) from that of the control at the highest two copper concentrations ($t > 2.66$, $df > 27$, $p < 0.05$; Fig. 21).

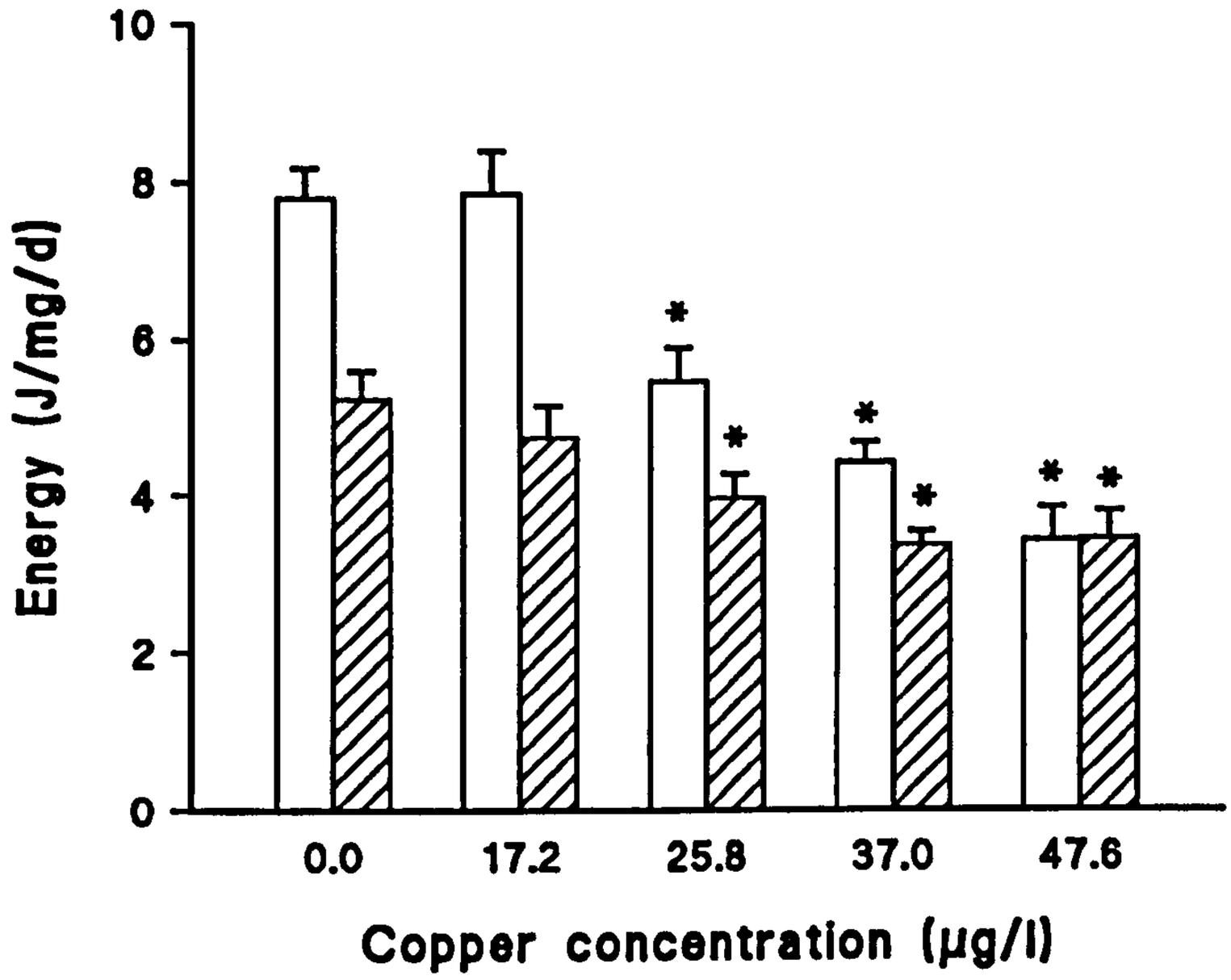


Fig. 21. Effect of copper on consumption rates (open bars) and faecal production (hatched bars) of female *G.pulex* exposed to copper for 6 days. Error bars represent 1 standard error, * data significantly different from control ($p < 0.05$).

b. Effect of female size on number and size of offspring.

There was a significant linear relationship between fecundity and female weight in the autumn experiment ($r^2 = 5\%$, $df = 158$, $p < 0.01$) and in the spring experiment there was also a statistically significant linear relationship between number of offspring per brood and female weight for both the current ($r^2 = 2.7\%$, $df = 152$, $p < 0.05$) and the subsequent ($r^2 = 33\%$, $df = 15$, $p < 0.05$) broods. Hence all results are expressed as size specific fecundity. In contrast, female weight had no significant effect on the size of offspring produced in any experiment ($r^2 < 1.4\%$, $df > 35$, $p > 0.05$).

c. Autumn broods.

Effects of copper on the size of offspring and the number of offspring produced per female when the female was exposed to copper for one complete instar were investigated. Effects of copper on the current brood only were determined in the autumn experiment as females failed to remate. Figures 22 and 23 show that there was no significant effect of copper on either size-specific fecundity ($F = 0.477$, $df = 4, 158$, $p > 0.05$) or size of offspring produced ($F = 2.175$, $df = 4, 715$, $p > 0.05$).

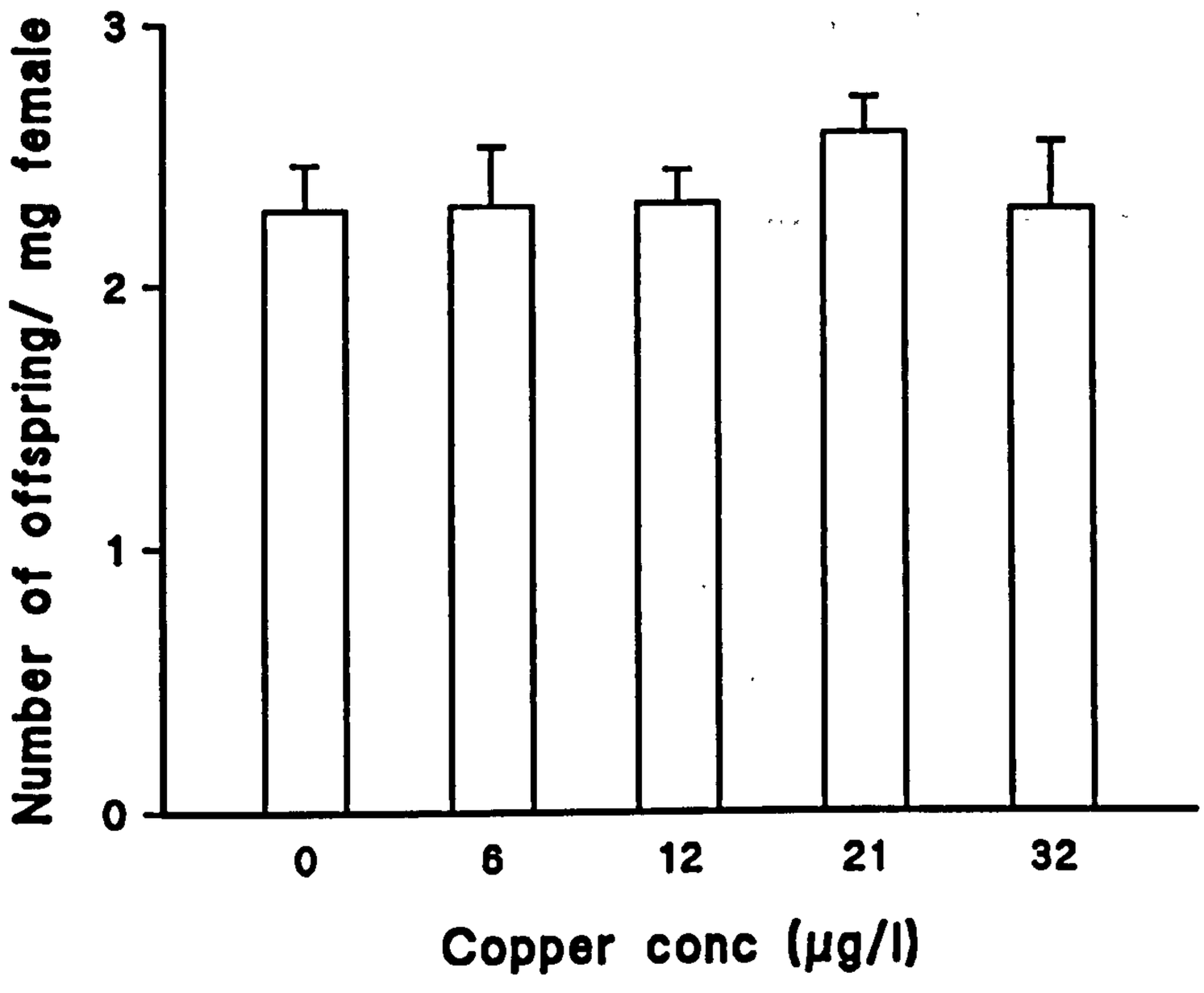


Fig. 22: Effect of copper on the number of offspring produced per mg of female from the current brood. Error bars represent 1 standard error.

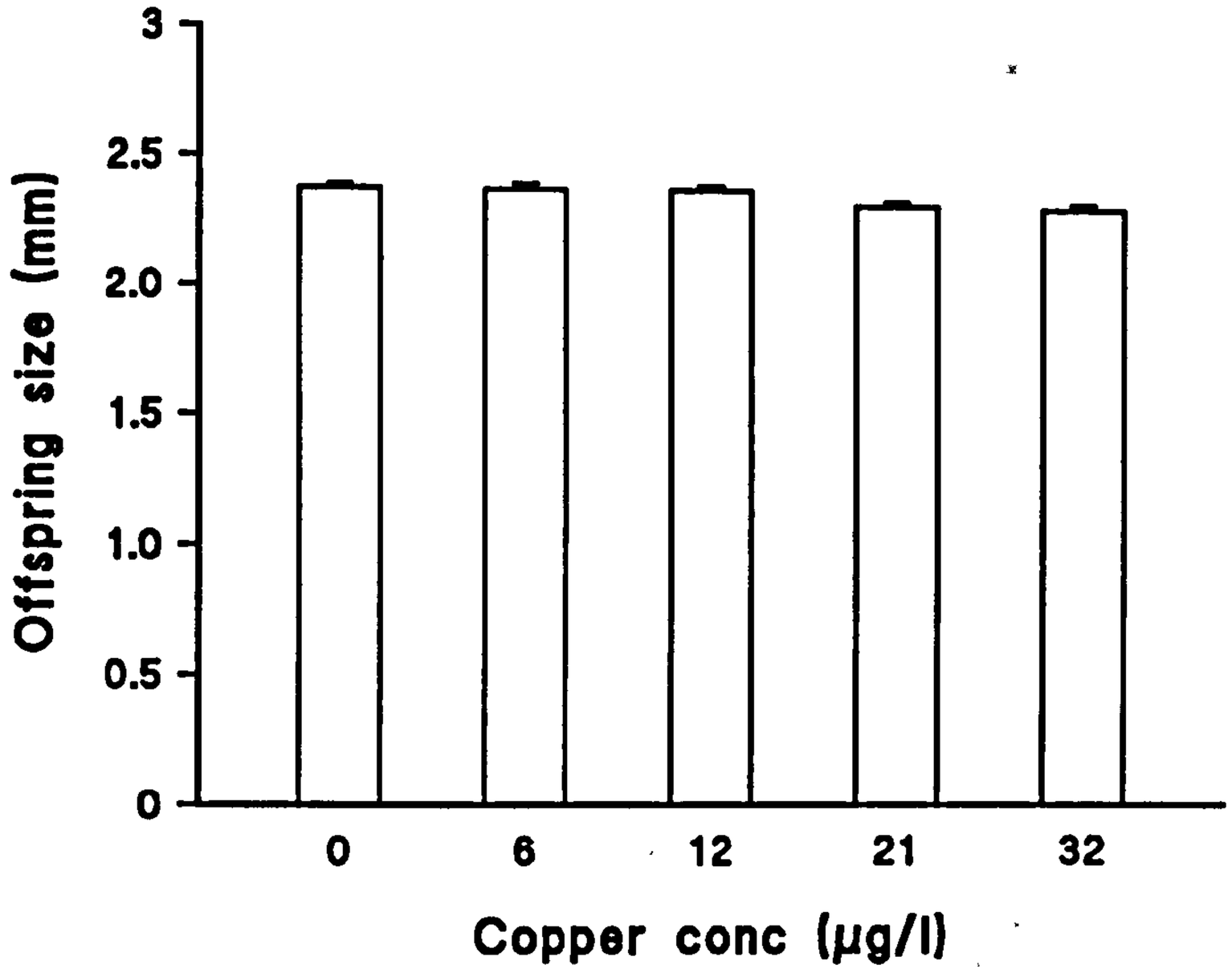


Fig. 23: Effect of copper on the size of offspring produced from the current brood. Error bars represent 1 standard error.

In contrast, abortion rates of females were elevated when they were exposed to copper, increasing from 11 to 58 percent over a copper concentration range of 0 to 32 µg/l. Further, there was a significant linear relationship between abortion rate and copper concentration ($r^2 = 77.2\%$, $df = 3$, $p < 0.05$) (Fig. 24).

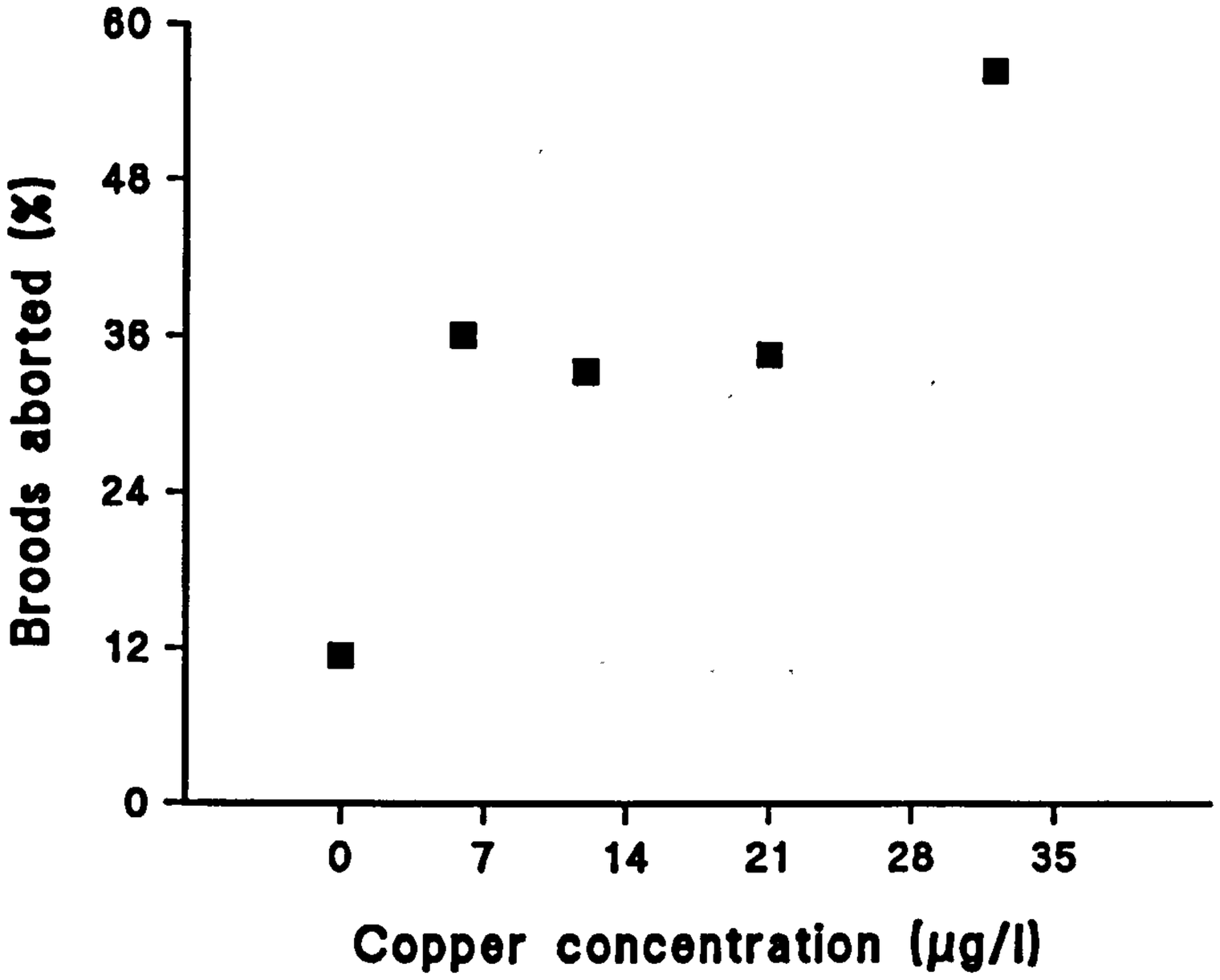


Fig. 24: Effect of copper on the percentage of females aborting their brood during one complete instar.

Abortion rates of all females were greatest during the first half of the instar (approximately day 0 to 10) with little or no abortion of broods occurring during the last half of the instar at any concentration over this range (Fig. 25).

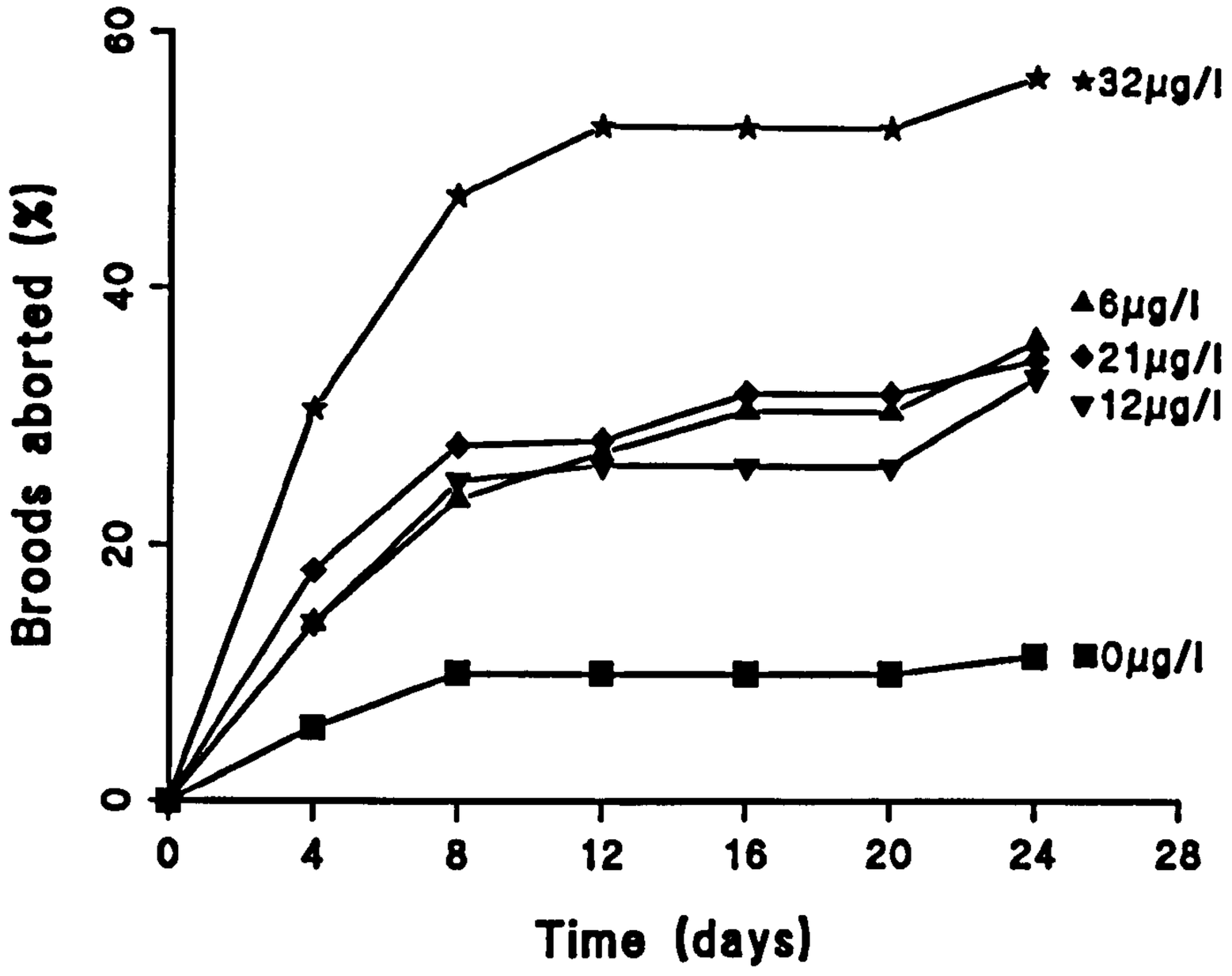


Fig. 25: Effect of copper on the percentage of females aborting over the period of one complete instar.

d. Spring broods.

Effects of copper on both current and subsequent brood were determined in experiments conducted in the spring although not for the same individual. As with the autumn broods there was no significant effect of copper on either the number of offspring produced per unit weight of female for the current ($F = 0.42$, $df = 4, 152$, $p > 0.05$), or the subsequent brood ($H = 4.35$, $df = 4, 16$, $p > 0.05$) (Fig. 26). Neither was there any significant correlation between copper concentration and number of offspring per unit weight of female for the current ($r^2 = 0.9\%$, $df = 153$, $p > 0.05$) or the subsequent brood ($r^2 = 2.9\%$, $df = 16$, $p > 0.05$).

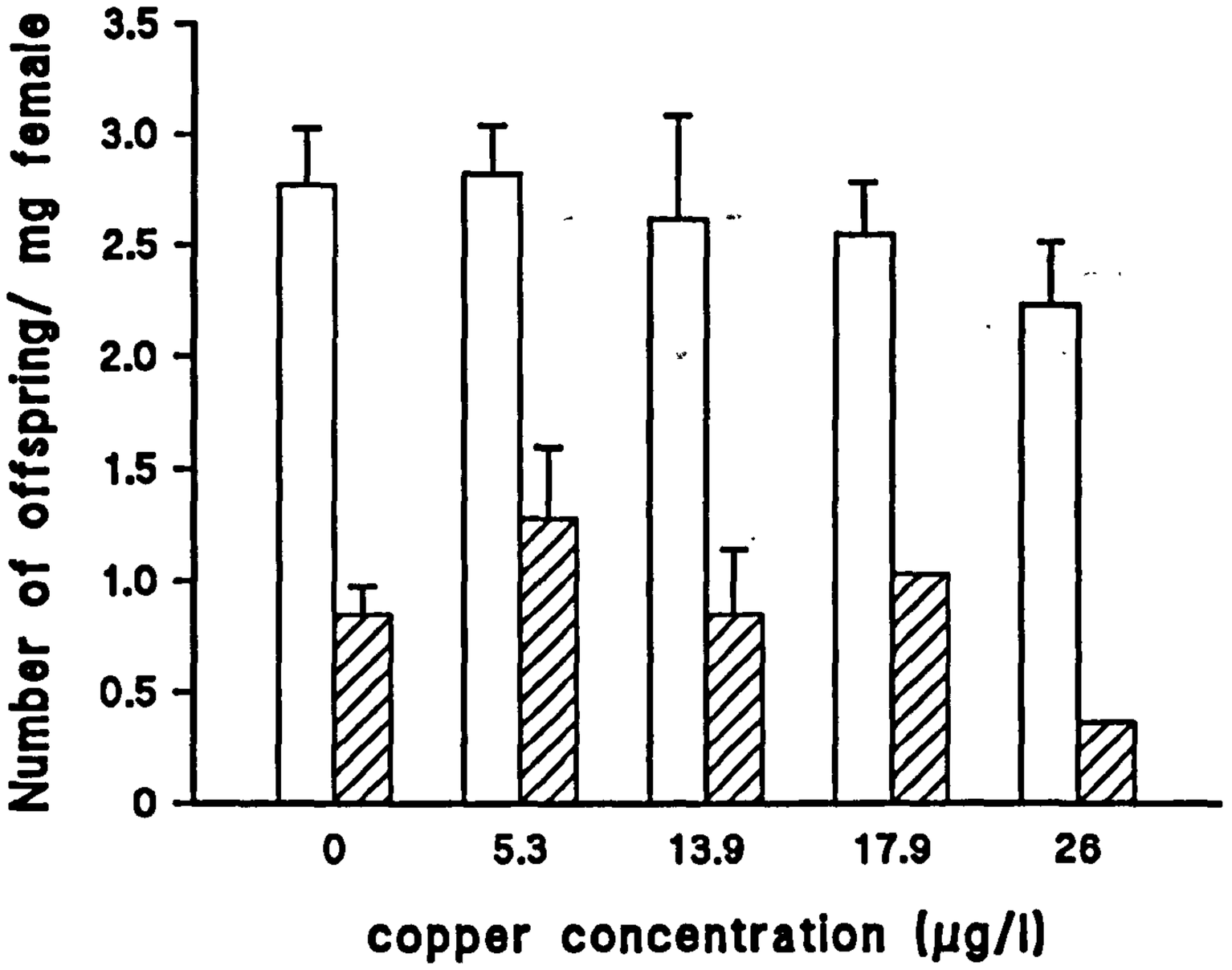


Fig. 26: Effect of copper on the number of offspring produced per mg of female from the current (open bars) and subsequent (hatched bars) brood. Error bars represent 1 standard error.

Similarly, there was no effect of copper on the size of offspring produced in the subsequent brood ($H = 1.35$, $df = 4, 36$, $p > 0.05$) and no significant correlation with copper concentration ($r^2 = 0.01\%$, $df = 36$, $p > 0.05$) (Fig. 27). Contrary to previous results however, there was a significant effect on the current brood ($F = 6.069$, $df = 4, 636$, $p < 0.001$)). Significantly smaller offspring than the control were produced by females exposed to 17.9 µg/l but not 26 µg/l copper and there was a significant correlation between copper concentration and the size of offspring in the current brood ($r^2 = 1.0\%$, $df = 637$, $p < 0.01$) (Fig. 27).

Fewer offspring were produced in the subsequent brood than in the current brood but they were larger in size; this was a general effect across all concentrations the reason for which is not apparent.

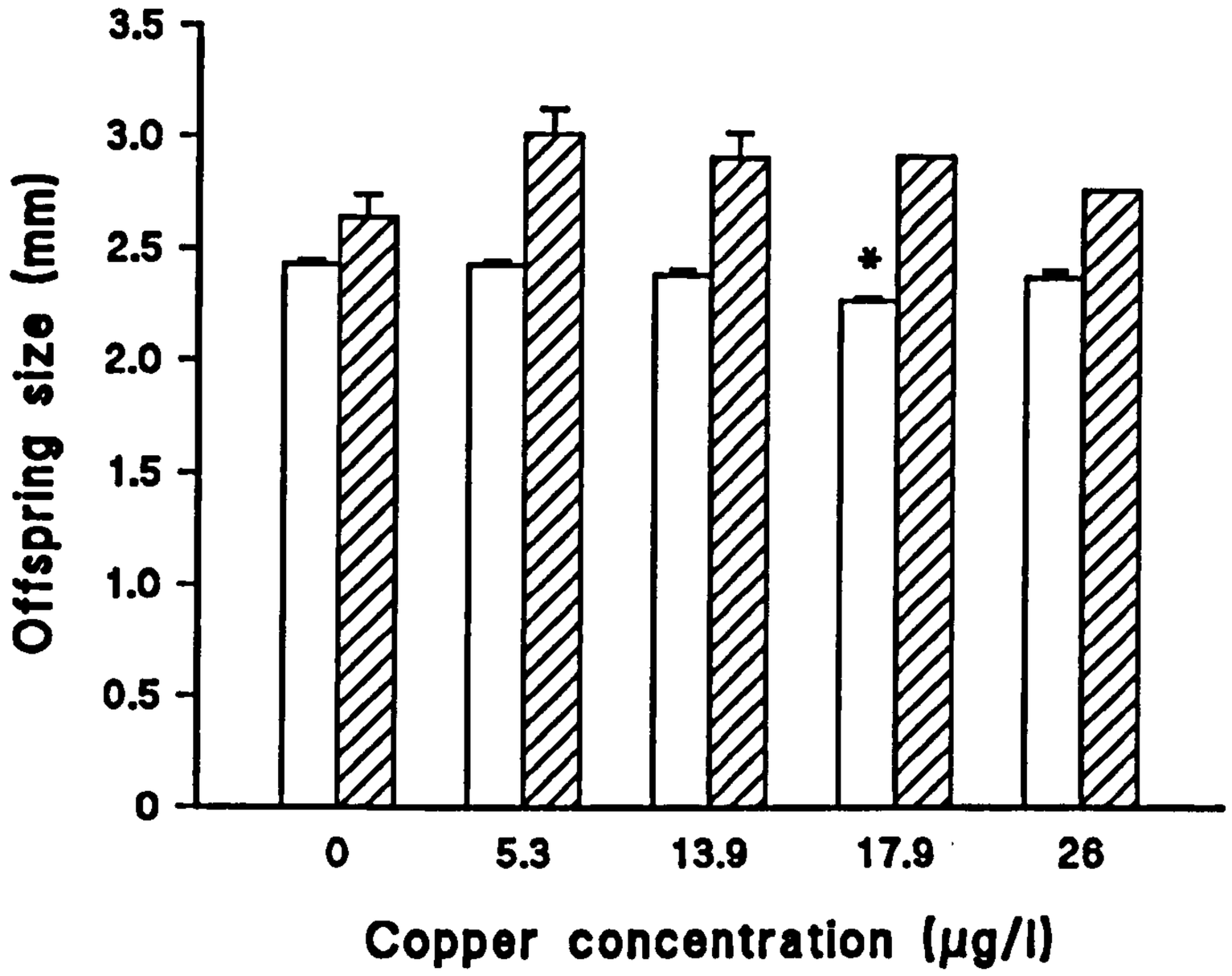


Fig. 27: Effect of copper on the size of offspring produced in the current (open bars) or subsequent (hatched bars) brood. Error bars represent 1 standard error, * data significantly different from control ($p < 0.05$).

Effects of copper on abortion rates of animals from autumn and spring experiments are remarkably similar. Analysis of variance of arcsine transformed data from the current brood showed a significant increase in the abortion rates of animals exposed to concentrations of 13.9 µg/l copper and above ($F = 23.581$, $df = 4, 34$, $p < 0.001$). Further, there was a significant relationship between abortion rate and copper concentration ($r^2 = 92.9\%$, $df = 3$, $p < 0.05$) (Fig. 28).

Abortion rates of the subsequent brood were also elevated at these concentrations but the regression of abortion rate against copper concentration was not statistically significant ($r^2 = 78.8\%$, $df = 3$, $p > 0.05$) (Fig. 28).

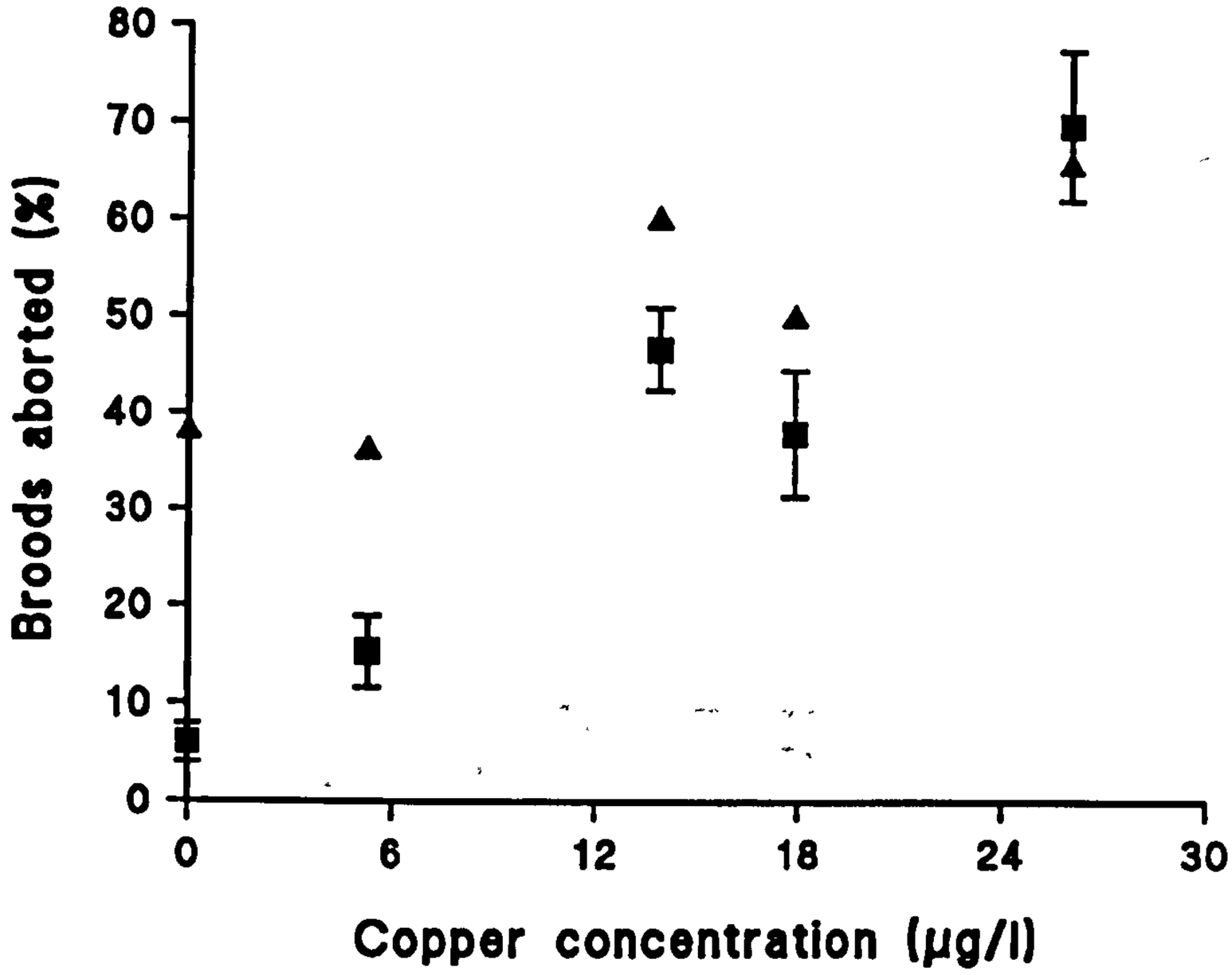


Fig. 28: Effect of copper on the percentage of females aborting their current (solid squares) or subsequent (solid triangles) brood during one complete instar. Error bars represent one standard error.

As in autumn experiments, abortion rates of all animals were highest during the first half of the instar (approximately day 0 to day 10), with little or no abortion of broods occurring during the second half of the instar at any concentration (Fig. 29).

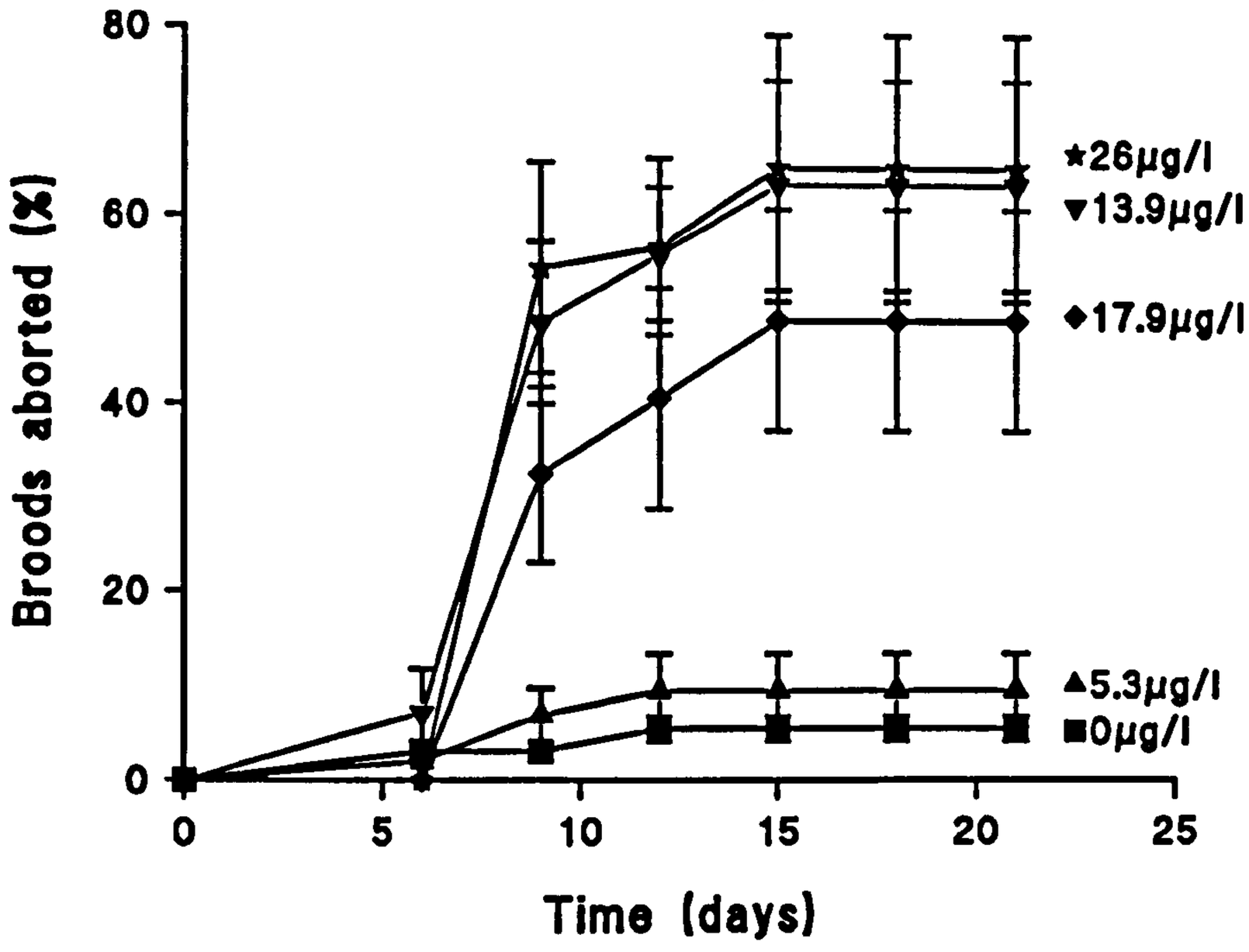


Fig. 29: Abortion rates of broods during exposure to copper over one complete female instar. Error bars represent 1 standard error.

In summary, copper had no effect on either the size or the number of offspring produced in either the current or the subsequent brood (with the exception of a significant reduction in offspring size at 17.9 µgCu/l but not 26 µgCu/l in the current spring brood). The major effect of copper on reproduction of *G.pulex* was an increase in the number of broods aborted with increasing copper concentration.

e. Brood cannibalism.

The major effect of copper on the reproduction of *G.pulex* was an increase in abortion rates. However, if eggs are prematurely released into the water this will represent a loss of resources. These resources could be recycled and hence the energy recaptured if the female consumed her aborted eggs. In flowing water, consumption of aborted eggs may be difficult as eggs would be swept downstream as soon as they were released. Hence

consumption of eggs directly from the brood pouch would be more profitable. Cannibalism experiments were therefore designed to investigate if females did consume aborted eggs and if so were they consumed directly from the brood pouch.

Animals placed in pots without a mesh divide could potentially consume eggs directly from the brood pouch or after they had been released into the water. Animals placed in pots with a mesh divide could only potentially consume eggs directly from the brood pouch as once eggs were released into the water they would fall into the lower chamber thus rendering tham inaccessible to the female.

Of the animals which were placed in pots without a mesh divide, 60 percent aborted and did not die. Of the latter 83 percent of animals consumed their brood. Of the animals placed in pots with a mesh divide, 45 percent aborted and survived, of these 33 percent of the broods fell into the chamber below, and 66 percent of the broods were consumed, presumably directly from the brood pouch.

f. Prediction of reproductive output.

The effects of copper on reproductive output of females were predicted from the amount of energy available for production (P; J/mg/d). The total amount of energy accumulated in an instar (Ei; J/mg) was calculated by multiplying P by 14.92 (the duration of the instar spent feeding; Table 8) and the values for the amount of energy lost due to moulting were obtained from Section 3.5. 1. c, Table 11. The joule equivalent for eggs was determined by microbomb calorimetry was equal to 25.15 J/mg (SE = 0.503, n = 5). The net amount of energy accumulated in an instar (Pr) was determined by subtracting the amount of energy lost in the moult from the total amount of energy accumulated in an instar. This value divided by the joule equivalent for eggs gives an estimated of reproductive output (RO; mg/mg) (Table 14).

Table 14. Effect of copper (Cu; µg/l) on the amount of energy available for production (P; J/mg/d) and lost via standard respiration (R; J/mg/d) and hence the total amount of enery accumulated in one complete instar (Ei; J/mg), energy required for moulting (Cm; J/mg), net amount of energy accumulated (Pr; J/mg) and the biomass produced through reproductive output (RO; mg/mg).

Cu	P	R	Ei	Cm	Pr	RO
0.0	0.498	0.177	7.430	4.416	3.014	0.120
13.0	0.833	0.191	12.427	4.458	7.969	0.317
23.0	0.022	0.217	0.322	4.536	-4.214	-0.168
27.0	0.039	0.139	5.765	4.302	1.463	0.058
30.0	-0.347	0.184	-5.170	4.437	-9.607	-0.382
36.0	-0.686	0.179	-10.241	4.423	-14.664	-0.583

Effects of copper on reproductive output, predicted from a measure of energy available for production, could then be compared to that observed in laboratory tests. Table 15 gives results for predicted and observed reproductive output of *G.pulex* exposed to copper. Concentrations in each test were slightly different so similar concentrations are boxed together to aid comparison of results.

Table 15. Reproductive output (Predicted RO) of females exposed to copper predicted from a measure of energy accumulated within an instar (E_i) and energy required for moulting (C_m) compared to that observed (Observed RO) for the current brood in the autumn (Autumn) and spring (Spring 1) and the subsequent spring brood (Spring 2).

Cu conc (µg/l)	Predicted RO	Observed RO		
		Autumn	Spring 1	Spring 2
0	0.120	0.110	0.147	0.059
5	/	/	0.151	0.110
6	/	0.120	/	/
12	/	0.115	/	/
13	0.317	/	/	/
14	/	/	0.134	0.061
18	/	/	0.108	0.092
21	/	0.108	/	/
23	-0.168	/	/	/
26	/	/	0.109	0.028
27	0.058	/	/	/
30	-0.382	/	/	/
32	/	0.099	/	/
36	-0.583	/	/	/

Positive values for reproductive output were predicted at copper concentrations below which consumption rates were significantly affected. Reproductive output of control

animals accurately predicts the values observed for the current broods of the autumn and spring experiment however, observed reproductive output of the subsequent brood is very much lower than predicted. The low reproductive output in the subsequent brood is a result of fewer offspring per brood being produced. However predicted estimates of reproductive output seriously overestimate those observed for animals exposed to copper concentrations close to 13 µg/l.

Reproductive output at copper concentrations of 23 µg/l and above (with the exception of 27 µg/l) was predicted to be negative. Hence death of animals would be expected to ensue at these concentrations. The experimental design here did not allow for determination of female survival as, upon aborting, females were removed from the toxicant. However, if abortion of the brood is a response to stress as an attempt to increase potential adult survival, then predicted effects of copper on reproductive output are likely to reflect observed effects on abortion rates.

Predicted reproductive output decreases with increasing copper concentration while observed reproductive output is maintained through all copper concentrations. However, observed reproductive output takes no account of abortion rates of females exposed to copper. As abortion rates were found to be the main parameter affected by copper in reproduction experiments, observed reproductive output was adjusted to account for abortion of females by multiplying the above values of observed reproductive output by the proportion of females which did not abort their broods (Table 16).

Table 16: Observed and predicted reproductive output (RO) of females accounting for proportion of females aborting their broods.

Cu conc (µg/l)	Predicted RO	Observed RO		
		Autumn	Spring 1	Spring 2
0	0.120	0.098	0.138	0.036
5	/	/	0.128	0.070
6	/	0.070	/	/
12	/	0.077	/	/
13	0.317	/	/	/
14	/	/	0.073	0.024
18	/	/	0.067	0.046
21	/	0.070	/	/
23	-0.168	/	/	/
26	/	/	0.032	0.012
27	0.058	/	/	/
30	-0.382	/	/	/
32	/	0.043	/	/
36	-0.583	/	/	/

Predicted reproductive output still compares favourably with that observed for the current broods of control animals. The predicted downward trend for reproductive output with increasing copper concentration is now observed with reductions in reproductive output occurring around 23 µgCu/l, the concentration at which reproductive output is predicted

to become negative. Predictions based on production data overestimate the effects of copper on reproductive output as at no concentration are negative values for reproductive output observed.

3.6. Discussion.

3.6. 1. Growth.

a. Growth rates.

Growth rates (Gs) of non-stressed *G.pulex* in this study are comparable to those determined by other workers (Table 17). Growth rates are dependent on diet, temperature, sex and stage of the life cycle and range from 0 to 4.67 mg/d.

Growth has been found to be a sensitive response criterion to copper in other species; e.g. growth of larval *Isognomon mathaei* (bivalve) was the most sensitive of the test criteria examined by Ringwood (1992) and Bellanger et al (1990) found that growth patterns of *Corbicula fluminea* were a clear and interpretable indicator of copper contamination in both artificial and natural streams. The growth of the nematode *Panagrellus silusiae* was blocked by copper at all stages of development (Haight, Mudry and Pastermak, 1982) and inhibition of growth due to copper contamination of the hydroid *Campanularia flexuosa* and of *Daphnia pulex* was also found to occur at lower concentrations than those which increased mortality (Borgmann et al 1980; Stebbing and Santiago-Fandino, 1983)

In other studies, however, growth has been found to be less sensitive to copper contamination than other parameters. The growth of *Tanytarsus dissimilis* was not reduced at exposure concentrations less than the LC_{50} (Anderson et al, 1980) and although copper dramatically reduced the growth of fathead minnows, experiments using trout resulted in control trout being smaller at the end of the test than those which were

exposed to copper. However, this difference was explained by differences in temperature as temperatures of controls were approximately $1^{o}C$ lower than other treatments (Spehar and Fiandt, 1986).

The study of Spehar and Fiandt (1986) highlights problems with the use of growth as a test criterion, as such long-term tests are subject to many confounding factors which may affect subsequent results. Ringwood (1992) stated that 'although growth rate was the most sensitive of the response criteria investigated, it was too tedious and time consuming to be used routinely.'

Table 17. Mean specific growth rates (Gs; % weight/ day) for *G.pulex*.

Gs	Dependent variables	Reference
0 - 1.69	diet	1
4.12 - 4.67	diet	2
0.0 - 6.65	diet/ stage in life cycle	3
0.44 - 1.23	stage in life cycle	4
0.28 -0.75	stage in life cycle	5
0.09 - 0.40	stage in life cycle/ temperature	6
0.66 - 1.18	sex	7
3.03	/	8
0.41 -1.03	individuals or groups	9

1. Willoughby and Sutcliffe, (1976); 2. Graca, (1990); 3. Sutcliffe et al, (1981); 4. Welton, (1979); 5. Iversen and Jensen (1977); 6. Welton and Clarke, (1980); 7. Nilsson, (1974); 8. Hynes (1955); 9. This study.

Copper caused a decrease in the growth rates of *G.pulex* when kept both individually and in groups although the reduction was only significant in grouped animals. A significant effect on the growth rate of grouped *G.pulex* was observed at a copper concentration of 19.44 µgCu/l, a slightly lower concentration than significant effects on consumption rates (the most sensitive of the energy budget parameters measured; i.e 23 µgCu/l, see Chapter 2). Effects of copper on growth rates were determined for juvenile animals while effects on consumption rates were determined for adults. It has been well documented that juvenile animals, including *G.pulex* are often more sensitive to stress than adults (Green et al, 1986; McCahon and Pascoe, 1988b). However, due to the lack of consumption data between the concentrations of 13 and 23 µgCu/l it is difficult to determine if there was any real difference between the sensitivity of these tests.

Although growth was suppressed by exposure to copper, survivorship of animals over the test period was low and variability resulted in a lack of statistically significant effects with animals kept individually. Hence the prediction of growth rates from a measurement of SfG may be a more useful method in determining the effects of a toxicant on the growth of a test species.

<u>b. Prediction of juvenile growth rates.</u>

The use of data determining the effects of copper on energy budget parameters for predicting subsequent effects on individual growth was assessed by comparison of observed growth rates with those predicted from production data.

At copper concentrations causing a significant reduction in consumption rates (i.e. 23 µgCu/l and above), energy required for moulting is predicted to exceed that available, hence animals would either not moult, and therefore not grow, or would die due to the stress of moulting (Tables 11 and 12). Indeed at copper concentrations just below this level (i.e. 19.44 µgCu/l) growth rates of groups (although not individuals) were observed

to be significantly reduced and a significant reduction in survival of both grouped and individual animals was observed when exposed to 32 µgCu/l (Figs 17 and 19).

Hence, although predicted and observed growth rates are difficult to compare, (due to different copper concentrations between the two groups and differences between the responses of animals held individually or in groups), it would appear that calculations, based on production estimates, accurately predict the trend for a reduction in growth and survival of *G.pulex* exposed to copper concentration of approximately 20 µg/l and over.

However, these techniques fall short of accurate predictions of actual growth rates, largely underestimating those observed. This would suggest error in the assumptions made in calculating predicted growth rates or in measured energy budget values. Error in these calculations may have been introduced due to many factors including:

1. An underestimate of the total energy accumulated during an instar (Ei). This could have been due to an overestimate of energy lost via feeding or active respiration for example. This was quite likely as values of Rf and Ra used in calculating production (P) were maximum values obtained.

2. Estimates of instar duration and period of time spent feeding within an instar were taken from the literature and may have been different for experimental animals in these tests.

3. C_m may have been overestimated, (e.g. an over-estimate of respiratory losses during moulting). Values again were taken from the literature.

4. Difficulties in direct comparison between predicted and observed results due to different copper concentrations being used.

5. Comparisons assume that energy available for production in juveniles (upon which growth measurements were made) is the same as for large adult males (upon which production estimates were based).

6. Energy equivalents for juveniles were based on one sample due to difficulties in obtaining sufficient biomass, therefore the reliability of this estimate is unknown.

In contrast to this study, predicted growth rates from scope for growth measurements of Nilsson (1974), over-estimated growth rates compared to those observed, this was probably due to Nilsson (1974) taking no account of active or feeding metabolic rates or of excretion.

3.6. 2. Reproduction.

a. Reproduction.

Copper appeared to have little effect on the either the number or the size of offspring produced by *G.pulex* in either the current (embryos in brood pouch during exposure of female to copper) or the subsequent brood (oogonia present in ovaries undergoing provisioning during exposure of female to copper). There was a slight indication of a decrease in the number of offspring produced in the subsequent brood when exposed to a copper concentration of 26 µg/l. This could be due to a lethal effect on the eggs whilst they are being provisioned. However, as this is based on just a single observation little certainty may be applied to this.

The lack of a direct lethal effect on the eggs themselves is in agreement with results of other workers. No effect of zinc or of ammonia was found on the number of eggs produced by *G.pulex* (Maltby and Naylor, 1990; Maltby, 1991). McCahon (1987) exposed eggs of *G.pulex in vitro* to cadmium and found that eggs were in fact the most

tolerant stage of the lifecycle with the chorion affording a high degree of protection. Green et al (1986) also found embryos of *Asellus aquaticus* to be more resistant to cadmium than 30 day old juveniles.

There was an indication that the size of offspring produced in the current brood was reduced as there was a significant reduction in size of offspring of females exposed to 17.9 µg/l copper in the spring. It is difficult to see how the size of offspring produced in the current brood could be reduced as these eggs are provisioned during the previous instar. It could be that copper causes the allocation of resources in the embryo to be diverted to processes other than growth (e.g. protein turn over) hence producing smaller offspring or, feeding of offspring within the brood pouch (Welton et al, 1983) is important for growth of the offspring and is inhibited by exposure to copper. However, as there was no effect of copper on the size of offspring produced in the current brood of animals exposed to copper concentrations up to 32 µg/l in the autumn or to those produced in the current brood of animals exposed to a higher concentration of 26 µg/l copper in the spring it is likely that the reduction in size of offspring of the current brood of females exposed to 17.9 µgCu/l in the spring was a result of natural variability rather than a real effect.

The major effect of copper on the reproduction of *G.pulex* appears to be an increase in the abortion rate of females. This is in agreement with the major effects of zinc and ammonia on reproduction in *G.pulex* (Maltby and Naylor, 1990; Maltby, 1991). Broods could be being aborted for various reasons including:

1. offspring are not viable, having been killed by copper whilst in the brood pouch;

2. copper toxicity to female results in abortion of young.

It is unlikely that abortion of broods is a result of the first of these possibilities as, there was no effect of copper on the number of viable young produced. Hence it would appear that the energy status of the female determines whether young will be aborted or not.

Reproduction is costly for the female in terms of energy - energy being committed to production of reproductive organs and gametes, provisioning, and energetic costs of carrying a brood. Williams' (1966) rule states that 'optimising current effort is to increase current commitment as long as current profits more than outweigh the future losses caused thereby.' Hence, it may be that when stressed by copper, continued commitment of resources to reproduction would jeopardize female survival, for example.

Hirshfield and Tinkle (1975) stated that 'the strategy of allocation of energy to reproduction in iteroparous species would depend on the degree to which the adults are able to predict the growth and survivorship of their young.' This statement was formalised in the model of Sibly and Calow (1984) which proposed a scheme to predict life history strategy favoured by natural selection under different conditions. This model classified two variables, S and G; S was an index of age-specific survival, and G and index of juvenile growth rate. One prediction arising from this model was that under conditions of low or variable juvenile survivorship (i.e. low S), iteroparous adults should invest less in reproduction, whereas under conditions of high juvenile survival they should invest more. In general juvenile *G.pulex* tend to be more sensitive to toxicity than are adults (McCahon and Pascoe, 1988b) therefore in view of the above predictions, in the face of copper stress, female *G.pulex* should invest less in reproduction in favour of their own survival. In *G.pulex* however, as survival of juveniles from the current brood is likely to be reduced, it might therefore be more profitable for the female to regain the energy already allocated to the current brood by ingesting the eggs and recycling the energy in order to increase the likelihood of adult survival and the probability of successful reproduction next time. This ingestion of aborted broods was observed to occur in *G.pulex* exposed to copper, females often removing eggs directly from the brood pouch.

The decision of which is the most profitable strategy, continued commitment to reproduction or abortion and consumption of the brood, may be determined by the energy status of the female. If, for example, energy available exceeds the energy required for moulting, the female may continue to invest in reproduction. If however, the energy required for moulting exceeds the energy available, a continued commitment to reproduction would result in the female dying at the next moult, hence energy allocated to reproduction may be recycled, trading off reproduction against the females own survival.

This type of strategy may only be useful for iteroparous animals. Calow (1973) suggests iteroparous parents maintain more strict physiological control on reproduction under adverse conditions than would semelparous animals. Under adverse conditions iteroparous insects (Bell and Bohm, 1975) and fish (Scott 1962) may resorb their gametes, while some animals have been found to resorb developing embryos (eg rabbits (Brambell, 1948)). Likewise the response of *G.pulex* to adverse conditions may be to consume developing eggs from the brood pouch.

It was noted that abortion rates were highest during the first half of the instar with little or no abortions occurring during the second half. Possible reasons for this include:

1. increased sensitivity of the female to copper during the first half of the instar;

2. increased energy demands on the female during the first half of the instar.

McCahon and Pascoe (1988c), studied the acute toxicity of cadmium to female *G.pulex* and found that females carrying stage one eggs in their brood pouch (i.e. one to three days post-fertilization, 11^{o}C), were more sensitive than animals carrying stage two to six embryos (i.e. four days post-fertilization to the hatch). It was postulated that this was due to the stage of the female moult cycle as it had earlier been determined by McCahon and

Pascoe (1988c), that toxicity of cadmium to *G.pulex* was significantly reduced within one week of moulting. This correlated with the finding of Wright (1980) that total recalcification of *G.pulex* following a moult occurs within 7 days at 50 mg/l calcium concentration of the water.

However, there is no evidence for copper regulation in *G.pulex*, therefore, copper uptake over the whole instar will be cumulative. If energy intake is related to total copper uptake, one might expect abortion rates to increase over the whole period of the instar.

The energy demands of the females during the first half of the instar may be greater than during the second half. Hence energy demand at this time may exceed energy available resulting in abortion of the current brood. Bradley et al (1991) determined that allocation of energy to reproduction in *D.magna* is discontinuous, developing oocytes being provisioned during the first half of the instar only. If the same pattern of allocation occurs in *G.pulex*, the energy demand at this time will be far greater than during the second half of the instar. Hence if animals have enough energy to continue brooding through the first half of the instar they are likely to be able to continue for the whole of the brooding period and abortion rates will be reduced during the second half of the instar. Further, females faced with toxic stress during the second half of the instar only, may be less sensitive than those faced with toxic stress during the first half only - in these circumstances the calcium status of the animals may then also contribute to enhanced sensitivity of the female during the first half of the instar.

<u>b. Prediction of reproductive output.</u>

Results from measurements of the effects of copper on production in *G.pulex* predicted a decrease in reproductive output with increasing copper concentration while first estimates of observed reproductive output remained approximately constant over all concentrations investigated (Table 15). However this measure of observed reproductive output was

misleading as the major effect of copper on reproduction in *G.pulex* was to cause an increase in the number of broods aborted hence causing a reduction in reproductive output not accounted for using equation 3.8. Observed reproductive output was therefore adjusted to account for abortion rates of females exposed to copper and the predicted downward trend for reproductive output with increasing copper concentration was then observed (Table 16).

Reductions in reproductive output were observed to occur around 23 µgCu/l, the concentration at which predicted reproductive output became negative. However, predictions based on production data overestimated the effects of copper on reproductive output as negative values for reproductive output were not observed even at the highest copper concentration (Table 14).

If, as it would appear, more energy is invested in reproduction than is available; this extra energy may come from mobilised body reserves. However, in view of the statements concerning optimal life history given previously (Section 3.6. 2. a) this would not appear a good strategy. If juvenile survival (S) is likely to be less than adult survival then iteroparous adults should seek to enhance chances of their own survival at the expense of reproduction. Indeed, the major effect of stressing females with copper was that the brood was sacrificed in an attempt to enhance adult survival.

It would therefore be more likely that the overestimate of the effects of copper on female reproductive output was due to error in the values used or assumptions underlying the calculations. Some reasons for potential error include points one to four, listed for predicted growth rates, (Section 3.6. 1. b). In addition to the above, comparisons between observed and predicted reproductive output assumed that energy available for production in females (upon which reproduction measurements were based) was the same as for large adult males (upon which production estimated were based).

Hence copper was found to cause a reduction in the growth rates of *G.pulex* at a copper concentration of 19.44 µgCu/l and a significant decrease in survival at 32 µgCu/l. This compares well both with effects of copper on consumption rates (the most sensitive of the energy budget parameters measured) and with trends expected using production data to estimate growth rates. Although it must be noted that concentrations between 0 and 19.44 µgCu/l were not investigated hence copper may decrease growth rates at concentrations below 19.44 µgCu/l. Quantitative predictions of growth rates however were not so accurate probably due to a number of errors in the values used and assumptions underlying predicted growth rate calculations.

Copper also affected reproduction with the most sensitive parameter being the abortion of broods by the female, which increased linearly with increased copper concentration. In the experiment where statistical analysis could be performed abortion rates were significantly increased by exposure of females to copper concentrations of 13.9 µg/l hence this parameter is more sensitive to copper than any of the energy budget parameters measured in Chapter 2. The predicted effect of copper on the reproductive output of females was however, over-estimated and as with growth rates this was likely to be due to a number of errors in the values used and assumptions underlying predicted reproductive output calculations

3.7. Summary.

1. Copper caused a decrease in the growth rate of *G.pulex* although significant reductions in the growth rate were only observed for animals held in groups.

2. Copper concentrations of 19.44 µg/l and above caused a significant reduction in growth rates of *G.pulex*. This compared well with the effects of copper on the most sensitive of the energy budget parameters measured in Chapter 2 (i.e. consumption rates), where a significant reduction was observed at 23 µgCu/l.

3. In addition to a significant reduction in growth rates of *G.pulex* at a copper concentration of 19.44 µgCu/l a significant decrease in survival was observed at 32 µgCu/l. This trend was accurately predicted using production data to estimate growth rates. However actual values of growth rates predicted from measurements of energy budget parameters underestimated those observed at all concentrations tested probably due to errors in the values used and assumptions made in calculating predicted growth rates.

4. Exposure of brooding females to copper had little effect on the number or size of offspring produced in either the current or the subsequent brood. The major effect of copper on the reproductive output of *G.pulex* was an increase in the abortion rate of females with a significant increase in abortion rates determined at copper concentrations of 13.2 µgCu/l and above.

5. It is likely that the increase in abortion rates of females was due to the effects of copper on their energy status. The iteroparous female may sacrifice and consume her brood when her own survival is threatened and the likelihood of survival of her offspring is low. In this way she may recycle energy already allocated to that brood and hence increase her own chances of survival and potential contribution of offspring to the population at subsequent reproductive events.

6. Allocation of energy from female reserves to reproduction appears to occur during the first half of the instar. When stressed by a toxicant during this period the female is likely to fall into negative energy balance. In response to this, abortion rates of the female are increased.

7. The downward trend in reproductive output over the range of copper concentrations investigated was predicted from production estimates. However, predicted values of

reproductive output fell significantly below those observed, This was probably due to a number of errors in the values used and assumptions underlying predicted reproductive output calculations.

CHAPTER 4

VALIDATION OF PREDICTIONS, BASED ON LABORATORY TESTS, USING ARTIFICIAL STREAMS

4.1 Introduction.

The 'scope for growth' bioassay, using *G.pulex*, has been modified from a standard laboratory test for field deployment (Maltby et al, 1990b), and is therefore potentially useful both for prediction and monitoring. Field deployment of the scope for growth bioassay was used to assess effects of a mixture of chlorinated ethers in artificial streams on the feeding rates of *G.pulex* (Maltby, 1992). Although no assessment of the predictive capability of this test was made by comparison with laboratory tests, the *G.pulex* feeding rate bioassay was found to be useful in assessment of impact. This test was more sensitive than community effects based on drift or population density as response criteria (Crossland, Mitchell and Dorn, 1992; Maltby, 1992). Hence, the scope for growth test is potentially useful in predictive assessment of the impact of a toxicant. However, it is necessary to validate results based on laboratory scope for growth experiments directly with equivalent tests in more natural systems.

Few studies have attempted to validate laboratory results in more complex systems and what studies there have been, have largely been inconclusive and contradictory. For example results from laboratory single-species tests have been reported to be similar to those from field studies (Hansen and Garton, 1982; Adams et al, 1983; Crossland, 1984; Crossland and Hillaby, 1985; Giddings and Franco, 1985; Larsen et al, 1986; Poirier and Surgeoner, 1988), more sensitive than field tests (Stout and Cooper, 1983; Clements, Cherry and Cairns, 1988; Fairchild et al, 1992), or less sensitive than field tests (Hansen and Garton, 1982; Adams et al, 1983; Crossland, 1984; Crossland and Hillaby, 1985; Giddings and Franco, 1985; Larsen et al, 1986; Poirier and Surgeoner, 1988).

Some of the above studies show greater predictive capability than others and we must consider what we judge to be acceptable correlation between laboratory and field results. For example, Adams et al (1983), state that a factor of 10 difference, between observed and predicted results, demonstrates a poor correlation, (their results in fact only vary by a factor of two). Although this statement appears to have no scientific backing. Chapman (1983) states that a factor of two can be viewed as general state-of-the-art variability and can therefore be used as a measure of the significance of other parameters that affect test results. The study of Giddings and Franco (1985), applies an arbitrary application factor of three percent to laboratory results before using them to predict effects in microcosms. This practice may be considered somewhat dubious as again there is no scientific basis for the magnitude of the application factor applied.

Previous work involving laboratory to field comparisons has either employed perturbation of natural streams or the use of artificial mesocosms. Both methods may be used for the validation of laboratory tests. They may also provide information on the nature and extent of adverse effects and allow the study of effects beyond the single-species level.

4.1. 1. Natural streams.

One way in which to validate the predictive capacity of laboratory bioassays is via direct perturbations of natural environments. Studies involving effects of a pollutant in natural systems have previously compared similar streams in the same area, one or more of which are dosed and one is left non-dosed as a control. Other studies have dosed at intervals down the same stretch of river and observed effects on the community compared to reference sites. Parameters measured include invertebrate drift, density, survival, leaf processing rates and organic matter transport (Winner et al, 1975; Hall et al, 1980;

Cuffney, Wallace and Webster, 1984; Wallace, Vogel and Cuffney, 1986; Ormerod et al, 1987; Poirier and Surgeoner, 1988; Wallace et al, 1989; Cuffney et al, 1990).

These direct field studies provide good ecological information and allow the study of ecosystem scale parameters such as population shifts, community structure changes or measures such as biomass, productivity, or nutrient processing. However, this type of study provides very low replicability and therefore problems in statistical analysis. Replication of treatments is in fact pseudoreplication (Hurlbert, 1984) as such systems have integral variability and no two natural systems will be entirely replicable. Moreover, controls will not replicate the state of test streams before treatment (Underwood, 1989). As natural systems are dynamic there is a high degree of environmental variability and, there has in fact been limited success in obtaining predictive information from field investigations (Touart and Slimack 1989). Direct perturbation of natural streams causes environmental damage and restoration is costly and may take years. There are also few streams suitable to use as undisturbed reference sites. Therefore what is required is a means of validating laboratory tests under both replicable and environmentally realistic conditions while not directly harming the natural environment itself.

4.1. 2. Mesocosms.

The use of artificial mesocosms in the form of artificial streams, ponds or enclosures provide a means of testing the accuracy of laboratory derived predictions.

Microcosms and mesocosms are often confused in the literature and there is no generally accepted definition of these terms (Gearing, 1989). Odum (1984) defined mesocosms as 'bounded and partially enclosed outdoor experimental set ups'.

As with the use of natural systems, use of mesocosms may indicate if concentrations producing adverse effects under laboratory conditions will adversely affect the same

organisms under field exposure conditions (Touart and Slimack, 1989). Mesocosms allow the control of many of the complex interactions between the biota and its variable habitat, (e.g. current, temperature etc). They may, therefore, be used to indicate real ecosystem changes which could be linked to direct cause and effect relationships of the effluent.

Mesocosms are relatively small. Therefore ecosystem level responses to a toxicant may be examined under both replicable and environmentally realistic conditions (as they should respond in a similar manner to natural systems) (Cairns and Pratt, 1989; Zieris, 1991). This is particularly useful in the screening of a substance prior to its release. Mesocosms allow the comparison of different concentrations of a single chemical or an array of different chemicals, while not contaminating the natural environment itself.

The extent of replication that may be attained is not as great as in laboratory tests but is greater than for natural systems. However, as in natural stream tests, there may be problems of pseudoreplication (Hurlbert 1984) in these systems as they are not entirely replicable and statistical significance may be found where in reality it does not exist between the assumed cause and effect. Simplification and miniaturisation of the natural system may introduce a degree of error in chemical and physical characteristics (May, 1975; Dudzik et al, 1979). Obviously as complexity increases, so will the degree of environmental realism but at the cost of replicability so control exercised by the operator is not as great as for microcosms (Gillett, 1988). However, although the production of mesocosms is costly, one may obtain information that can not be obtained from single-species laboratory tests and which should not be obtained from the natural systems we wish to protect.

4.2 Objectives.

This chapter investigates the correlation between laboratory and field results, based on energy budget parameters measured in Chapter 2. In the validation of these tests, an

insight may be gained into effects of copper on *G.pulex* in more natural systems and if effects are the same as those observed in laboratory tests. This would allow determination of whether the laboratory-based scope for growth test on individuals can indeed be extrapolated to predict how populations of *G.pulex* might respond, when exposed to copper, in natural systems.

In order to investigate this the following questions will be addressed:

1. Are the direct effects of copper on the survival and energetics of *G.pulex*, as determined in the laboratory, validated in artificial streams?

2. How do the effects of copper on the relationship between energy budget parameters and body concentrations of animals deployed in the artificial streams compare with those of animals used in laboratory tests?

3. Can observed effects of copper on individuals, be extended to effects at the population level of organisation?

In order to address these questions energy budget experiments were conducted in artificial streams which simulated the natural environment. The use of such systems allowed a comparison of effects of copper observed in the laboratory with effects under more natural conditions. It also allowed the investigation of consequent effects at higher levels of organisation.

4.3 Materials and Methods.

4.3. 1. Artificial streams

The artificial streams were located in the grounds of the Leeds Sewage Works, Kent, in the South East of England (NGR TQ824 535). The experiments were conducted during May, 1991.

The artificial streams consisted of six, 5 metre long, 0.35 metre wide, 0.25 metre deep stainless steel troughs each holding 240 litres of water (Fig. 30 and 31). Each stream was divided into pool and riffle sections, separated by stainless steel mesh plates (see Crossland, Mitchell and Dorn, 1992, for details). The substrate, which lay in half the pool section and in the whole length of the riffle section, was a coarse gravel mixture. (Table 18).

Table 18. Size distribution of substrate particles in the artificial streams.

Particle size range	percentage of total within range
>8 mm	34
2-8 mm	62
1.4-2 mm	1
0.25-1.4 mm	2
<0.25 mm	0.4

Natural stream water was supplied from an adjacent stream via a header tank at a rate of 10 litres per hour, providing one complete change of water every 24 hours. Water was recirculated via a semi-recirculating loop at a rate of 5500 litres per hour. Flow rates in the pool sections were approximately 5 cm/s and approximately 20-25 cm/s in the riffle

section. A degree of temperature control was obtained via a heat exchange mechanism operating to cool recirculating water (Fig. 30 and 31).

The streams were set up as largely detritus-based systems with packs of leaf litter added as the food source, hence *G.pulex* was a potentially important organism in the functioning of the artificial stream ecosystems (Chapter 1, Section 1.2). Some algal covered stones were added for grazers, but algal growth was limited by covering the streams with a mesh canopy (Fig. 31).

The artificial streams were seeded with invertebrates over a one week period. Benthic invertebrates were collected by 'kick sampling' at two sites on a tributary of the River Len, stream running through the village of Hollingbourne, near Leeds, Kent (NGR TQ836 549 and TQ840 550), four to five weeks before commencing dosing. The invertebrate community present in the streams was characterised by *Polycelis* sp., *Nais* sp., *Gammarus pulex*, Chironomidae (unidentified sp), and *Elmis* sp.

Toxicants were held in a reservoir and were added to the streams along with the input of natural stream water. Copper was added as copper sulphate (BDH; Analar grade). Stream 1 was not dosed and maintained a background concentration of approximately 0.5 µgCu/l (SE = 0.02). Other streams were dosed with concentrations of 2 (Stream 3), 6 (Stream 4), 20 (Stream 2), 60 (Stream 5) and 200 (Stream 2) µgCu/l. The amount of copper required to obtain these nominal levels in the streams was dissolved in three litres of stream water. At the beginning of the experimental period, the copper solution was initially added to the bottom of the appropriate stream at the start of the recirculation system. Simultaneously, copper sulphate solutions were continuously pumped through the streams from the reservoirs.

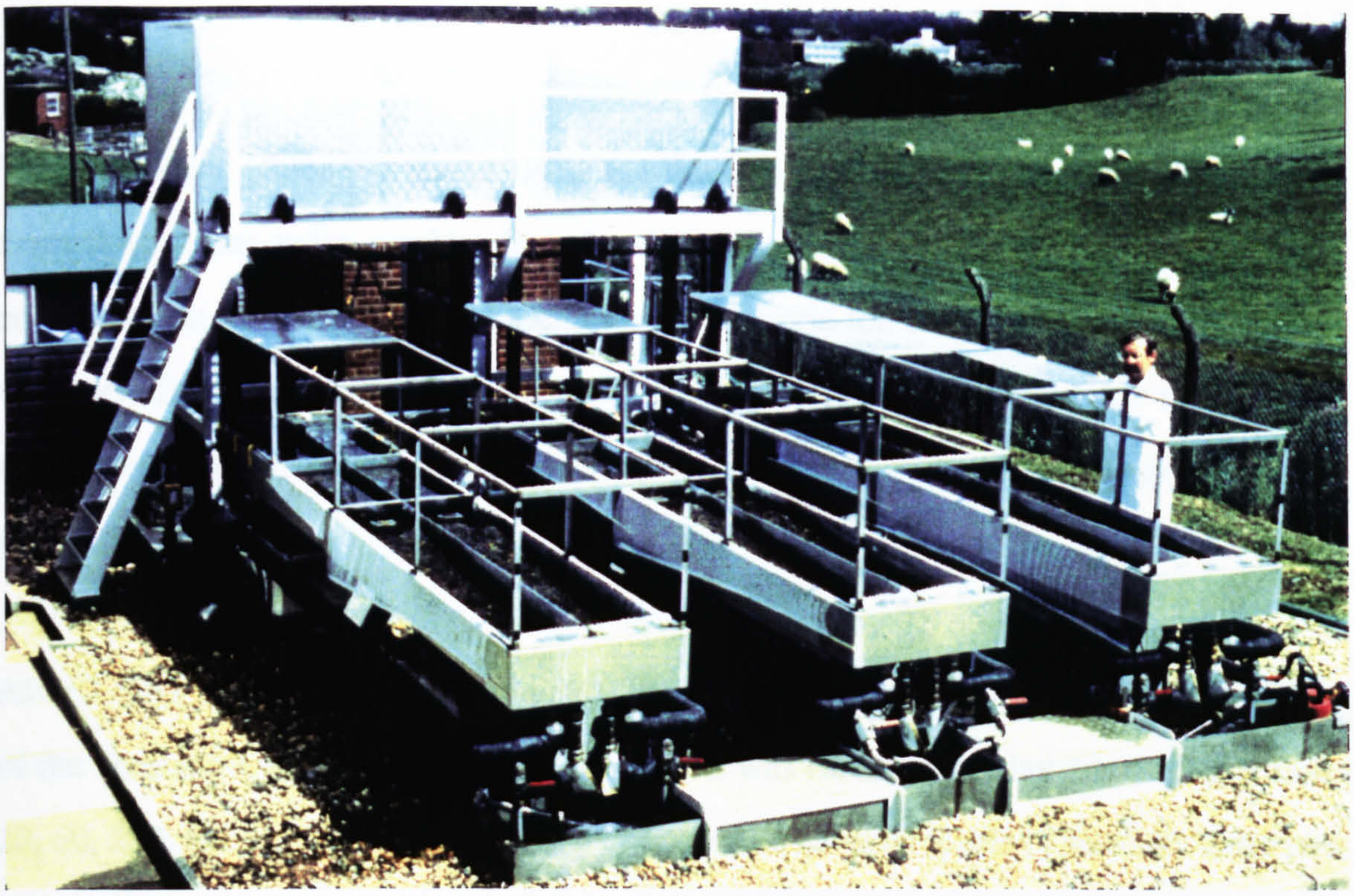


Fig. 30. The artificial streams (shown without mesh canopy).

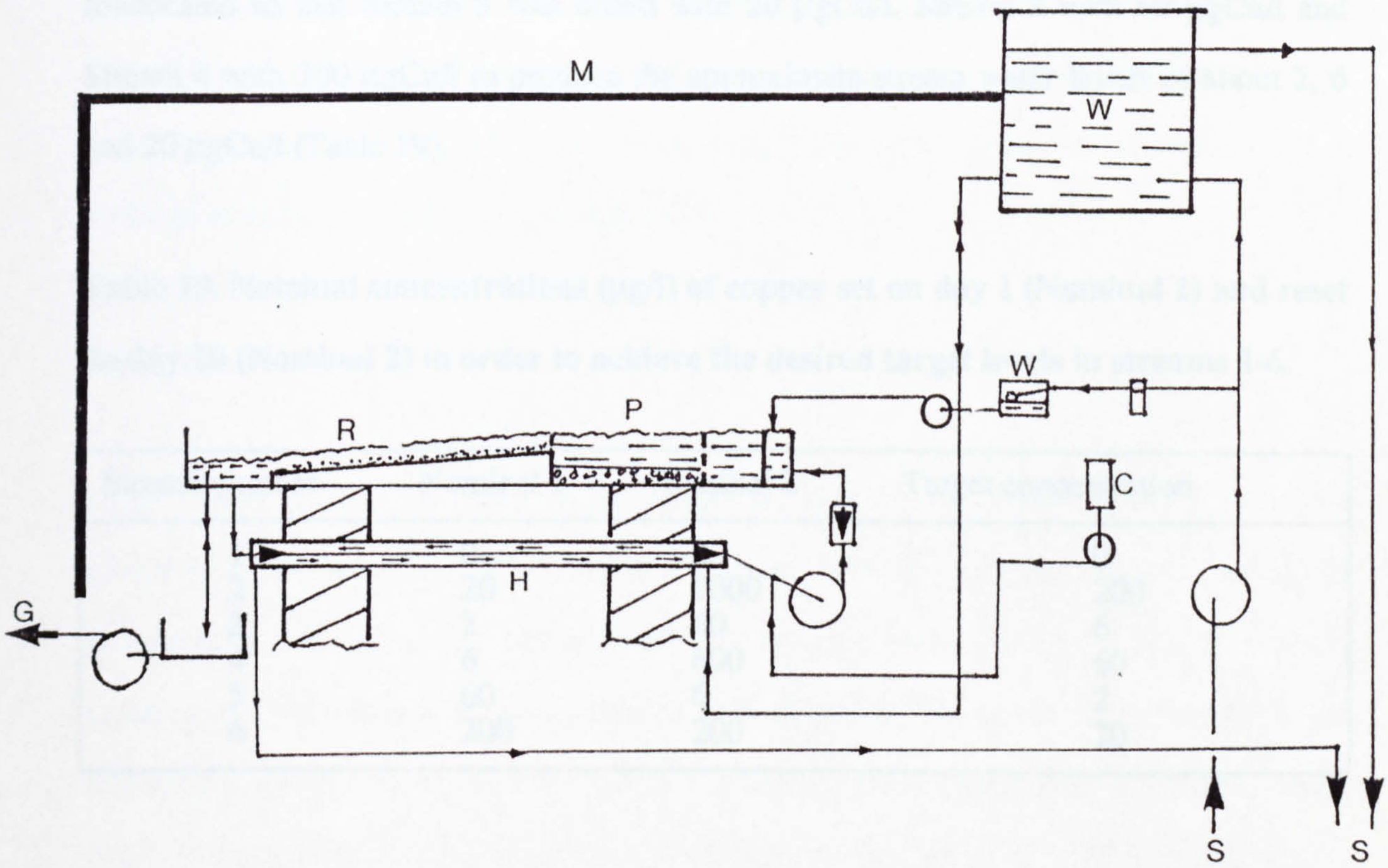


Fig. 31. Schematic representation of the artificial streams: water reservoir, W; chemical reservoir, C; mesh canopy, M; pool section, P; riffle section, R; heat exchanger, H; stream water, S; to sewage works, G. Arrows represent direction of water flow.

Water samples, (three per stream), filtered through 0.22 or 0.45 µm filters, were taken from the streams twice weekly. Samples were analysed for cupric ion (free and labile complexes) at the Shell Research Centre using a cupric ion electrode. Triplicate samples were acidified with 0.25 ml of Aristar grade nitric acid and analysed for total copper (total free, associated and complexed forms) by atomic absorption spectroscopy at Cardiff University.

After 7 days of dosing however, the streams had only reached between 10 to 35 percent of the nominal levels. The dosing of the streams was modified on day 10, dosing with 0, 20, 60, 200, 600 and 2000 µgCu/l to try to obtain the nominal levels (Table 19). Only two of the streams were actually redosed, Stream 4 with 600 µgCu/l and Stream 2 with 2000 µgCu/l. The control stream remained as such, while the remaining streams were reallocated so that Stream 5 was dosed with 20 µgCu/l, Stream 3 with 60 µgCu/l and Stream 4 with 200 µgCu/l to produce the approximate stream water levels of about 2, 6 and 20 µgCu/l (Table 19).

Table 19. Nominal concentrations (µg/l) of copper set on day 1 (Nominal 1) and reset on day 10 (Nominal 2) in order to achieve the desired target levels in streams 1-6.

Stream number	Nominal 1	Nominal 2	Target concentration
1	0	0	0
2	20	2000	200
3	2	60	6
4	6	600	60
5	60	6	2
6	200	200	20

As the levels of copper dosing were altered during the experimental period at which *G.pulex* was exposed, the experiments concerning survival and consumption rates were repeated, after redosing. The first test and its repeat will hence be referred to as 'trial 1' and 'trial 2' respectively. Animals deployed in Stream 2 and Stream 4, which were

redosed during exposure, were discarded due to the high variation in copper levels to which they were exposed during trial 1.

Water temperatures were continuously monitored in the natural supply stream and both temperature and dissolved oxygen (DO) concentrations were monitored continuously in all 6 artificial streams with thermistors and YSI Model 58 DO meters coupled to a Grant Squirrel data logger. Weekly measurements of total chlorine, nitrate, phosphate and conductivity were made in the natural, control (Stream 1) and the highest dosed stream (Stream 6 then 2), using a portable, battery powered, Hach spectrophotometric DREL/5 kit for water analysis. Water hardness was determined by EDTA titrimetric methods, using Solochrome Black 6b as the indicator. Total organic carbon (TOC) was determined by acidifying samples with HCl to reduce the pH to < 2 and stored at 4^{o}C until they were analysed with a Dohrmann DC-80 auto-analyser. The samples were purged with CO_2-free nitrogen gas for 5 minutes before injection into the auto-analyser. 500 ml of water were filtered through 0.8 µm filter papers which were then dried to constant weight in order to determine levels of suspended solids.

4.3. 2. Survival.

Large, male *Gammarus* were collected from Crags Stream (NGR SK497 745) (see Chapter 2, Section 2.3. 1). Survival of these animals was investigated using 25 individually caged *G.pulex* per stream. The animals were placed in 5 x 5 x 5 cm cylindrical PVC cages with mesh covered ends (Fig. 32). The mesh pore size was 1 mm allowing a through-flow of water while not allowing the *Gammarus* to escape. These individual cages were deployed in each of the streams in a larger 30 x 10 x 25 cm holding cage.

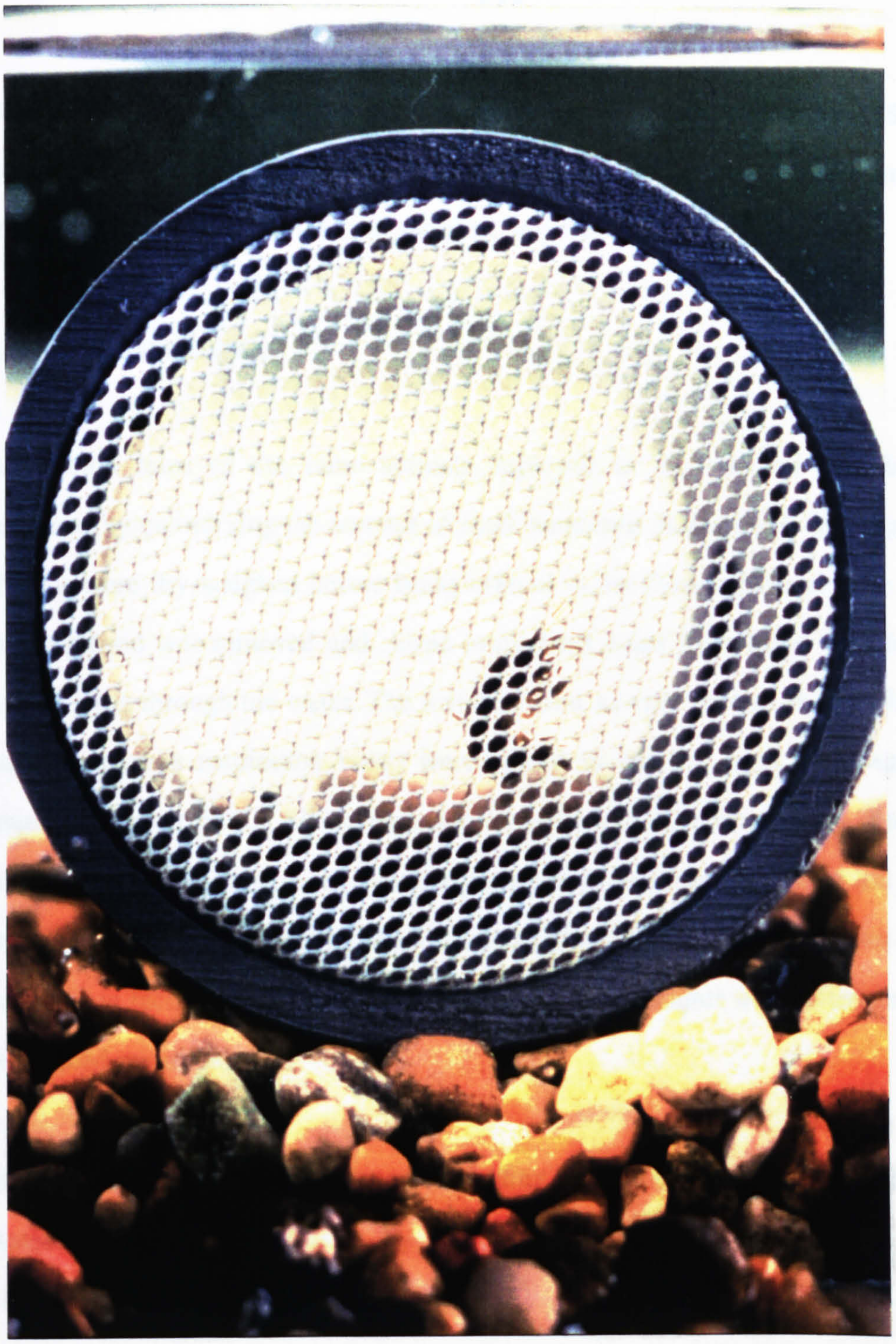


Fig. 32. Cage, containing animal and *Cladosporium* inoculated alder leaf discs as food, used for the deployment of *G.pulex* in the artificial streams.

Five alder leaf discs, prepared in the same manner as described in Chapter 2 (Section 2.3. 2. b), were placed in each cage for food. The animals were deployed in the streams for 6 days after which they were removed and their survival noted.

4.3. 3. Consumption rates.

Consumption rates of the same animals deployed in the survival experiment were determined by quantifying the amount of food consumed during the 6 day exposure. The leaf discs were dry weighed before being offered to the *Gammarus* and after 6 days the remaining food was removed and rinsed in distilled water by holding with forceps and gently moving through the water. The leaf discs and animals (except those animals being used to determine respiration rates) were placed in labelled trays and were dried to constant weight at 60^{o}C. All animals and discs were then reweighed to determine the final animal and leaf weight. Five cages per stream containing leaf discs but no animal were used as controls to compensate for any weight changes of the leaf discs that might occur, due to factors other than *Gammarus* feeding. All weights were determined using a Mettler 30M micro-balance.

Consumption rates were determined according to the equations already outlined in Chapter 2 (i.e. eqn 2.1 and 2.2).

4.3. 4. Absorption rates.

Absorption rates could not easily be measured in animals in the streams due to the inability to collect faeces. Therefore faecal production was quantified using the following equation relating consumption rates (C; J/mg/day), to faecal production (F; J/mg/day), obtained in Chapter 2 (i.e. eqn 2.14):

$$F = 0.631 \times C + 0.191$$

$$(r^2 = 85.3\%\ df = 121)$$

Absorbed energy (A; J/mg/d) was then calculated as the difference between C and F.

4.3. 5. Standard respiration rates.

As measurements of respiration rates for each treatment take a full day and time was limited, metabolic costs were only measured in a subset of control animals and animals deployed in the top two dosed streams during the first trial.

Respiration rates were measured by flow-through respirometry in filtered stream water using a Radiometer oxygen electrode and a Strathkelvin oxygen meter (model 781) using the same methodology as described in Chapter 2 (Section 2.3. 4 c). Animals were then placed in labelled trays and dried at 60°C to determine animal dry weights.

4.3. 6. Body copper concentration.

Ten dry weighed animals and 10 dry weighed samples of leaf material per treatment were digested in primar grade nitric acid (Fisons) in a Tecam DG1 block digester using methodology described in Chapter 2 (Section 2.3. 7). The amount of total copper in the animal and leaf material digests was analysed by Furnace Atomic Absorption Spectroscopy (FAAS) and the concentration of copper in the animals and leaves determined using equation 2.12 (Chapter 2).

4.3. 7. Population density.

Animals from the pool and riffle section of each stream were sampled, counted and measured, prior to exposure (day 0) and at the end of the trial period (day 28). Samples from the riffle section were also taken in the middle of the exposure period (day 14).

Animals were sampled by means of lift out mesh substrate samplers, enclosing a 188.7cm^2 area (Designed by Shell, manufactured by House and Co.). There were six samples per stream, three from the riffle and three from the pool section. Samples were returned to the laboratory and animals separated by floatation using 30% sucrose solution. *Gammarus* were divided, on the basis of number of antennal segments, into adults (19 or more segments) and juveniles (less than 19 segments) and numbers present in each group recorded and density (no./cm^2) calculated. An estimate of total number of individuals per stream was obtained by multiplying mean density by total stream area.

4.4 Statistical analyses.

All results were tested for normality using the n-scores test before conducting further analysis. Water quality parameters between the control and highest dosed stream and between artificial streams and the natural stream were compared with a two sample t-test.

Energy budget parameters and population density were analysed using one way analysis of variance and the Tukey multiple range test. Determination of significant differences between groups were assessed by applying the twosample t-test at $p < 0.05$.

The survivorship data of animals deployed in the artificial streams was not distributed in a manner suitable for probit analysis. Therefore, estimation of LC_{50} values were obtained using least squares regression techniques of arcsine transformed data and prediction of copper concentration causing 50 percent mortality.

Regression lines for consumption rates and for body copper concentration against copper concentration of the media were also obtained using least squares regression, and compared by analysis of covariance.

All tests were performed using either Minitab, Statgraphics or Fig P software packages.

4.5 Results.

4.5. 1. Copper concentrations.

Actual levels of copper in the stream water increased with time up to and beyond the nominal levels. The animals were therefore exposed to an increasing range of copper concentrations over the experimental period (Table 20).

Cupric ion concentrations were also measured in three of the streams (Table 21). Cupric ion levels in the 200 µg/l nominal level stream on day 12 gave a curious result where cupric ion concentration appeared greater than total copper levels. This is probably due to problems in analytical techniques and thus this data point was not subsequently used in statistical analysis.

Table 20. Actual concentrations of total copper (μg/l) in the artificial streams from day 1 to day 28. Vertical line after day 8 represents time at which redosing occurred in streams nominally at 60 and 200 μg/l on day 10.

Nominal (μg/l)	Days from start of dosing 1	4	6	8	12	15	21	25	28
0	0.5	0.5	0.6	0.6	0.5	0.3	0.5	0.5	0.9
2	1.2	1.4	1.8	2.1	2.8	2.4	2.2	2.5	2.7
6	6.4	6.7	7.9	9.7	8.8	12.5	11.0	10.6	12.0
20	13.5	18.0	19.2	24.3	23.0	24.8	23.4	29.9	33.0
60	0.7	0.7	0.7	0.8	44.6	54.8	91.2	121.8	120.0
200	2.5	3.2	4.0	4.3	200.0	330.0	317.0	450.8	490.0

Table 21: Cupric ion concentration (μg/l) as percentage of total copper concentration (μg/l).

Target conc. (μg/l)	Days from start of dosing 12	15	21	25	28	32
20	91	48	61	60	79	69
60	78	64	63	69	83	74
200	215*	73	84	80	90	63

* Anomalous reading not used in statistical analysis.

There was no significant difference between the proportion (arcsine transformed data) of total copper present in the form of cupric ions in the top three dosed streams (Table 21). (Paired t-test $t < 2.11$, $df = 7$, $p > 0.05$).

Hence total copper and cupric ion concentration were proportional, and the use of total copper in comparisons of toxicity was not complicated by possible effects of different proportions of cupric ion. For the purposes of this study, the levels of total copper in the system will be used. Total copper levels of the streams may be directly compared to those in the laboratory where the facility for measurement of cupric ion did not exist.

4.5. 2 Water quality.

Water quality measurements for control, highest dosed and supply streams, are summarised in Tables 22, 23 and 24.

Table 22. Water quality parameters of the control stream.

Water quality parameter	Days from start of dosing				
	5	13	19	26	33
Temperature(^{o}C)	16.2	18.8	11.3	15.3	14.3
Dissolved oxygen (mg/l)	10	9.2	10.5	9.9	10.9
pH	8.4	8.5	8.2	8.2	8.8
Total chlorine (mg/l)	0.00	1.00	0.03	0.02	0.04
Nitrate (mg/l)	25.5	33.0	26.4	33.0	26.4
Phosphate (mg/l)	0.20	0.12	0.03	0.15	0.95
Conductivity (mhos/cm)	550	550	580	550	550
Total Hardness (mg$CaCO_3$/l)	244	256	310	288	292
Suspended solids (mg/l)	3.2	2.4	4.0	1.2	0.8
Total organic carbon (mg/l)	1.6	3.1	1.9	1.3	1.4

Table 23. Water quality parameters of the highest dosed stream.

Water quality parameter	Days from start of dosing				
	5	13	19	26	33
Temperature(^{O}C)	16.3	17.2	11.1	15.0	14.1
Dissolved oxygen (mg/l)	9.4	8.5	10.3	9.3	9.9
pH	8.3	8.4	8.2	8.3	8.8
Total chlorine (mg/l)	0.01	0.07	0.01	0.03	0.03
Nitrate (mg/l)	24.4	33.0	30.8	35.2	27.3
Phosphate (mg/l)	0.25	0.26	0.05	0.18	0.22
Conductivity (mhos/cm)	575	600	630	620	580
Total Hardness (mg$CaCO_3$/l)	240	254	312	350	316
Suspended solids (mg/l)	3.6	3.2	1.4	0.8	0.4
Total organic carbon (mg/l)	3.5	3.2	3.6	1.4	1.1

Table 24. Water quality parameters of the natural stream.

Water quality parameter	Days from start of dosing				
	5	13	19	26	33
Temperature(^{o}C)	15.6	17.5	10.4	14.5	13.3
Dissolved oxygen (mg/l)	-	-	-	-	-
pH	8.3	8.3	8.0	8.1	8.2
Total chlorine (mg/l)	0.00	0.05	0.03	0.02	0.20
Nitrate (mg/l)	24.2	30.8	29.9	33.0	19.8
Phosphate (mg/l)	0.10	0.08	0.22	0.14	0.75
Conductivity (mhos/cm)	590	525	620	620	650
Total Hardness (mg$CaCO_3$/l)	256	260	340	340	370
Suspended solids (mg/l)	11.6	11.0	7.2	15.0	5.6
Total organic carbon (mg/l)	1.9	2.1	14.9*	2.0	1.5

(* 14.9 anomalous data point not included in statistical analysis)

Water hardness ranged between 240-370 mg/l $CaCO_3$, pH between 8.03 and 8.78 and TOC between 1.1 and 3.6 mg/l. This compared to 200 mg/l $CaCO_3$, pH 7.92, and TOC 0.2 to 1.3 mg/l for artificial pond water used in laboratory tests.

Except for conductivity, there was no significant difference in water quality between the control and the top dosed stream ($t < 1.47$ $df = 7$ $p > 0.05$). Conductivity was significantly greater in the top-dosed stream probably due to the increase in copper ions ($t = 3.65$, $df = 6$, $p < 0.01$). Fluctuation in measured conductivity of the natural stream resulted in no significant difference between the natural stream and either the control or top-dosed stream ($t < 2.04$, $df > 4$, $p > 0.05$).

There was no significant difference between water quality parameters of either the control or highest dosed stream and the natural stream except in the case of suspended solids which were considerably lower in the artificial streams ($t = 4.38$, $df = 5$, $p < 0.01$). This decrease in suspended solids in the artificial streams may have resulted from a settling out of particles whilst water was contained in the holding reservoir.

4.5. 3. Survival.

Survival of caged animals in both the first and second trial was ≤ 90 percent in all treatments except at 439.42 µg/l (SE = 48.41), in trial 2, where it fell to 24 percent (Fig. 33). This distribution of data did not lend itself to probit analysis due to the lack of data in the middle range of survivorship, and a heavy bias towards 100 percent survival. Therefore prediction of LC_{50} was based on linear regressions of arcsine transformed data.

There were significant regressions for copper concentration against survival both for trial 2 ($r^2 = 93.2\%$, $df = 4$, $p < 0.01$) and for trials 1 and 2 combined ($r^2 = 86.6\%$, $df = 8$, $p < 0.001$). The LC_{50} for trial 2 was predicted to be 302.5 µg/l (95% C.I. 207.6, 397.4) while that for combined results from trial 1 and 2, was 283.8 (95% C.I. 203.1, 364.5). Due to the lack of data over the concentration range 114.22 - 439.42 µg/l in artificial stream tests, this estimate is likely to be conservative. The LC_{50} estimate from the artificial stream test may be compared to that of fed animals in laboratory test (Chapter 2, Section 2.5. 1) where a much lower 144 hour LC_{50} estimate of 44.63 µgCu/l (95% C.I. 39.64, 49.63) was obtained.

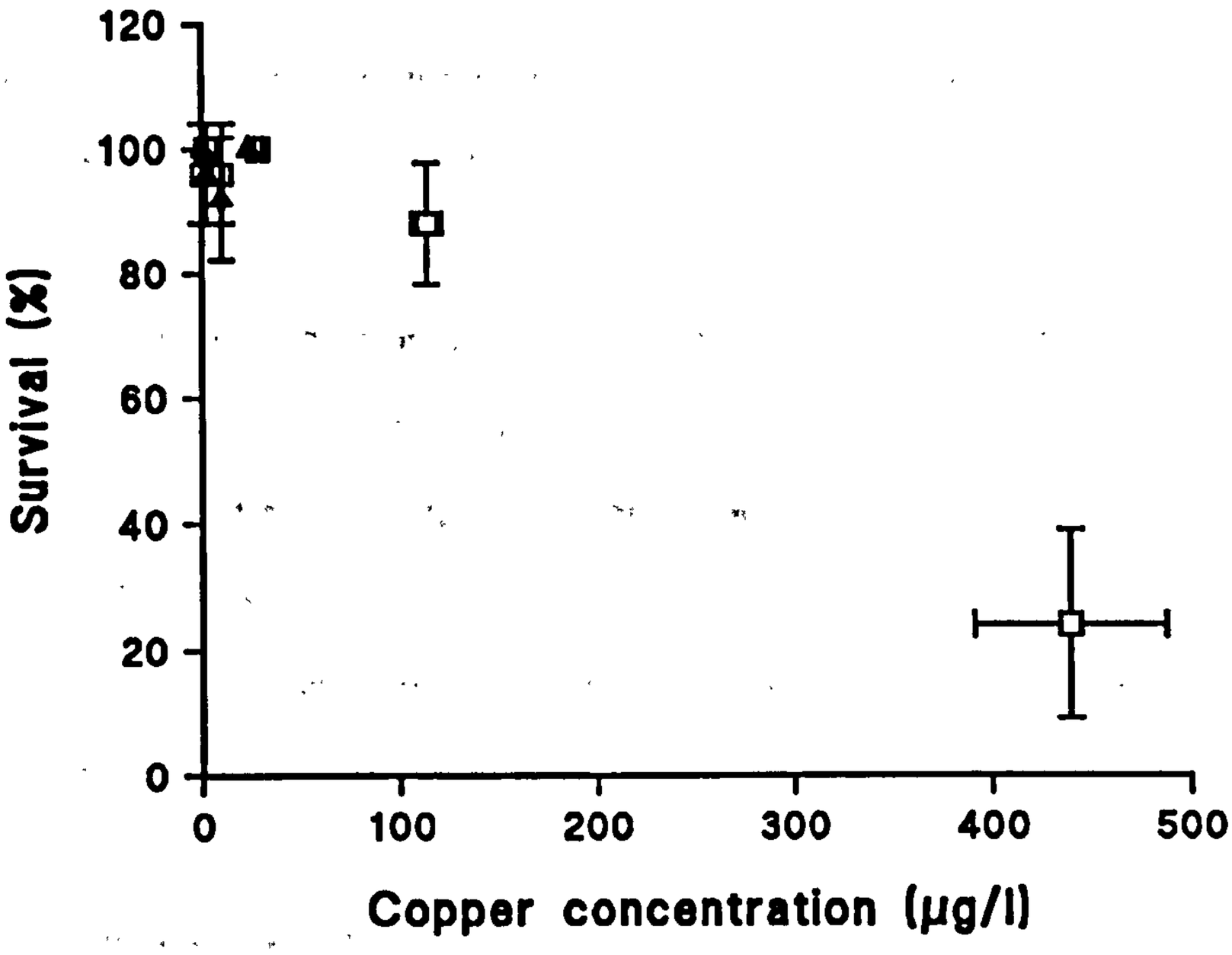


Fig. 33. Effect of copper on survival of *G.pulex* in artificial streams during trial 1 (solid triangles) and trial 2 (open squares). Error bars represent 1 standard error.

It would appear that animals in laboratory tests were more sensitive than those deployed in the streams as there is a 90 percent survival rate at a concentration of 114.22 µg/l (SE = 7.6217) in the streams whereas in the laboratory at a concentration of just 36 µg/l survival falls to 64 percent. Further, analysis of covariance for arcsine transformed data of treatments below 100 µg/l shows a significant difference (ANCOVA: $F = 96.31$, $df = 1$, $p < 0.001$) between laboratory and field response to copper in terms of survivorship with laboratory test animals appearing to be more sensitive.

4.5. 4. Consumption rates.

There was a general decrease in consumption rates with increasing copper concentration of the water in both the first and second trial in the streams. The only exception to this general trend was the slight increase in consumption rates in the second trial with animals

in Stream 5 (copper concentration 2.59 µg/l SE = 0.22) consuming more than animals in the control stream (copper concentration 0.5 µg/l (SE = 0)) although this increase was not significant ($t > 0.79$, $df = 27$, $p > 0.44$).

There was a significant reduction in consumption rates ($F = 16.238$, $df = 5, 92$, $p < 0.001$) between mean total copper concentrations of 9.92 (SE = 0.72) and 23.83 µg/l (SE = 0.42) in the first trial, and at slightly higher concentrations of between 28.98 (SE = 2.066) and 114.22 µg/l (SE = 7.684) in the second trial ($F = 12.658$, $df = 5, 91$, $p < 0.001$) (Fig. 34). A significant effect of copper on consumption rates of animals deployed in the stream with a copper concentration of 28.98 µg/l was probably not detected in trial 2 due to the low consumption rates of the control animals in that trial, as consumption rates of animals exposed to 28.98 µg/l (trial 2) and 23.83 µg/l (trial 1) were remarkably similar (Fig. 34).

The negative consumption values observed for animals in the highest dosed stream was obviously an experimental error and may have been caused by insufficient rinsing of the leaves when removed from this stream (Fig. 34).

Although the consumption rates of control animals in trial 2 were unusually low and significantly different from those of control animals in trial 1 ($t = 3.11$, $df = 32$, $p < 0.01$), consumption rates of stressed animals from trial 1 and trial 2 were not significantly different ($t < 1.58$, $df > 28$, $p > 0.05$) and hence the two data sets are combined for all subsequent analysis and discussion.

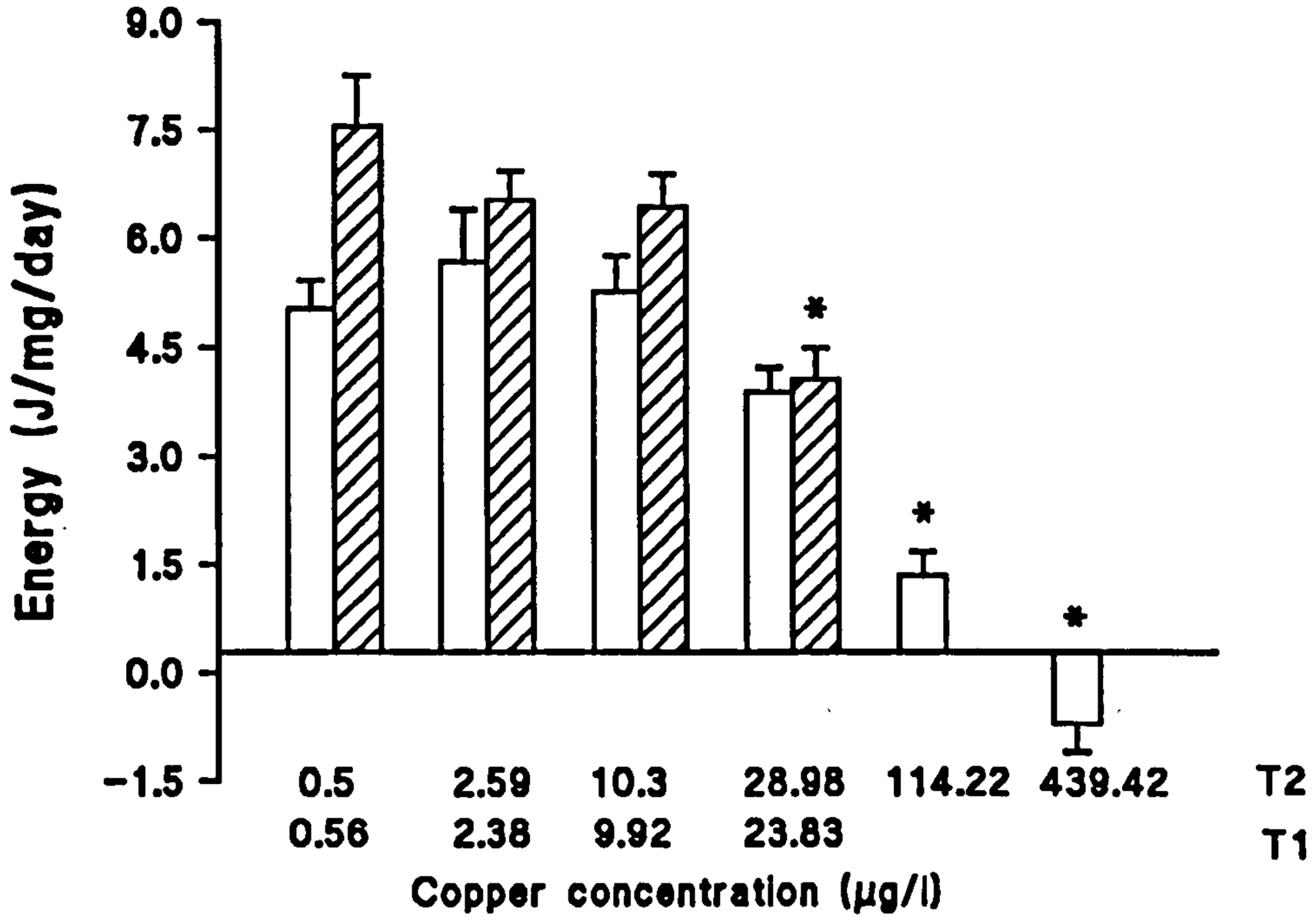


Fig. 34. Consumption rates of *G.pulex* exposed to copper in artificial streams in 2 replicate trials 1 (T1; hatched bars) and 2 (T2; open bars). Error bars represent 1 standard error, * data significantly different from control ($p < 0.05$).

The relationship between consumption rates and copper concentration in the artificial stream tests was curvilinear, (Fig. 35). There was a significant linear relationship between log consumption rates (C; J/mg/d), and log copper concentration (Cu; µg/l) such that:

$$\ln C = 1.84 - 0.206 \ln Cu$$

$$(r^2 = 22.3\%, df = 182, p < 0.001)$$

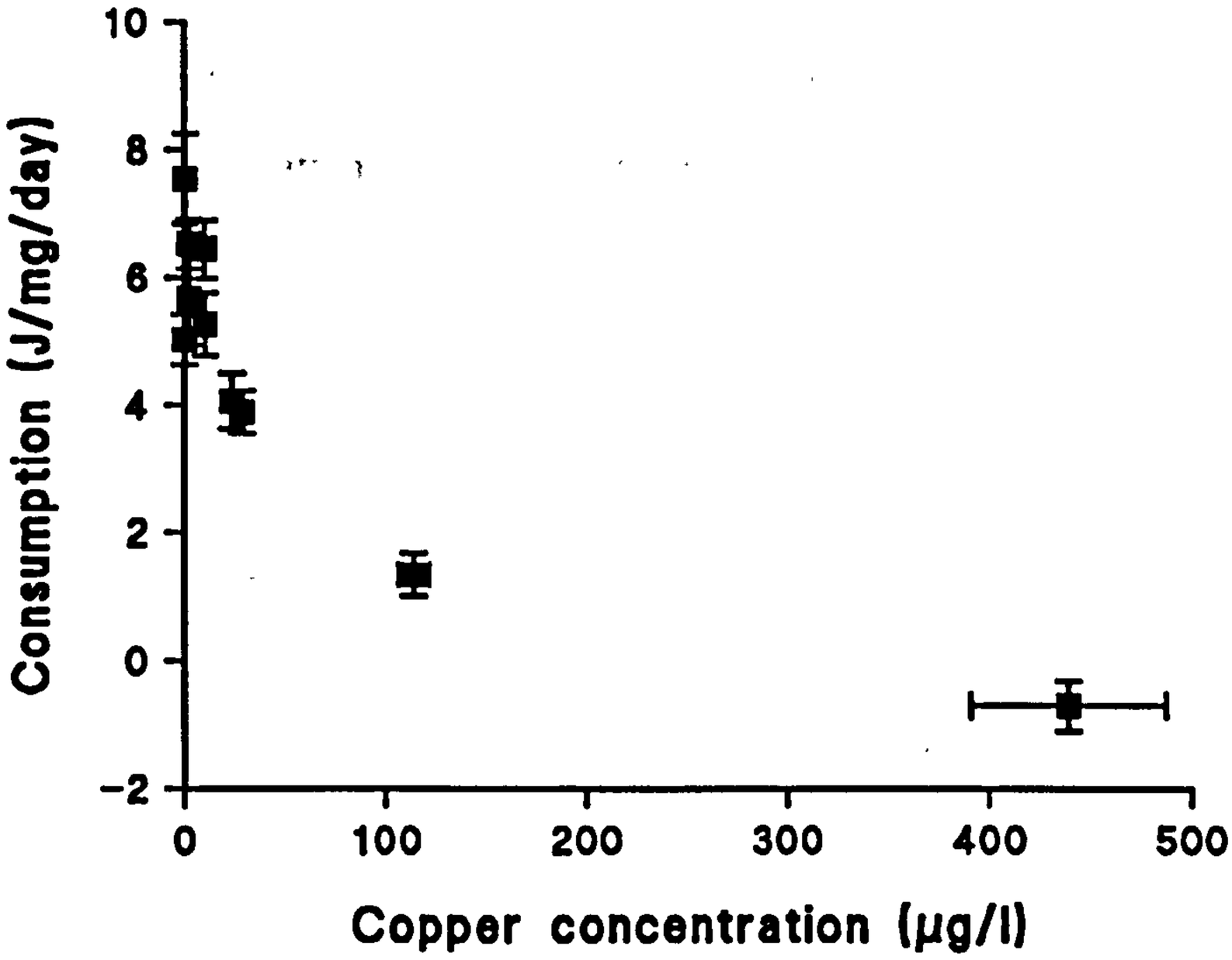


Fig. 35. Curvilinear relationship between consumption rates (J/mg/day) and copper concentration (µg/l). Error bars represent 1 standard error.

Consumption rates of animals deployed in laboratory tests were investigated over a much narrower concentration range. Therefore, laboratory and field results were only compared over the concentration range 0 to 40 µg/l, i.e. the linear part of the curve (Fig. 36). Resulting regression equations for the two data sets were:

$C = 6.51 - 0.092\ Cu$ Artificial streams

($r^2 = 13.5\%$, $df = 169$, $p < 0.001$)

$C = 5.83 - 0.084\ Cu$ Laboratory

($r^2 = 27.7\%$, $df = 119$, $p < 0.001$)

Analysis of covariance showed there was no significant difference between the response to copper, of consumption rates, of animals deployed in artificial streams and those used

in laboratory tests (ANCOVA: $F = 0.12$, $df = 1, 293$, $p > 0.05$) (Fig. 36). Hence animals in laboratory tests and in the artificial streams responded in the same way in terms of effect of copper concentration on consumption rates.

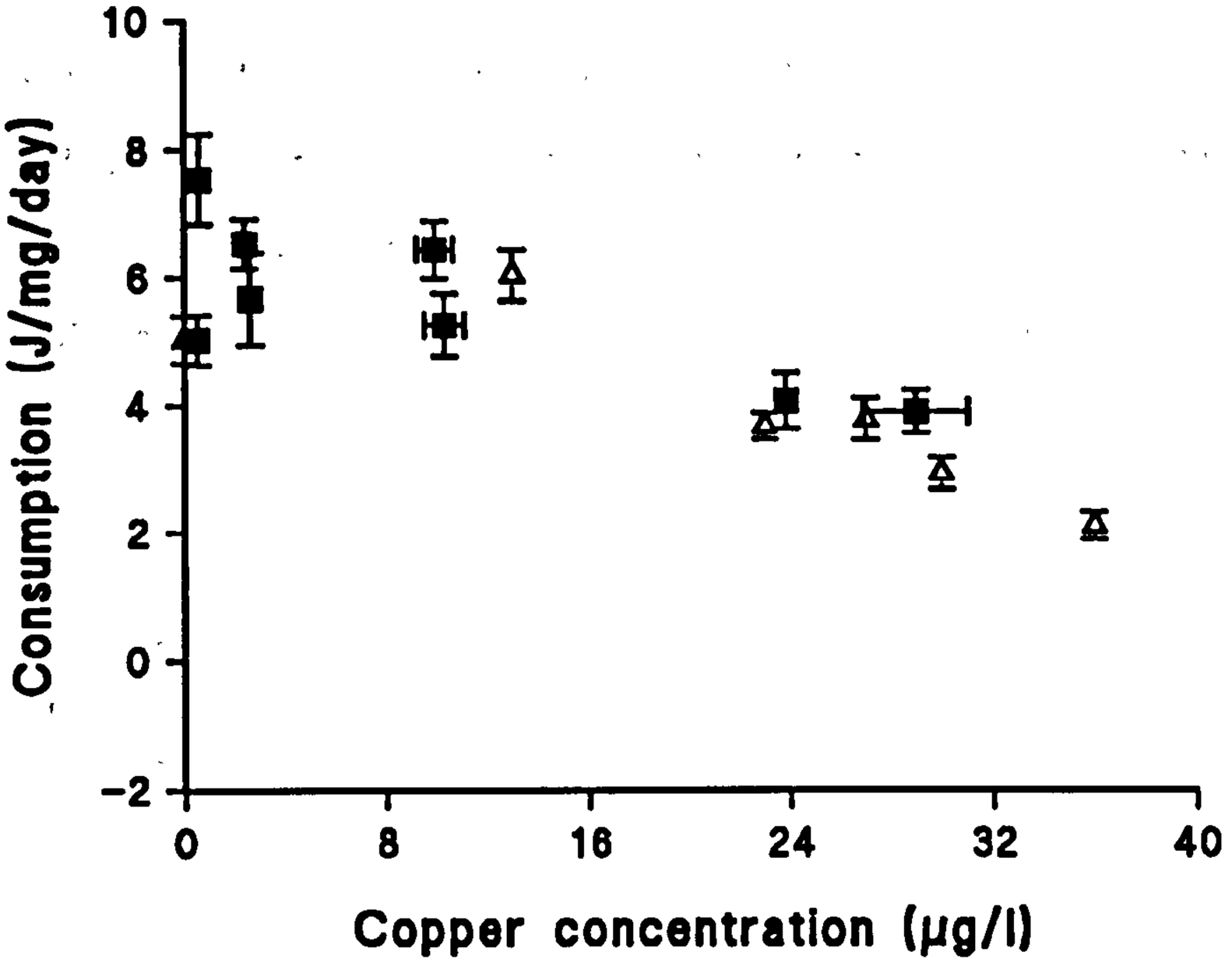


Fig. 36. Consumption rates (J/mg/day) of G.pulex exposed to low levels of copper (µg/l) in artificial stream (solid squares) and laboratory (open triangles) tests. Error bars represent 1 standard error.

4.5. 5. Absorption rates.

Due to the linear relationship between consumption rates and faeces production (eqn 2.14), significant decreases ($F = 16.238$, $df = 5, 91$, $p < 0.001$) in absorption rates were detected at the same copper concentration as significant reductions in consumption rates (i.e. 23, 83 and 114.22 µgCu/l in trial 1 and 2 respectively) (Fig. 37).

4.5. 6. Standard respiration rate and scope for growth.

Laboratory results predicted that copper would have no effect on respiration rates of animals deployed in the streams. There was in fact no significant effect ($F = 1.491$, $df = 2, 31$, $p > 0.05$) of copper on the respiration rates of animals exposed to mean total copper concentrations of 0.56 µg/l (SE = 0.03 (control)), 23.83 µg/l (SE = 0.42), or 110.44 µg/l (SE = 61.7) (Fig. 37). Animals in the latter treatment group were subjected to an extreme difference in copper concentrations (i.e approximately 4 to 200 µgCu/l) as this stream was redosed during the test. It was therefore assumed that respiration rates were constant in animals from all streams.

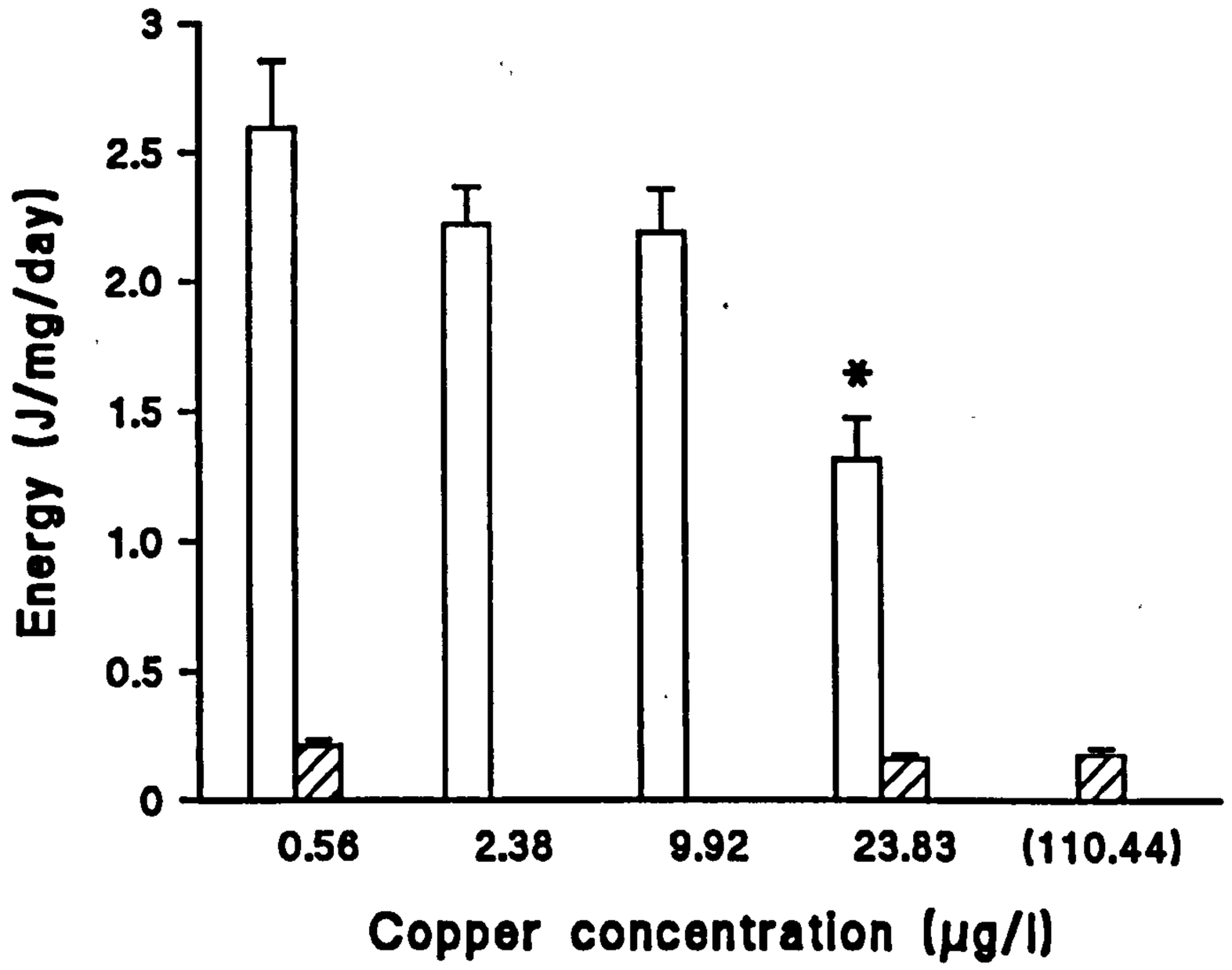


Fig 37. Effect of copper on absorption (open bars) and respiration rates (hatched bars) of animals deployed in the artificial streams during trial 1. Error bars represent 1 standard error, * data significantly different from control ($p < 0.05$).

Scope for growth is the difference between energy absorbed and energy respired. A significant decrease in scope for growth was observed at a mean total copper concentration of 23.83 µgCu/l (SE = 0.42) in the first trial ($F = 22.371$, $df = 2, 31$, $p < 0.001$; Fig. 37). This decrease in scope for growth strongly reflects the effects of copper on consumption rates suggesting that information gained from the effects of copper on feeding rates alone would be adequate in the assessment of impact, no further information being gained from a full estimation of scope for growth.

4.5. 7. Body copper concentration.

There was no significant difference ($F = 0.477$, $df = 1, 18$, $p > 0.5$) between the mean copper concentration of control animals deployed in the artificial streams, i.e. 35.38 µgCu/g, and those used in laboratory tests, i.e. 33.62 µgCu/g (Fig. 38).

There was a significant positive linear relationship between *G.pulex* body concentration and copper concentration of water in both the artificial streams ($r^2 = 69.9\%$, $df = 38$, $p < 0.001$) and laboratory test media ($r^2 = 41.0\%$, $df = 57$, $p < 0.001$), (Fig 38). However the form of this relationship was significantly different between the two data sets (ANCOVA: $F = 78.96$, $df = 1, 99$, $p < 0.001$; Fig. 38). The body concentration of animals deployed in the stream tests increase to a greater extent than animals used in laboratory tests, indicating that copper was taken up more easily by animals in the artificial streams compared to those in the laboratory.

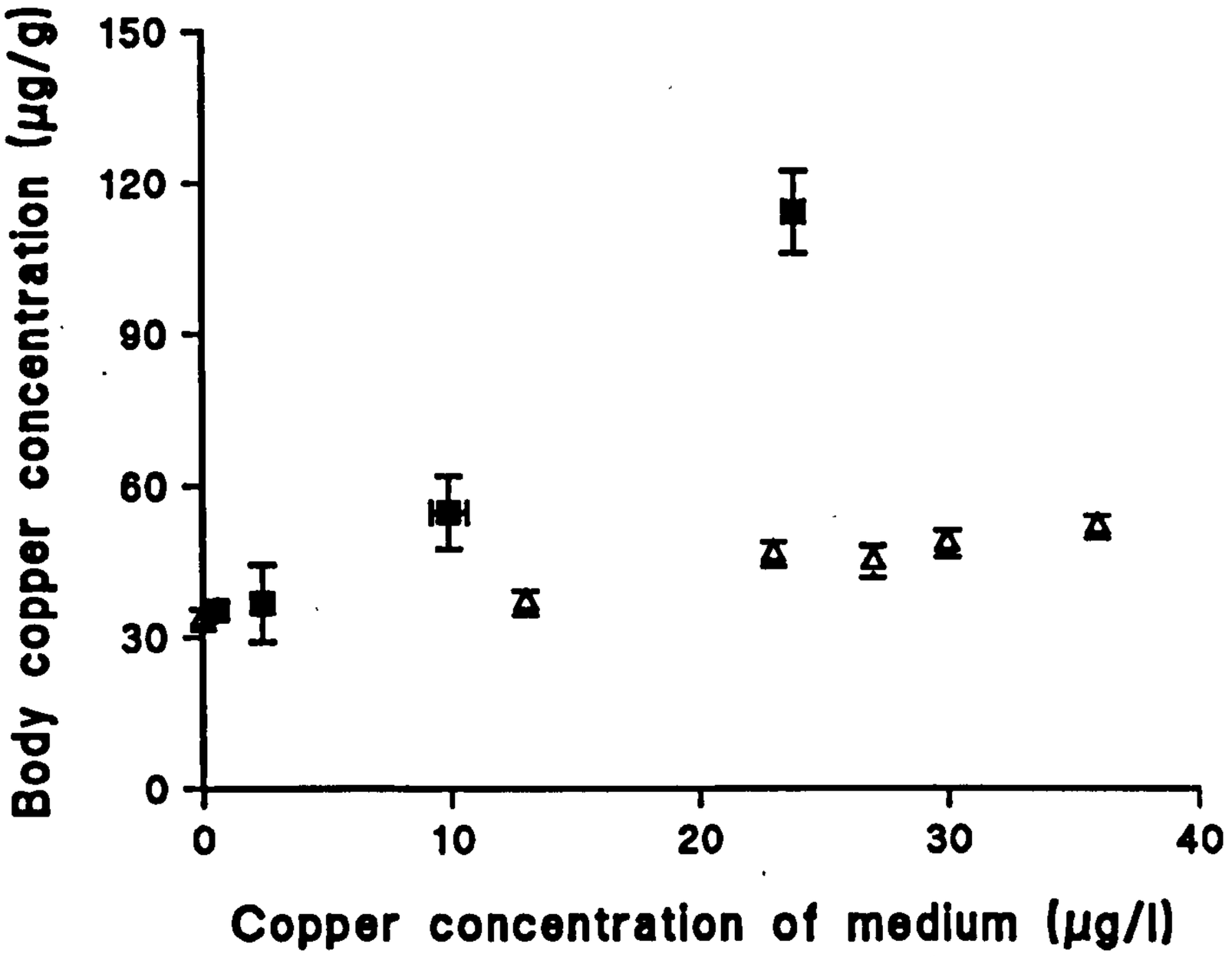


Fig 38. Body concentration of animals (µg/g) exposed to copper (µg/l) in artificial stream (solid squares) and laboratory (open triangles) tests. Error bars represent 1 standard error.

There was a linear relationship between leaf copper concentration and copper concentration of the media (concentrations up to 25 µgCu/l) in both the 2 artificial stream trials and in laboratory tests ($r^2 > 75.4\%$, $df > 38$, $p < 0.001$). Analysis of covariance also indicated that while there was no significant difference between the copper concentration of leaf material offered to *G.pulex* in the two artificial stream trials (ANCOVA: $F = 0.44$, $df = 1, 78$, $p > 0.05$), there was a significantly greater concentration of copper in leaves from the artificial streams than in those used in laboratory tests (ANCOVA: $F = 68.21$, $df = 1, 98$, $p < 0.001$). Hence presence of food in the gut may contribute to the difference in body concentrations between animals in laboratory and artificial stream tests.

Although animals deployed in the artificial streams have a greater body burden of copper than do animals in laboratory tests, it has already been shown (Section 5.5. 4, Fig. 36) that

consumption rates of animals deployed in the two tests respond in the same way to copper in the water. Therefore there appears to be poor correlation between total body loading and toxicity.

As in laboratory tests (Chapter 2, Section 2.5. 8), there was a significant correlation between body concentration and consumption rates (r^2 = 43.9%, df =1, 38, $p < 0.001$). However, the relationship between body concentration and consumption rates in laboratory and field tests was significantly different (ANCOVA: F = 4.34, df = 95, $p < 0.05$).

4.5. 8. Population density.

There was no significant difference between the population density (number / cm^2) of either adult (F = 0.735, df = 5, 13, $p > 0.05$) or juvenile (F = 1.87, df = 5, 13, $p > 0.05$) *G.pulex*, in the pool section of each of the artificial streams, one day prior to dosing with copper (Fig. 39). The same was true for the population density of both adults (F = 1.133, df = 5, 13, $p > 0.05$) and juveniles (F = 1.032, df = 5, 13, $p > 0.05$) in the riffle sections of the artificial streams (Fig. 40).

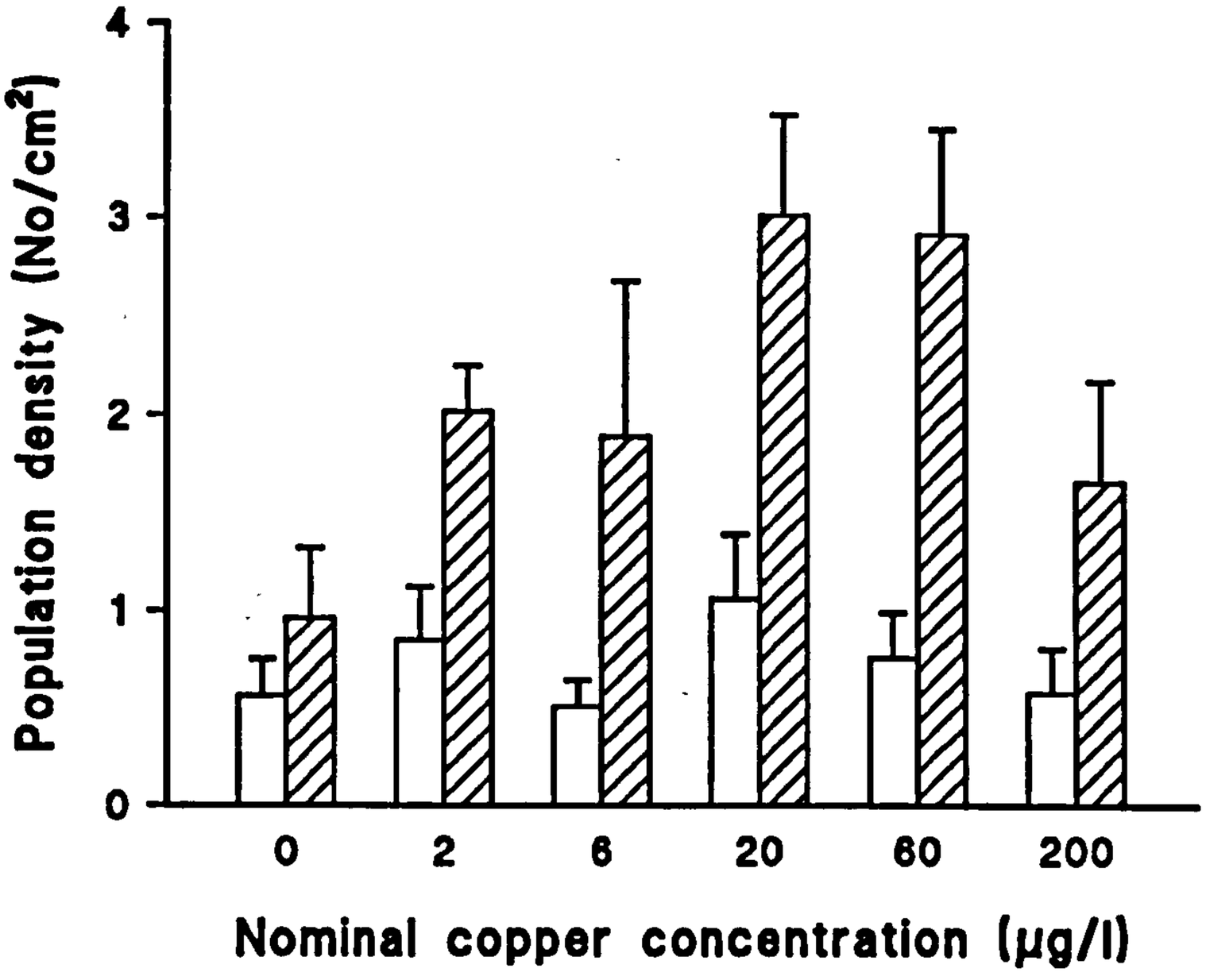


Fig. 39: Population density of adult (open bars) and juvenile (hatched bars) *G.pulex* in the pool section of artificial streams one day prior to dosing. Nominal copper concentrations indicate concentrations of subsequent dosing. Error bars represent 1 standard error.

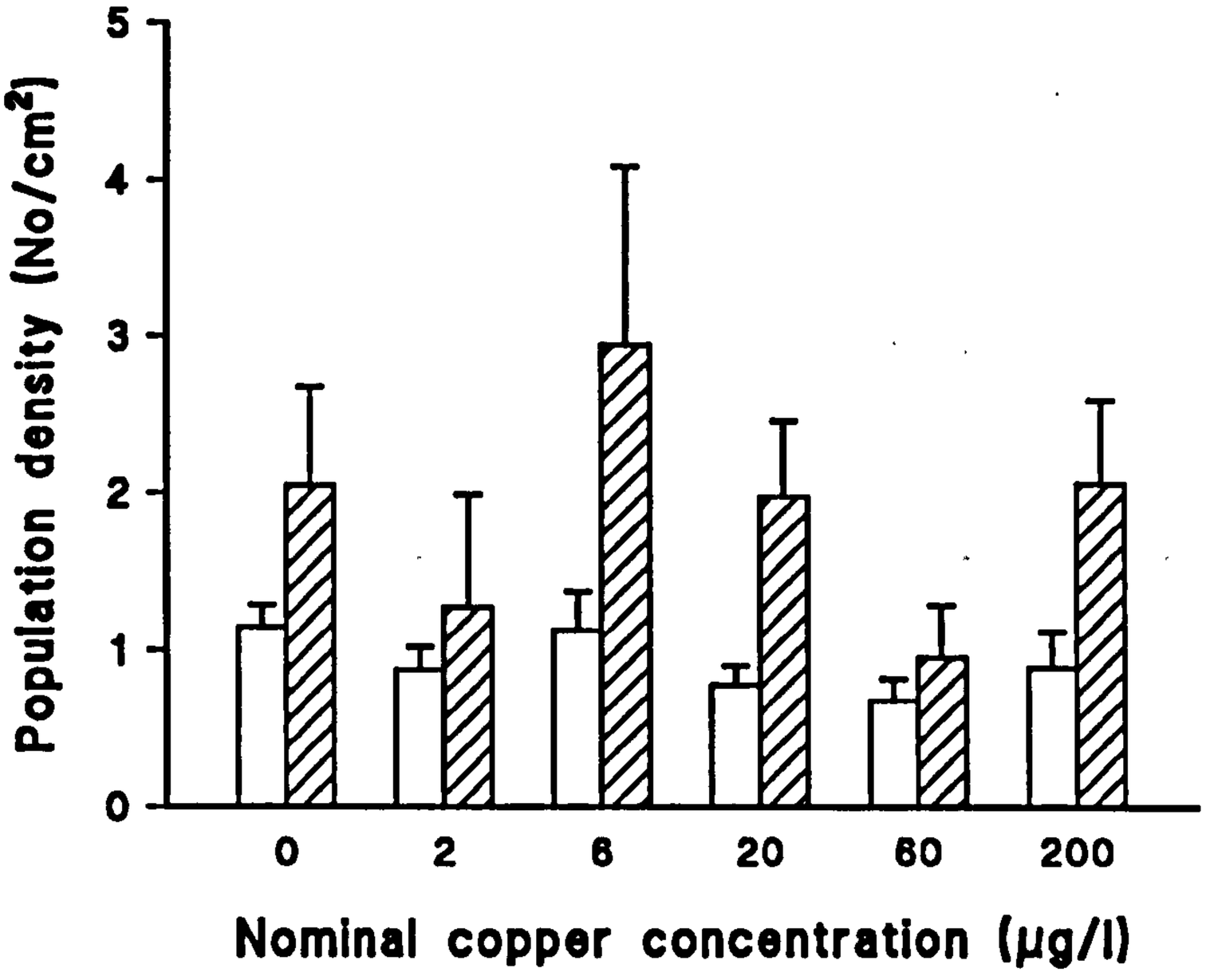


Fig 40: Population density of adult (open bars) and juvenile (hatched bars) *G.pulex* in the riffle section of artificial streams one day prior to dosing. Nominal copper concentrations indicate concentrations of subsequent dosing. Error bars represent 1 standard error.

Samples were taken from the riffle section after 14 days of dosing and from both the pool and riffle sections after 28 days. Mean copper concentrations in each stream over this period are given in Table 25:

Table 25 : Mean copper concentrations (µg/l) in the artificial streams during 14 and 28 day periods.

Day	nominal	Actual (µg/l)	SE
14	0	0.5	0.045
	2	1.95	0.247
	6	8.66	0.92
	20	20.46	1.79
	60	22.05	10.41
	200	90.67	57.63
28	0	0.54	0.05
	2	2.12	0.19
	6	9.51	0.74
	20	23.23	1.98
	60	48.36	17.28
	200	200.2	67.91

After 14 days of dosing with copper the population density of both adults and juveniles ($F > 7.488$, $df = 5, 13$, $p < 0.01$) was significantly lower in the riffle section of streams dosed with concentrations of 20.47 µgCu/l and over than in the control stream (Fig. 41).

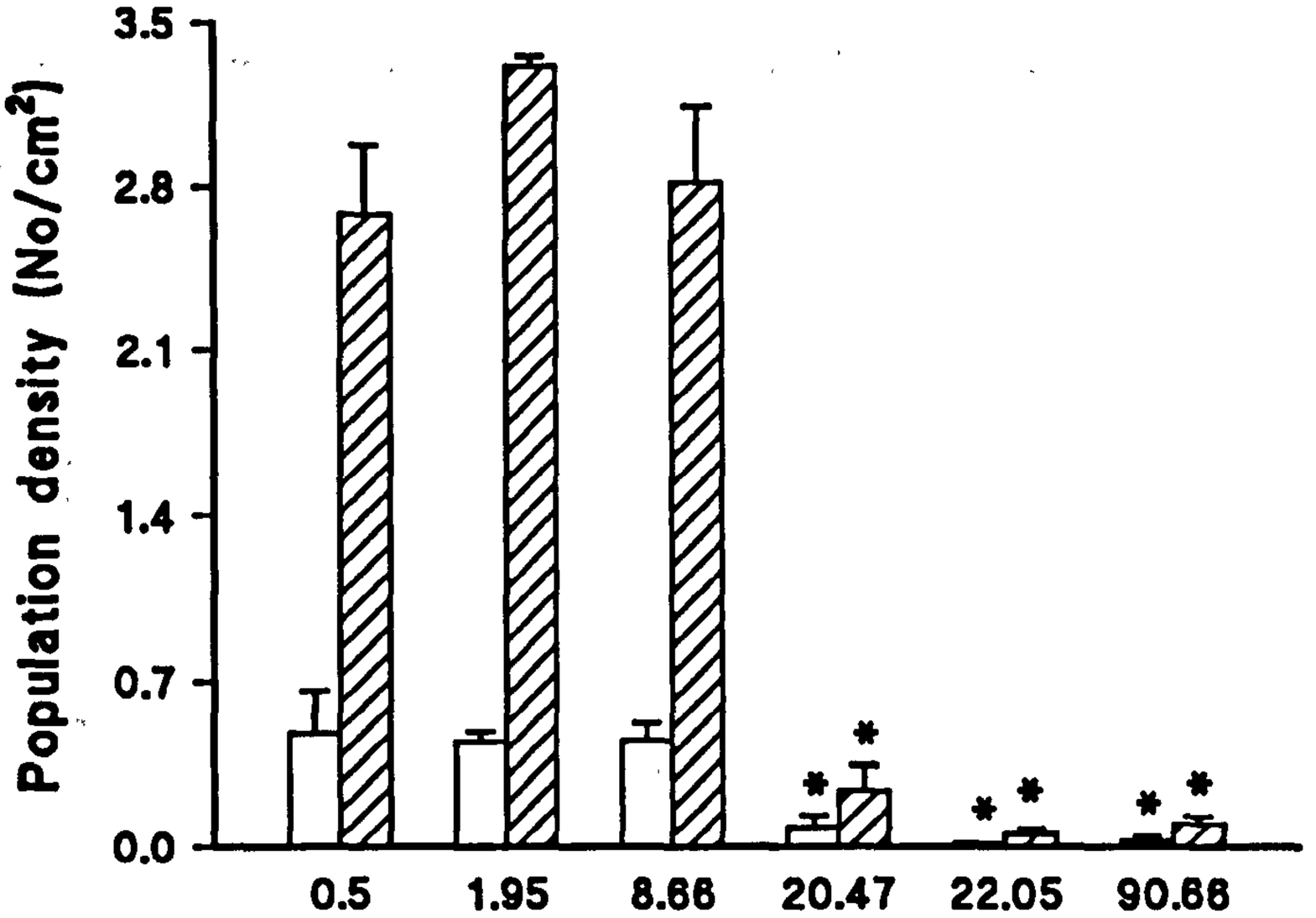


Fig. 41: Population density of adult (open bars) and juvenile (hatched bars) *G.pulex* in the riffle section of artificial streams 14 days after dosing. Error bars represent 1 standard error, * Significantly different from control $p < 0.05$.

At the end of the trial, after 28 days of dosing, the population density of adult and juvenile *G.pulex* in the pool section of streams dosed with copper concentrations greater than or equal to 48.36 µgCu/l was significantly lower than in the control stream ($F > 11.284$, $df = 5, 13$, $p < 0.001$; Fig. 42). In the riffle sections both adult and juvenile population densities were significantly lower than in the control stream in streams dosed with nominal concentrations of 23.36 µgCu/l and above ($F > 11.108$, $df = 5, 13$, $p < 0.001$; Fig. 43).

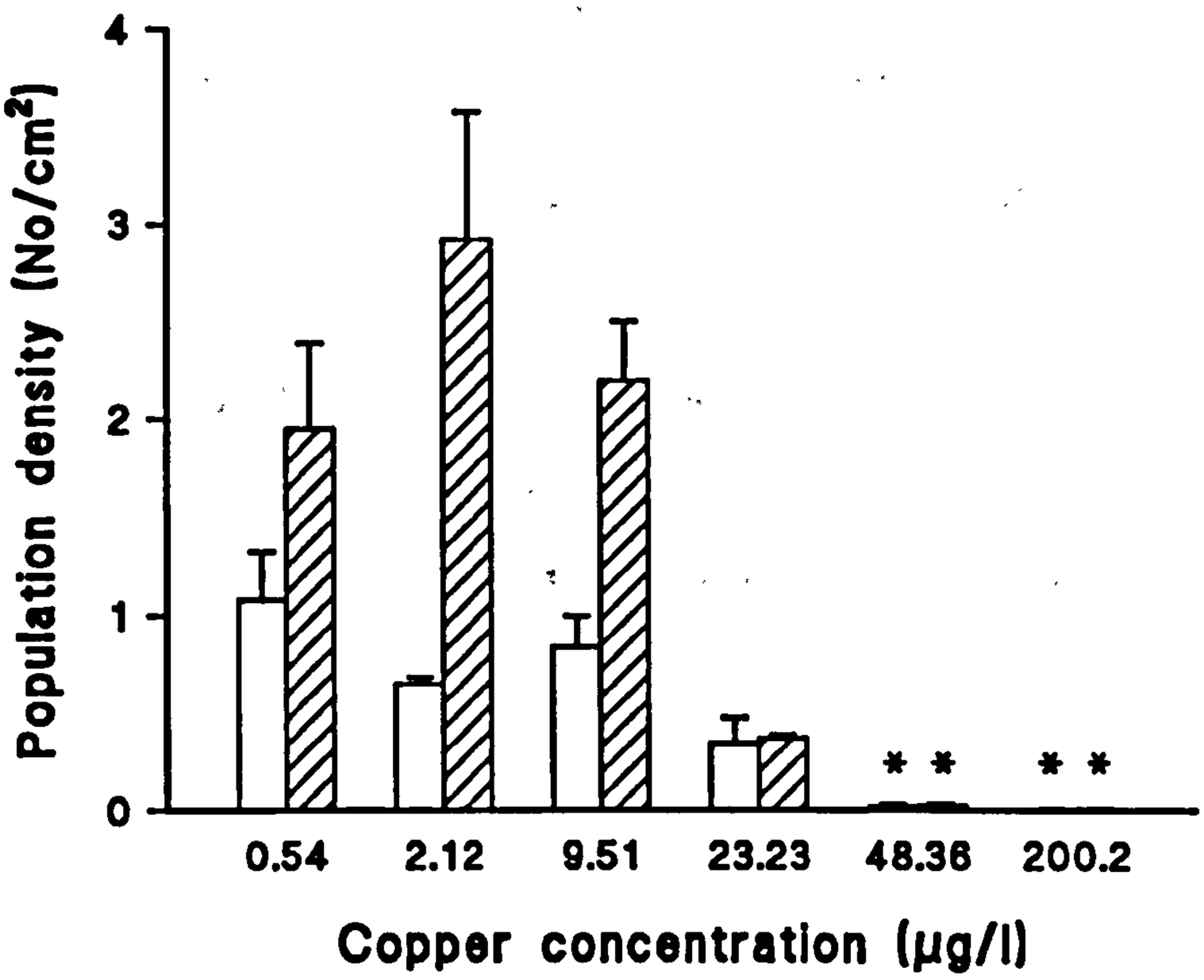


Fig. 42: Population density of adult (open bars) and juvenile (hatched bars) *G.pulex* in the pool section of artificial streams 28 days after dosing. Error bars represent 1 standard error, * data significantly different from control ($p < 0.05$).

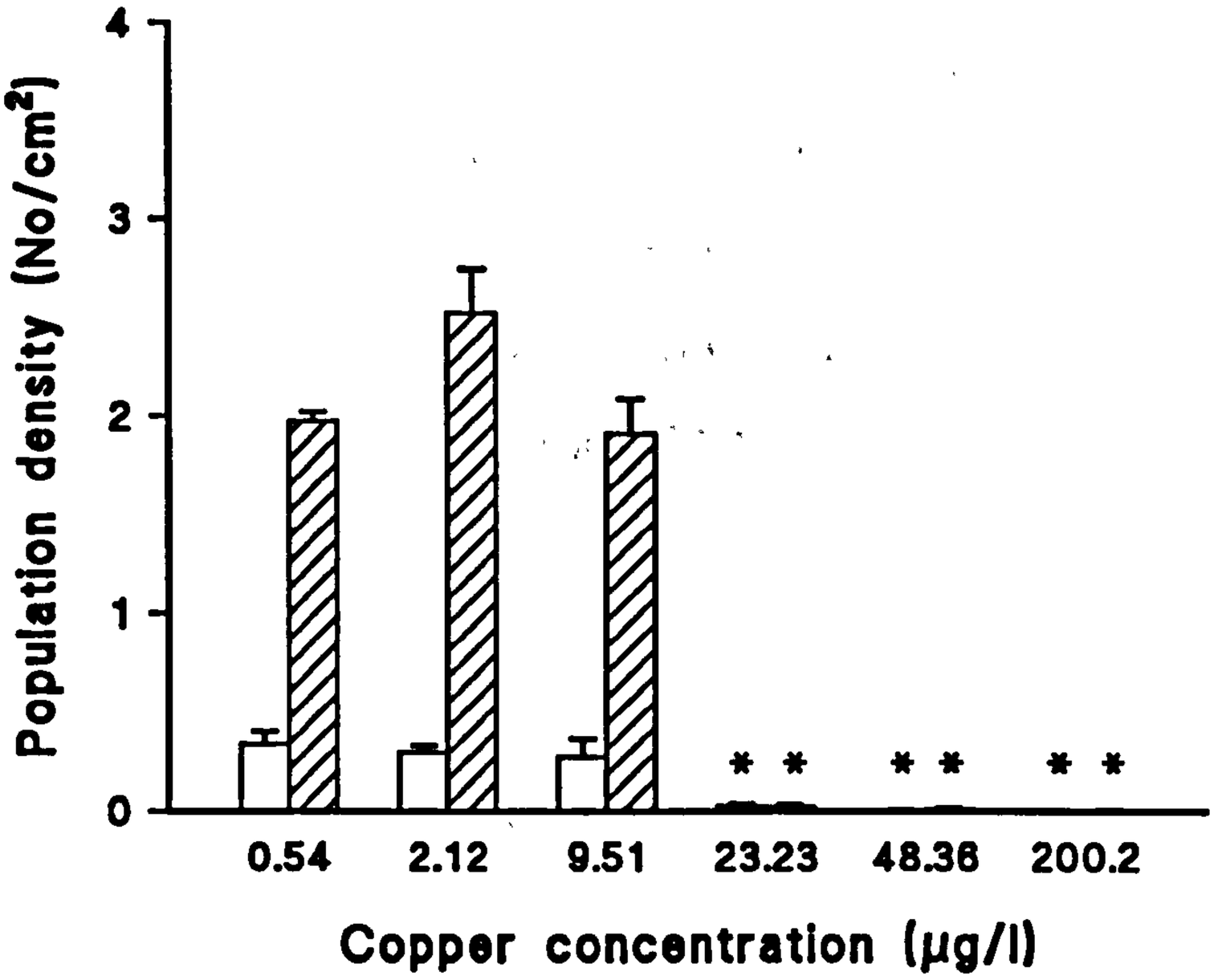


Fig. 43: Population density of adult (open bars) and juvenile (hatched bars) *G.pulex* in the riffle section of artificial streams 28 days after dosing. Error bars represent 1 standard error, * data significantly different from control ($p < 0.05$).

Another way of displaying this data is as a derived total population size of *G.pulex* in each stream. This was acheived by multiplying the number of animals per cm^2 by the area of the stream bed. Prior to dosing of the artificial streams with copper, the absolute population size of adult and juvenile *G.pulex* ranged between 13121 and 17565 and between 21749 and 48161 individuals respectively. The streams were dominated by juvenile *G.pulex* with population size ranging between 1.7 to 2.8 times that of adults in each stream (Fig. 44).

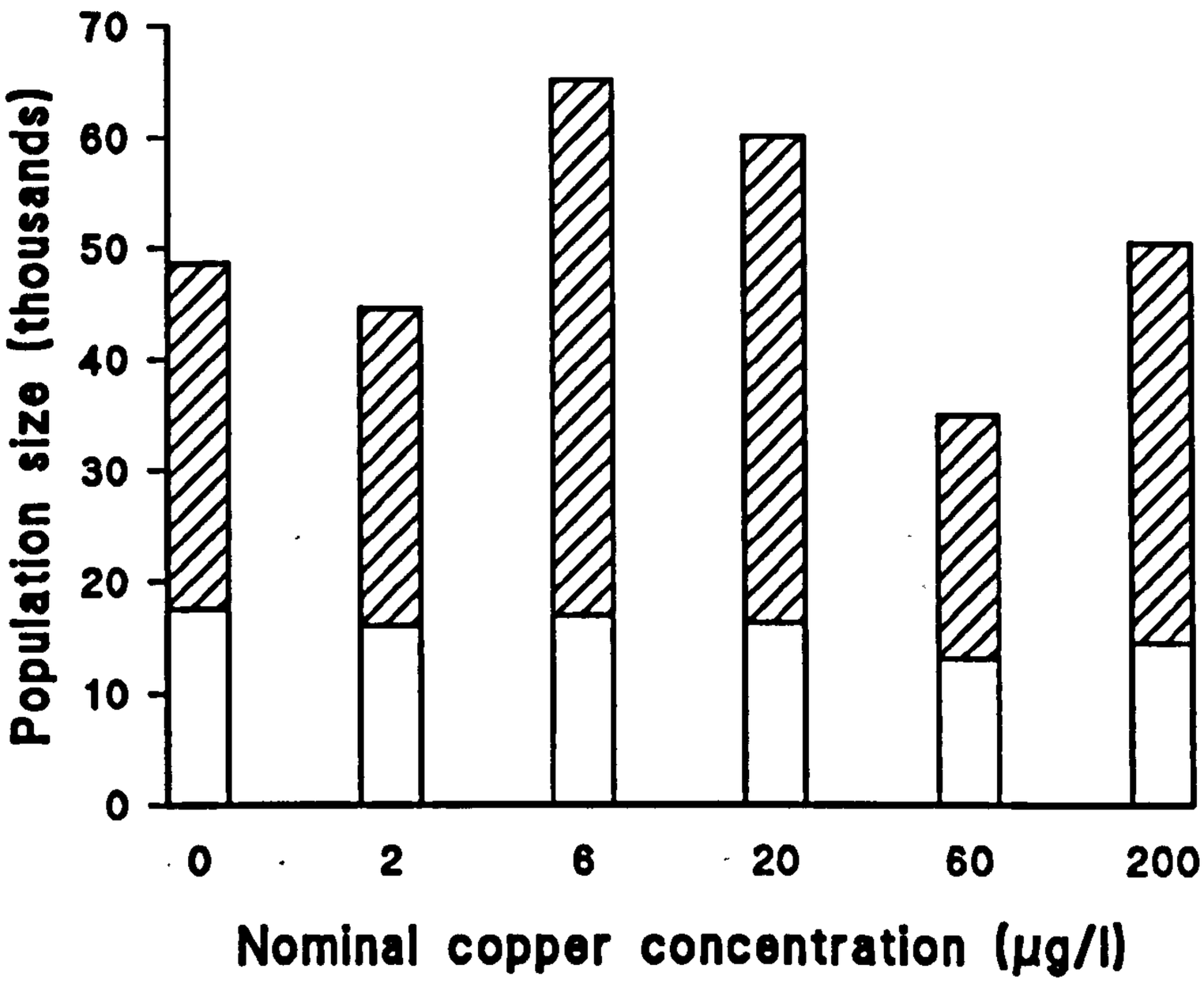


Fig 44. Estimated population densities of adult (open bars) and juvenile (hatched bars) *G.pulex* in the artificial streams on day one, prior to dosing with copper. Nominal copper concentrations indicate concentrations of subsequent dosing.

After 28 days of exposure, the absolute population size in the control and streams dosed with lowest concentrations of copper were little affected but, there was a significant reduction in population size in streams dosed with 23.23 µgCu/l and above. Proportionally juveniles appear to be more sensitive than adults (Fig. 45).

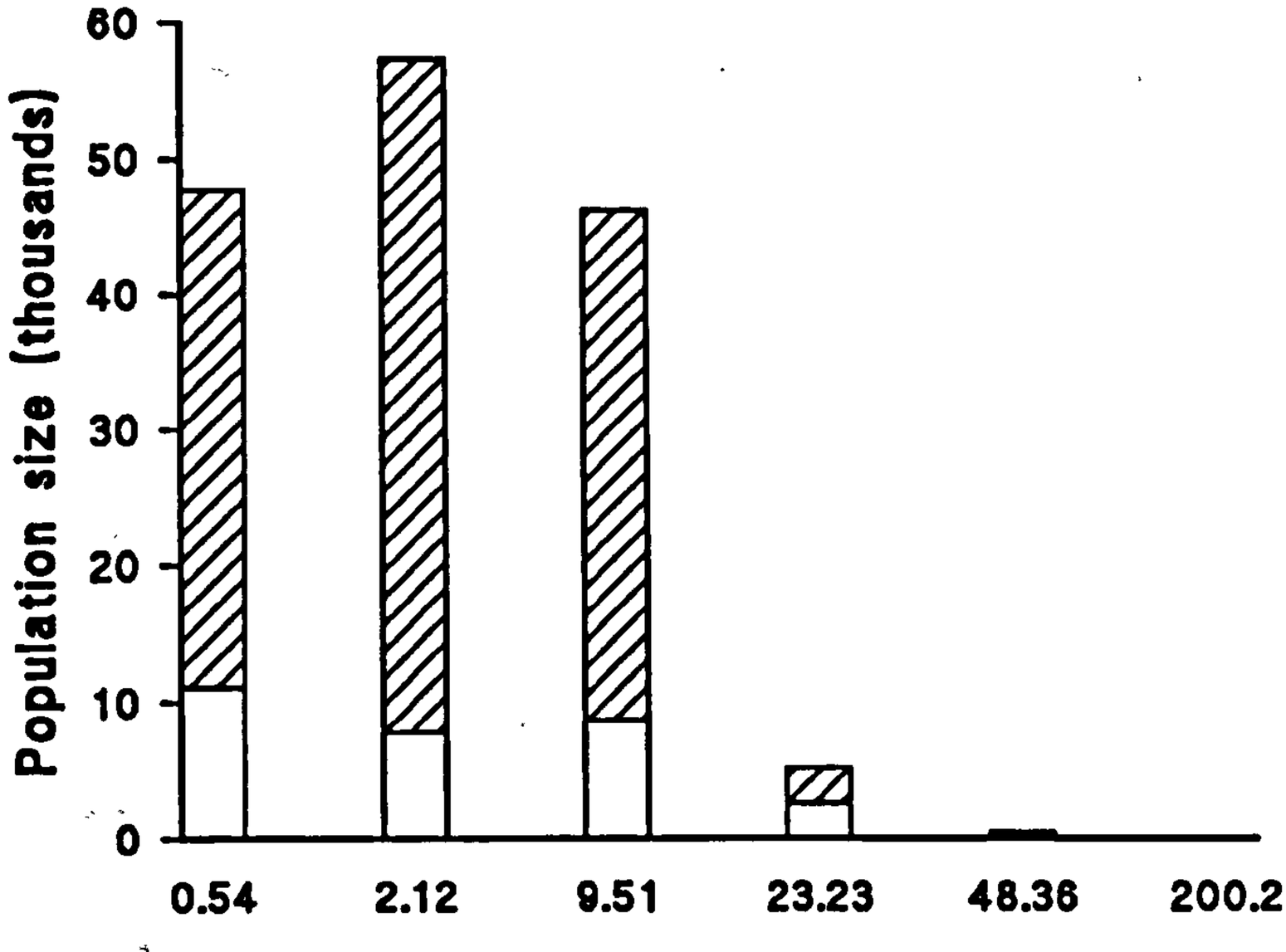


Fig 45. Population densities of adult (open bars) and juvenile (hatched bars) *G.pulex* after 28 days exposure to copper.

Estimation of the actual percentage of initial population remaining after 14 days exposure to copper, in the riffle section of the artificial streams showed that the numbers of adults decreased in all streams to less than 50 percent of initial densities (Fig. 46). This decrease was significantly greater in streams dosed with 23.23 µgCu/l and over where the density fell to less than 10 percent of initial levels. The population density of juveniles was maintained or increased (100 to 260 percent of initial levels) in the control and 2 lowest dosed streams but decreased to less than 12 percent in streams dosed with 20.47 µgCu/l and over (Fig. 46).

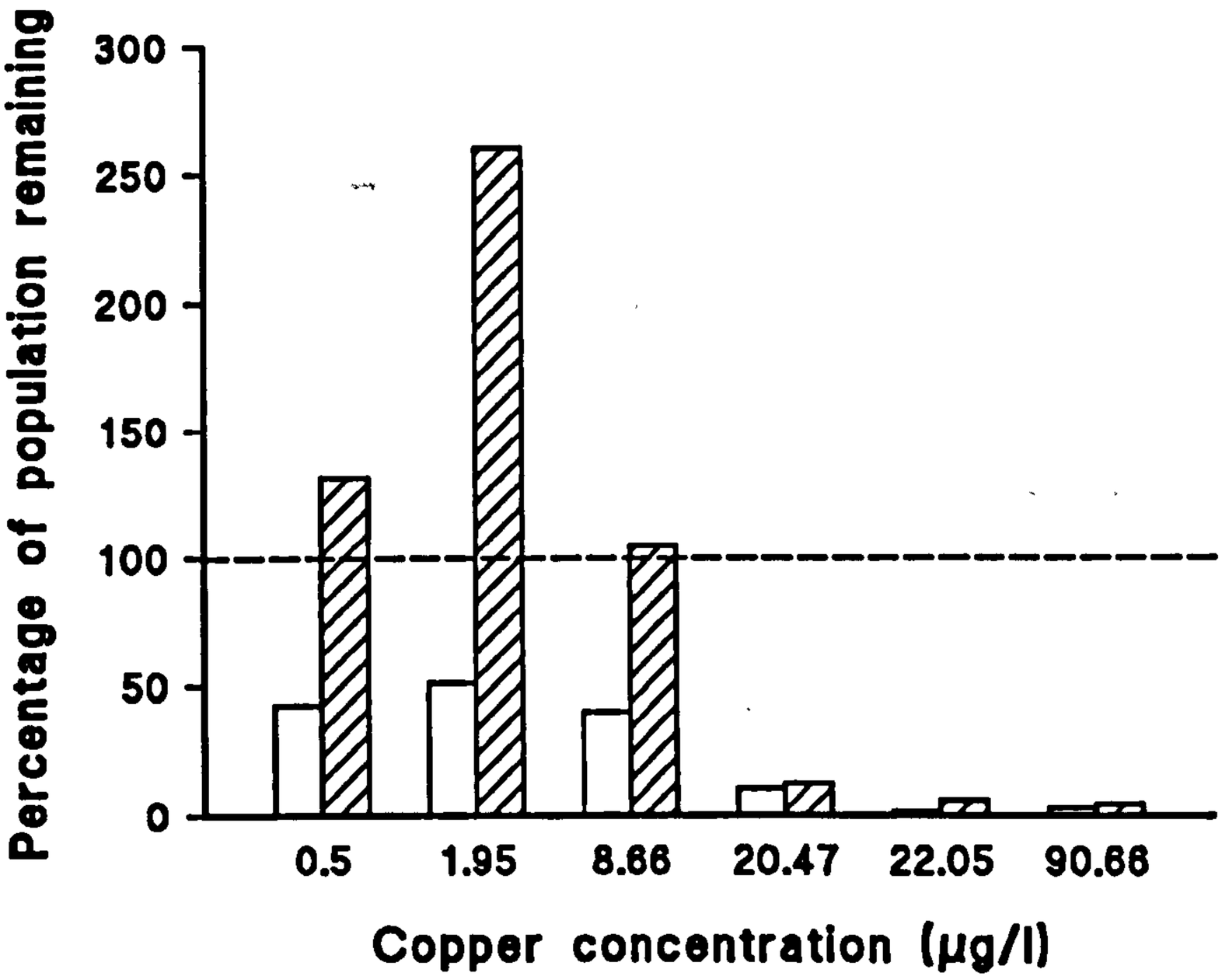


Fig. 46: Percentage of original adult (open bars) and juvenile (hatched bars) population in sample remaining after 14 days exposure to copper in the riffle section of the artificial streams. Line denotes maintenance of population density to initial levels.

Estimation of the actual percentage of initial population remaining after 28 days exposure to copper, in the pool section, showed a maintenance or increase in population densities of juveniles and adults in the control and two lowest dosed streams (75 to 200 percent) (Fig. 47). A decrease in population density of adults to 32 percent of initial levels was found in streams dosed with 23.23 µgCu/l and less than 2 percent in the top 2 dosed streams (48.36 and 200.2 µgCu/l). Juveniles appeared to be slightly more sensitive than adults with densities falling to less than 12 percent in streams dosed with 23.23 µgCu/l and over (Fig. 47).

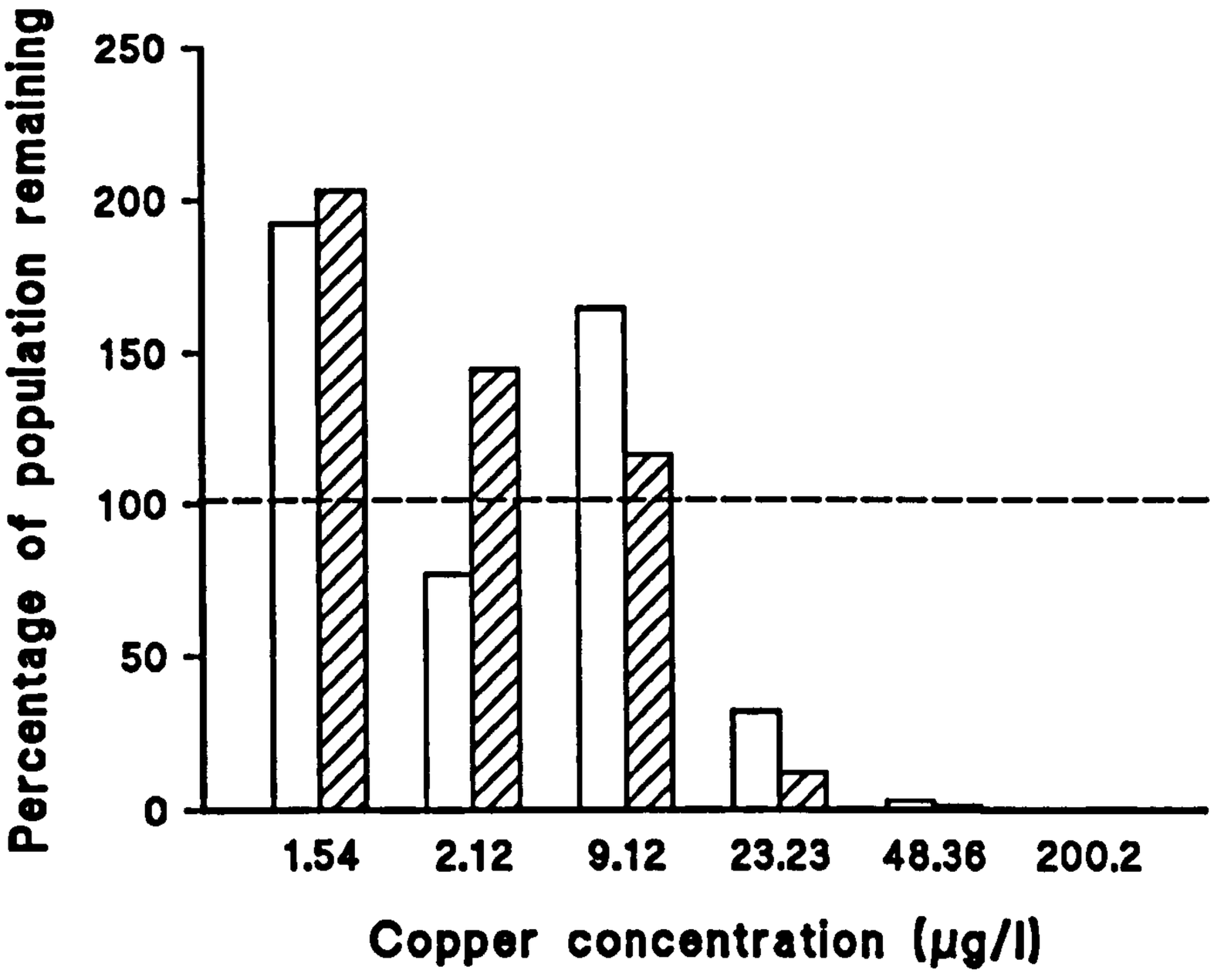


Fig. 47. Percentage of original adult (open bars) and juvenile (hatched bars) population in sample remaining after 28 days exposure to copper in the pool section of the artificial streams. Line denotes maintenance of population density to initial levels.

In the riffle section the population density of adults fell to approximately 30 percent in the control and two lowest dosed streams although once again densities were affected most at copper concentrations of 23.23 µgCu/l and above where they fell to less than 3 percent of original levels (Fig. 48). Juvenile *Gammarus* in the riffle section of the control stream were present in approximately the same densities as on one day prior to dosing while in the lowest dosed stream (2.12 µgCu/l) the density has approximately doubled. At concentrations of 23.23 µgCu/l and over the population density fell to less than 10 percent of initial levels (Fig. 48).

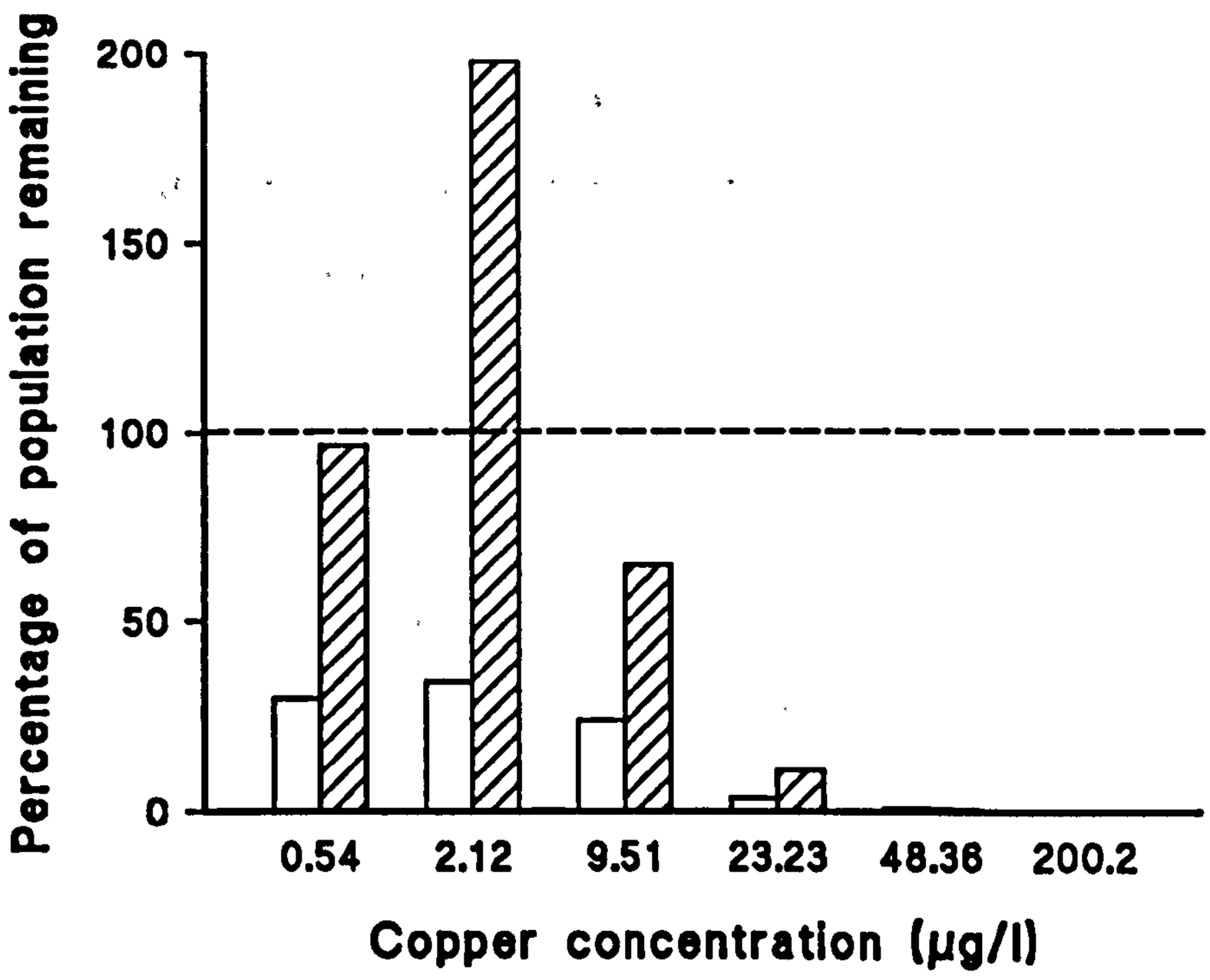


Fig 48. Percentage of original adult (open bars) and juvenile (hatched bars) population in sample remaining after 28 days exposure to copper in the riffle section of the artificial streams. Line denotes maintenance of population density to initial levels.

4.6 Discussion.

The streams were set up as predominantly detritus based systems with dominant invertebrates being *Polycelis* sp., *Nais* sp., *Gammarus pulex*, Chironomidae (unidentified sp), and *Elmis* sp.. *G.pulex* was shown to both grow and reproduce under the conditions set up in the artificial streams, as indicated by increases in both adult and juvenile populations in the control stream.

The water quality in the artificial streams approximated that of the adjacent natural stream, varying only in one measured parameter; suspended solids. The amount of suspended solids in the artificial streams was less than in the adjacent natural stream, probably due to settling in the holding reservoir. Although this may influence the toxicity of metals by altering partitioning between water and sediment (Hall et al, 1985) it did not present a problem in interpretation of results between artificial streams as it did not vary among the artificial streams.

Suspended solids and sedimentary material did however, bind copper in the artificial streams and this made establishment of required copper concentrations difficult. There is therefore, need for greater understanding of the dynamics of toxicants in such systems in order to attain water concentrations required.

The only water quality parameter that did vary between the artificial streams was conductivity but this could probably be attributed to the large number of copper ions in the higher dosed streams.

The aim of this part of the study was to validate results from laboratory tests in a more ecologically relevant system. Hence the validity of laboratory test results, based on various response criteria in *G.pulex*, was determined by comparison with results obtained from the artificial streams (Table 26).

Table 26: Validation of laboratory tests based on various response criteria. NS denotes no significant difference in response of animals between laboratory and stream tests; * denotes significant difference at $p < 0.05$.

Response criterion	Significance		Test
1. Survival	*	$p < 0.001$	Ancova
2. Consumption	NS	$p > 0.05$	Ancova
3. Standard respiration	NS	$p > 0.05$	One-way Anova
4. Scope for growth	NS	$p > 0.05$	two-sample t-test
5. Body Cu concentration	*	$p < 0.001$	Ancova

The two response criteria that were significantly different between laboratory and field tests were the 144 hour LC_{50} and accumulation of body copper concentrations. The 144 hour LC_{50} estimate was 44.63 µg/l for fed animals in laboratory tests compared to 283.8 µg/l for animals deployed in the artificial streams. Hence estimates from laboratory tests would be conservative rather than under-protective.

The applicability of LC_{50} estimates from the stream data is questionable due to the lack of data between approximately 100 and 400 µg/l. This bias may further distort an already derived value. However, analysis of covariance indicates laboratory animals are far more sensitive, in terms of survivorship, than animals deployed in the artificial streams.

Possibilities for differences between laboratory and stream LC_{50} and body copper concentration results, in these tests, include: seasonality / temperature, condition, stress, body size, water quality and bioavailability.

Seasonality / temperature.

Laboratory tests were conducted during February while tests in the artificial streams were run in May. Seasonal effects may therefore have influenced the sensitivity of animals. Khangarot and Ray (1987), for example, found a seasonal fluctuation in the sensitivity (LC_{50}) of *Lymnaea luteola* to zinc. SfG in the scallop was also found to vary seasonally in field tests of MacDonald and Thompson (1986). Both these studies attributed seasonal effects primarily to changes in water temperature. An increase in temperature was found to render phenol less acutely toxic to *Asellus aquaticus* (Green et al, 1988), cadmium or copper toxicity was also found to increase with increasing temperature (Braginski and Shcherban, 1978). Laboratory tests in this study were conducted at a constant temperature of 15^{o}C, while the temperature of the artificial streams was similar but more variable ranging from 11.1 to 18.8^{o}C.

Condition. (nutritional status/ moult/ reproductive stage/ disease).

Although animals were collected from the same site they may not necessarily have been of the same physiological condition. The nutritional status of an organism can significantly modify the apparent toxicity of a chemical (Lanno et al, 1989). This is because the nutritional status is intimately related to nutritional effects on metabolic rate. McCahon and Pascoe (1988c) found a significant reduction in the toxicity of cadmium to *G.pulex* within one week of moulting, this time coincides with recalcification of the cuticle (Wright, 1980). Finally although reproductive stage (McCahon and Pascoe (1988c)) and disease state (Guth et al, 1977; Boyce and Yamada, 1977) can affect the toxicity of metals, these parameters were not a problem in this study as all animals were healthy and large males only were the standard for all tests.

Stress.

Stresses imposed on experimental animals may have included effects of handling, transport, caging, different media etc. Increased sensitivity of laboratory tests was interpreted by Clements et al (1990) to be due to the inability of certain taxa to acclimate

to laboratory conditions. However, acclimation processes would also be necessary to acclimate to conditions of the artificial streams.

Body size.

With morphologically similar life stages, body size can influence acute toxicity several fold (Anderson and Spear, 1980 a, b; Green et al 1986). McCahon and Pascoe (1988b) found the toxicity of cadmium to juvenile *G.pulex* was approximately 250 times greater than to adults. However, in this study large males, (approximately 10 mg), were used in all tests hence any effects of differing body size will have been eliminated.

Water quality.

Many water quality characters can affect metal toxicity. Major factors influencing copper toxicity include: water hardness, pH, and organic compounds.

An increase in water hardness caused a significant reduction in the acute toxicity of copper to *G.pulex* (Stephenson, 1983). Effects of copper on invertebrate abundance in low alkalinity and hardness artificial streams, were significantly greater than in high alkalinity and hardness streams (Clements et al, 1989). Laboratory media had a hardness of 200 mg$CaCO_3$/l compared to slightly higher and more variable values of 240 to 350 mg$CaCO_3$/l in the artificial streams. Hence the differences in water hardness may have contributed to the differences in LC_{50} and body copper concentrations between laboratory and field tests. The reduction in the toxicity of copper caused by increased calcium and magnesium ions may be due to the complexing of toxic cations or the competition for active sites in the animals (Wright, 1980; Zitko and Carson, 1976)

pH is important in controlling the amount of ionic copper in solution. Ionic copper concentration has been shown to decrease by one order of magnitude for every 0.5 increase in pH over pH 6 (Stumm and Morgan, 1981). The pH of the water in the artificial streams (pH 8.2 to 8.8) was in fact slightly higher than that of APW (pH 7.8)

and hence the greater survivorship of animals in the artificial streams could not be explained by this variable.

Organic compounds may decrease toxicity by complexing with metals and rendering them non-toxic. Humic acid has been shown to have a strong ameliorating effect on copper toxicity (Winner, 1984; Winner and Gauss, 1986) The TOC in the streams (2.44 mgC/l) was indeed slightly higher than in laboratory tests (0.80 mgC/l) and may have contributed to the lower lethal toxicity of copper to *G.pulex* in the artificial streams.

Bioavailability.

The significant difference between the body copper concentration of animals deployed in the artificial streams compared to those used in laboratory tests indicates a greater degree of bioavailability of copper in the artificial streams.

There are several possibilities for this increased bioavailability including differences in body size, condition, season and / or temperature, food / water partitioning and water quality.

Differences in body size, as discussed in Chapter 2 (section 2.4), may be dismissed as only large animals were used in this study (mean weight = 10.5 mg SE = 1.8) and there was no significant ($p > 0.05$) relationship between dry weight and body loading.

Factors affecting the condition of animals which may alter metal uptake include such parameters as reproductive stage (Moore, Rainbow and Hayes, 1991; Rajan et al, 1991), disease state (Krakk, 1992), and stage of the moult cycle (Wright, 1980; Nugegoda and Rainbow, 1987). Although it is known that reproductive stage and disease state can affect accumulation of metals, as stated previously, these parameters were not a problem in this study. It was not known however, whether animals in the artificial stream tests moulted during the trial or not, and if this would have had an effect on body copper concentration.

Wright (1980) found that because of the possible link between cadmium and calcium uptake mechanisms in *G.pulex* the rate of uptake of cadmium was linked to the calcium status of the animal and cadmium uptake is considerably enhanced following the moult when there was a 10-fold increase in calcium influx. However, Weeks and Moore (1991) found no significant change in mean body copper (or zinc) concentration of four species of Talitrid amphipods throughout the moulting cycle and hence stated that copper and zinc must be conserved at the moult in the 4 species studied.

Over the 6 day test period it is likely that a number of animals moulted in the stream and laboratory test, this factor would have been randomised between the tests and although increased temperature causes increased moulting rates, (Zauke, 1982; Nugegoda and Rainbow, 1987), temperatures in the two tests were similar and unlikely to have a major effect.

Although temperature regimes were comparable, laboratory and field tests were run during different months (February and May respectively). Zauke, (1982) found cadmium concentration in *G.pulex* was not seasonally affected and in this study, Figure 39 illustrates that there was very little difference between body copper concentration of control animals for these two months. Therefore seasonal effects were also negligible in this population of *G.pulex* at least for the months of February and May.

Smock (1983) and Rainbow and Moore (1986) concluded that clearing of the gut was not necessary before analysis of body metal concentrations of the mayfly *Stenonema modestum* or tailitrid amphipods, respectively. However, in this study, metal analysis of leaves (which would have been the major component of gut contents in experimental animals) showed that the copper concentration of leaves from the artificial streams were significantly greater than those from laboratory tests. Hence gut contents of animals

deployed in the artificial streams could, in this instance, have contributed to the difference the overall body concentrations of animals in these tests.

The increased body copper concentration of animals deployed in artificial stream tests could be due to interactions between copper ions and other chemical constituents of the water. Winner and Gauss (1986) found changes in water hardness, and humic acid concentration changed bioaccumulation and/ or chronic toxicity of three metals including copper. Table 27 gives a summary of the findings of Winner and Gauss concerning the effects of water hardness and humic acid concentration on bioaccumulation and toxicity of copper to *Daphnia.*

Table 27. Effects of water hardness and humic acid concentration on body copper loading and NOEC in *Daphnia* (data from Winner and Gauss 1986)

Effect		Increased water hardness ($mgCaCO_3$)/l)	
		58-115	115-230
Body loading	-HA	Decrease	No additional effect
	+HA	Decrease	Increase
NOEC	-HA	No change	No additional effect
	+HA	No change	Decrease

+HA = Humic Acid added at a concentration of 1.5 mg/l; -HA = No humic acid added; NOEC = No observed effect concentration (based on survivorship).

Mean water hardness in both laboratory (200 $mgCaCO_3$/l) and artificial streams (282 $mgCaCO_3$/l) falls into the category of 'hard water' although artificial stream water is harder than laboratory water. Total organic carbon (a proportion of which would be humic acids) in the artificial streams has a mean value of 2.44 mgC/l while in laboratory media the TOC is lower with a mean concentration of 0.80 mgC/l. The effects of a

combination of these differences in water hardness and total organic carbon, for example, could have resulted in the increased body loading and no change in chronic toxicity observed in animals deployed in the artificial streams compared to animals in laboratory tests.

Winner and Gauss (1986) state that there is a poor correlation between bioavailability, bioaccumulation and toxicity in multicellular animals because of complex storage, transformation and excretion processes in these animals. It may be that, although animals in the artificial streams gain a greater body load, this load is not all in a form which is toxic. If the copper becomes complexed to another substance it may be that this substance can enter the organism more easily, and therefore increase the total body load, but this extra complexed copper may be in an non-metabolically available, nontoxic form or in a form which is more easily sequestered and detoxified. Hence although the total copper body load of animals in the artificial streams was greater than those in laboratory tests, the toxic load may be the same and hence elicit the same response.

In contrast to effects of copper on survival and body copper concentrations of animals in laboratory and field tests, there was no significant difference between results based on consumption rates, respiration rates and scope for growth as response criteria (Table 26). Consumption rates were the most sensitive of the energy budget parameters measured to copper contamination. Copper caused a significant reduction in consumption rates of animals used in laboratory tests at a concentration of 23 µgCu/l and of animals deployed in the artificial streams at copper concentrations of 23.83, and 114.22 µgCu/l in trial 1 and 2 respectively. The reduction in sensitivity of consumption rates to copper in trial 2 was a result of consumption rates of control animals being unusually low. Correlation between effects of copper on consumption rates of animals from artificial stream and laboratory tests was remarkably good with analysis of covariance indicating there was no significant difference between the responses of the two sets of animals.

Respiration rates of animals deployed in the artificial streams were comparable to those used in laboratory tests. Both tests show this parameter to be insensitive to copper stress (Chapter 2, Section 2.5. 2. c). However as respiration rates remain constant while absorption decreases when animals are exposed to copper, the relative amount of energy required for respiration increases with exposure. Hence, overall efficiency is reduced by copper due to a relative increase in energy demands for maintenance (Baird et al, 1990).

Scope for growth, of animals deployed in the artificial streams was derived from measurements of consumption rates and respiration rates and from estimates of faecal production. Effects of copper on scope for growth were exhibited at the same concentrations as effects on consumption rates. In laboratory tests, variability in the data caused a slight decrease in the sensitivity of absorption rates and hence significant effects on SfG were observed at slightly higher copper concentrations than they were on consumption rates. It is therefore difficult to compare results of measured values from laboratory tests with derived values in the streams, based on those laboratory tests. However, in neither laboratory nor stream tests is the SfG test more sensitive than simply measuring consumption rates. Hence measurement of consumption rates are sufficient in assessing effects of copper on *G.pulex*, with no additional information being gained from a full measurement of scope for growth.

Hence, the degree of agreement between laboratory and stream results in these tests, was dependent upon the response criterion used. Adams et al, (1983), also found that the effect parameter used influenced the degree of agreement between laboratory and field results. Adams et al, (1983), observed better agreement between survival data than growth rates of fathead minnows. These differences between laboratory and field growth rates were attributed to differences in available food and space between the two test systems.

As well as validating laboratory tests by comparison with results obtained in artificial streams, it is important that such tests should be indicative of effects at higher levels of organization and hence be valid in the sense of protection of the ecosystem. This was approached by determining the effects of copper on the population density of *G.pulex* in the artificial streams and assessing the ability of laboratory tests, based on consumption rates as a response criterion, to predict effects of copper at the population level of organisation.

It was shown that *G.pulex* was able to establish in the artificial streams and increase or maintain densities in low dosed streams throughout the dosing period (28 days). This indicates the general health of the populations in these streams as animals have been able to both grow (indicated by increased adult densities) and reproduce (indicated by increased juvenile densities). The density of juveniles was always higher than that of adults a fact also noted for a natural stream by Welton (1979).

Adult densities in the riffle section were generally low, while, juveniles appeared able to maintain their densities in the riffle sections, at least in the low dosed streams. It may be that smaller animals are able to find more refuges in the riffle area and are not subject to such great forces of water flow as larger animals might be. Further, particles of fine particulate organic matter may become trapped in pockets of slack water in the riffle area which may be profitable for exploitation by juvenile *Gammarus* but not for adults.

Good correlation between effects observed at the individual and population level of organisation in the artificial streams was exhibited. A significant reduction in population density of *G.pulex* in the riffle section of the artificial streams (20.47 µgCu/l) was observed at similar concentration to a significant reduction in consumption rates (23 µgCu/l) while reduction in population density in the pool section was observed at a higher concentration (48.36 µgCu/l) than predicted from consumption rates. Hence estimates of toxicity based on consumption rates as a response criterion would be protective at the

population level of organisation i.e. population density of *G.pulex* was not significantly affected at concentrations below those significantly affecting consumption rates of *G.pulex* in laboratory tests.

There are models which make explicit links between energy budget descriptions of individual physiology and the dynamics of their populations, mostly using *Daphnia* as models, via quantitative predictions on growth and reproduction (Kooijman and Metz, 1984; Kooijman, 1986; Metz et al, 1988; Gatto et al, 1989; Lynch, 1989; Nisbet et al, 1989; M^{c}Cauley et al, 1990; Gurney et al, 1990; Calow and Sibly 1990). Hence predictions of fecundity and mortality rates can give measures of population rates of increase (r). However most of these models need more development before they may be fully evaluated and many of the parameters involved in these models are outside the scope of this study.

Densities of *G.pulex* in the low dosed streams increased due to reproduction and copper undoubtedly influenced reproduction of females in higher dosed streams due to a reduction in energy accumulation. However, reductions in population density of *G.pulex* in this study were likely to be primarily due to an increase in *G.pulex* drift. Differences in population density of *G.pulex* in the streams was significant within 14 days of dosing. This time period was unlikely to be sufficient for changes in population density to be manifested mostly through changes in production (growth and reproduction). Hence effects of copper on population density were detected at the same concentration causing a significant increase in *G.pulex* drift (Mitchell, pers comm) and were indeed predictable from effects of copper on consumption rates.

The validity of laboratory tests in more natural environments was therefore dependent upon the response criterion used. Tests based on energy budget parameters as response criteria accurately reflected results obtained in laboratory experiments, while those based on survival and body concentration were significantly different. As well as being valid in

a more natural environment, tests based on energy budget parameters were indicative of effects of copper at the population level of organisation indicating that effects observed on such response criteria were ecologically relevant.

4.7 Summary.

1. Correlation between laboratory and field results, based on energy budget parameters (i.e. consumption rates, respiration rates and scope for growth) were good, while those based on survivorship and body copper concentration were poor.

2. No further information was gained from a measure of scope for growth than from a measure of consumption rates. Therefore, of the parameters measured, tests based on consumption rates alone would be sufficient for determining the sensitivity of an individual to copper.

3. Whole body copper concentrations were significantly different between animals exposed to copper in laboratory tests and those deployed in the artificial streams. These differences were probably due to different concentrations of copper present in food in the gut and physicochemical characteristics of the water in the artificial streams. These physicochemical characteristics may cause copper to be more easily taken up from solution, but may render the copper less toxic, causing toxic effects of copper on consumption rates in *G.pulex* to be observed at the same concentration as in animals used in laboratory tests.

4. Copper caused a decrease in the population density of *G.pulex* with juveniles being more sensitive than adults. Effects of copper on the population density of *G.pulex* in the riffle section appeared to be greater than effects in the pool section of the artificial streams. The observed decrease in population density was likely to be primarily a result of copper-induced increase in drift rate.

5. The concentration at which population density was significantly decreased was similar to that causing a significant decrease in consumption rates. Hence, tests based on effects of copper on consumption rates of individuals were indicative of subsequent population level effects.

CHAPTER 5

EFFECTS OF COPPER ON LEAF PROCESSING.

5.1 Introduction

Because of its importance in energy flow in heterotrophic streams, leaf decomposition provides a useful ecosystem level measure of pollution effects (Gray and Ward, 1983). Litter decomposition is vital for the functioning of detritivorous stream systems and any reduction in decomposition, caused by a pollutant, may affect other biotic components by decreasing nutrient and energy transfer (Pritchard and Bourquin, 1985). Measures of pollutant-induced changes in leaf processing provide a link with effects on the functioning of the shredder community and may allude to potential effects on the rest of the detritivorous community.

Decomposition has been described as the metabolism of organic compounds to inorganic forms or the incorporation into living biomass (Boulton and Boon, 1991). It proceeds via a complex series of interactions between many groups of organisms in conjunction with physical and chemical factors.

After leaching, autumn shed leaves undergo 'conditioning' whereby the leaf material is colonised by microorganisms increasing the palatability of the leaf material to invertebrates (Cummins, 1974). Initially fungi are more active than bacteria in the conditioning process (Barlocher and Kendrick 1973 a; MacKay and Kalff 1973; Kostalos and Seymour, 1976; Suberkropp and Klug, 1976; Pattee et al, 1986). The aquatic hyphomycete fungi are of particular importance (Barlocher and Kendrick, 1973 a, b, c, 1974; Suberkropp and Klug, 1976; Suberkropp et al, 1983; Anderson and Sedell, 1979; Cummins and Klug, 1979; Findley et al, 1986a). Colonisation of leaf material by aquatic

hyphomycetes occurs very rapidly once leaves fall into the stream (Nilsson, 1964; Chergui and Pattee, 1991).

Microorganisms cause weight loss of leaves and an increase in their nitrogen and protein content (Kaushik and Hynes, 1968, 1971). There may also be an increase in nitrogen content due to the production of non-labile humic nitrogen due to reactive phenolic and carbohydrate groups forming condensation products with amino acids, yielding precursors to nitrogenous humic polymers (Rice, 1982). The chemical composition of the leaf material is altered during softening of the leaf material by the action of fungal enzymes which attack pectins, hemicelluloses and cellulose (Suberkropp and Klug, 1976, 1980; Charmier and Dixon, 1982 a, b; Charmier et al, 1984).

The degree of microbially-mediated decomposition depends on a number of factors including: temperature (Barlocher and Kendrick, 1974; Charmier and Dixon, 1982;), pH (Suberkropp and Klug, 1980), leaf species (Whitkamp, 1966; Sinsabaugh et al, 1981; Charmier and Dixon, 1982), and chemical content of the water, eg. Leaves in nitrate enriched water decompose more rapidly due to an increase in microbial processing in response to nitrogen enrichment (Barlocher and Kendrick, 1974; Meyer and Johnson, 1983)

In addition to the direct role of aquatic hyphomycetes in the decomposition of leaf material, they also play an important role in improving the palatability of leaf litter for invertebrates (Findley et al 1986a,b). Shredder invertebrates comminute coarse particulate organic matter (CPOM) into fine particulate organic matter (FPOM) and dissolved organic matter (DOM) (Petersen and Cummins, 1974; Sedell et al, 1975; Cummins et al, 1980; Webster and Benfield, 1986). FPOM is then utilized as the food source for collector invertebrates (see Chapter 1). Hence shredder invertebrates are key in the breakdown of CPOM and in the transfer of energy to other trophic groups.

There is a general peak in the acceptability of leaf material as a food source to invertebrates as conditioning progresses (Barlocher, 1985). Once this peak has been reached, any additional exposure to microbes decreases the leaves attractiveness and the leaves are said to be 'post-conditioned' (Barlocher, 1985). The preference of detritivores for optimally conditioned leaves is also indicated by a time lag before colonisation of leaf material by invertebrates after entry of leaf material into a stream (Sedell et al, 1975). Leaf-eating invertebrates selectively ingest areas rich in fungal cells (Barlocher, 1980). Percentage breakdown of leaf material by invertebrates depends on the duration of fungal conditioning and on the species of fungi and invertebrates involved (Petersen and Cummins, 1974). For example, Chergui and Pattee (1991) found the proportion of breakdown caused by *Melanopsis praemorsa, Physa acuta* and *Hydropsyche maroccana* were respectively, 38, 21 and 13% in willow and 40, 15 and 8% in orlander leaves. Further when willow was inoculated individually with 4 species of fungi the amount consumed by the 3 invertebrates increased with time of fungal conditioning and according to the fungus and invertebrate species.

The importance of shredders in the decomposition of leaf litter has been demonstrated by insecticide-induced decreases in shredder abundance causing a decrease in leaf litter decomposition rates, seston concentration and export. Further, with the subsequent recovery of the stream invertebrates after insecticide application there was a restoration of decomposition rates, seston concentration and export (Cuffney et al, 1984; Wallace et al, 1986). Other studies have manipulated the shredder abundance by removal and again showed breakdown rates were greatly affected by invertebrates (Petersen and Cummins, 1974; Sedell et al, 1975). There are studies which suggest shredders are of little importance in leaf breakdown (Matthews and Koweleski, 1968; Kaushik and Hynes, 1971; Stout and Cooper, 1983; Beiser et al, 1991). However these studies were largely conducted in streams where CPOM and shredder invertebrates were not generally important and the shredder community was poorly adapted to exploit artificially elevated input of CPOM.

5.2 Objectives.

In the preceding chapters, the effects of copper on the physiology of an important leaf-shredding invertebrate, *Gammarus pulex*, have been determined. Because of the key role of *G.pulex* in the freshwater detritivorous system, it can be argued that a decrease in consumption rates and population density caused by copper contamination would have a significant effect on the processing of leaf litter through the system. Further, because of the intimate relationship between shredders and aquatic hyphomycete fungi, any effects of copper on the aquatic hyphomycete community may have additional, indirect effects on shredder leaf processing, or a direct effect on microbial leaf processing. Hence the studies reported in this chapter were designed to examine the effects of copper on:

1. processing of preconditioned leaf litter by the combined effects of microbes, invertebrates and physical factors;

2. functioning of the microbial community;

3. structure of the aquatic hyphomycete community;

4. growth, biomass production, sporulation, germination and copper uptake of selected aquatic hyphomycete species in pure culture techniques;

5. the feeding behaviour of *G.pulex* indirectly via changes in the quality of its food.

5.3 Materials and methods.

The effects of copper on leaf weight loss, associated microorganisms and invertebrates in outdoor artificial streams were examined at the same time as the experiments described in Chapter 4 were performed. The outdoor artificial streams comprised 6 stainless steel

channels incorporating both pool and riffle sections. The substrate was a coarse gravel mixture detailed in Chapter 4 (Table 4.18). One stream served as a control while the remaining 5 were dosed with nominal copper concentrations of 2, 6, 20, 60 or 200 µg/l (Chapter 4, Table 4.19). Details of the stream design and physicochemical parameters are given in Chapter 4 (Sections 4.3. 1, 4.5. 1. and 4.5. 2).

5.3. 1. Leaf weight loss due to combined effects of micro-organisms, invertebrates and physical processes.

Effects of copper on the weight loss of conditioned leaf material due to the combined effects of micro-organisms, invertebrates and physical processes was investigated by measuring weight losses of individually tagged leaves deployed in the artificial streams. Individually dry weighed alder leaves, tagged and numbered with plastic popper fasteners were placed in 12 x 12 cm, 350 µm pore size bags and were incubated in the supply stream for one week (preconditioned leaves). The pore size of the leaf bags allowed aquatic hyphomycete spores and other microbes to reach the leaves but excluded invertebrates.

After one week of conditioning, the tagged leaves were removed from the bags and tethered individually onto strands of fishing line. 15 leaves were tethered in the pool section and 15 in the riffle section of each artificial stream. After 4 hours the invertebrates associated with a random subsample of 2 leaves in each section of each stream were sampled using a 425 µm pore size sieve. The invertebrates were identified and quantified on site and were then released back into the streams. 27 hours after deployment the leaves were removed, rinsed thoroughly in distilled water, dried to constant weight at 60°C and reweighed on a Sartorius pan balance (accuracy ± 0.001 mg).

5.3. 2. Leaf weight loss due to micro-organisms.

a. Weight loss of leaves deployed in the artificial streams.

Effects of copper on microbially-mediated weight loss of preconditioned leaf material (Section 5.3. 1) and leaf material which was not first preconditioned in the supply stream (referred to as sterile leaves from here on) was investigated by deploying leaves enclosed in fine mesh bags.

One hundred replicates of approximately 1 gramme of dry-weighed alder leaves, with stalks removed, were allocated into 12 x 12 cm, 350 µm pore size leaf packs. As the pore size of the mesh was only 350 µm, aquatic hyphomycete spores and other microbes could reach the leaves but invertebrates were excluded, hence any weight loss would be principally due to microbial processing.

70 leaf packs were incubated in the supply stream for a week prior to deployment in the artificial streams to allow colonisation by a natural microbial flora. After this period five leaf packs containing preconditioned leaves were allocated to the pool section of each stream and five to the riffle section. The remaining 10 packs containing conditioned leaf material were rinsed in distilled water, dried at 60^{o}C and reweighed to determine weight loss during the conditioning period. The remaining 30 leaf packs containing sterile leaves were divided between the pool sections of the artificial streams, so that five packs, containing sterile leaves, were allocated to each stream.

The leaf material in each pack was exposed to copper in the artificial streams for one week. The artificial streams were dosed with nominal copper concentrations of 2, 6, 20, 60, and 200 µgCu/l and one stream remained undosed as a control (Actual copper concentrations are given in Chapter 4, Table 20). After one week of exposure, all the leaf

packs were then removed and the leaf material rinsed thoroughly in distilled water, dried to constant weight at 60^{o}C and reweighed on a Sartorius pan balance as above.

b. Weight loss of leaves deployed in laboratory tests.

In order to determine if copper had an effect on leaf processing over a longer time period (six to seven weeks) than was assessed in the artificial streams (one week), laboratory tests were conducted. 90 leaf packs, containing approximately one gramme of dried alder leaf material, were prepared in the same way as above (Section 5.3. 2. a). Leaves were preconditioned for one week in a relatively clean site (see Appendix 7 for physicochemical data) on the River Don in Yorkshire (NGR SK 216029). Leaf packs were randomly allocated to three 30-litre glass tanks containing 18 litres of APW and 2 litres of stream water from the River Don. 30 leaf bags per concentration were exposed to nominal copper concentrations of 0, 100, and 150 µg/l. Solutions were changed weekly and copper concentrations determined using AAS. Five bags from each concentration were removed weekly for six to seven weeks, rinsed in distilled water, dried to constant weight at 60^{o}C and reweighed. This procedure was repeated using sterile leaf material.

5.3. 3. Effects of copper on the structure of the microbial community.

Thirty six leaf bags (350 µm pore size) containing three to four alder leaves were prepared in order to investigate the effect of copper contamination on the structure of the fungal assemblage colonising leaf material which had been deployed in the artificial streams. Twenty four Leaf bags were conditioned in the supply stream as above (Section 5.3. 1). Both conditioned and sterile leaf material was deployed in the artificial streams for one week. Two bags containing conditioned leaves were placed in the pool and two in the riffle section of each artificial stream. Two leaf bags containing sterile leaves were placed in the pool section only. After deployment leaves were examined qualitatively by scanning electron microscopy and semi-quantitatively by assessing 'relative importance

values' (RIVs) (Shearer and Lane, 1983; Shearer and Webster, 1985). These methods are detailed below:

a. Scanning electron microscopy.

Small leaf squares were cut from conditioned and sterile leaves which had been exposed to copper in the artificial streams for one week. The squares were mounted in pairs and were frozen in liquid nitrogen at -176^{o}C. They were then transferred to a Phillips 501B Electron Microscope and heated up to -70^{o}C in order to etch away any surface water. The sample was then removed from the microscope and put into a low temperature cryo unit, (EMscope SP 200), and was gold coated for three minutes at 30 mAmps. The sample was then returned to the microscope and examined at an accelerating voltage of 15 KV. The images were captured on Type 52, 4 x 5 Polaroid film.

A semi-quantitative measure of fungal cover was determined by dividing pictures into a grid of 54, 1cm^2 units and assessing the percentage hyphal cover in each unit. Hence an approximation of the overall percentage cover was determined.

b. Relative importance values.

Relative importance values (RIVs) of aquatic hyphomycetes colonising leaf material were calculated using methods developed by Shearer and Lane (1983) and Shearer and Webster (1985). A relative importance value for each species is calculated as the sum of the relative leaf frequency (A), and the relative disc frequency (B) (eqn 5.1).

$$RIV = A + B \qquad \text{eqn 5.1}$$

The number of leaves on which a species is found (L_n) divided by the number of leaves examined (L) gives the leaf frequency value (L_f) (eqn 5.2); this number divided by the

sum of leaf frequency values of all species found in that sample (ΣL_f) gives the relative leaf frequency value (A) (eqn 5.3).

$$L_n / L = L_f \qquad \text{eqn 5.2.}$$

$$L_f / \Sigma L_f = A \qquad \text{eqn 5.3.}$$

The sum of the number of discs on which each species was found gives the disc frequency (D_f) from which mean disc frequency (XD_f) may be calculated by dividing by the number of discs examined (D) (eqn 5.4); the mean disc frequency of a species (XD_f) divided by the sum of the mean disc frequency values of all species found in that sample (ΣXD_f) gives the relative mean disc frequency value (B) (eqn 5.5).

$$D_f \ / \ D = XD_f \qquad \text{eqn 5.4}$$

$$XD_f \ / \ \Sigma XDf = B \qquad \text{eqn 5.5}$$

Hence A values may be used to indicate the patchiness of a species between different leaves while B values may indicate the degree of patchiness within one leaf. RIVs range in value from 0 to 2 and indicate the overall relative importance of a species compared to the importance of all other species found.

For both conditioned and sterile leaf types, ten, 16 mm diameter discs per leaf were cut from five leaves per stream giving a total of 50 discs per treatment. Each individual disc was incubated in a 9 cm diameter sterile petri dish containing distilled water for two days, at 15^{o}C. The fungi on the discs were stained and killed with lactophenol cotton blue (BDH) and 10 percent lactic acid (BDH) before being mounted on microscope slides and examined at 100 x magnification for presence of aquatic hyphomycete spores using a Wild light microscope. Presence or absence of aquatic hyphomycete species, identified

by their spore morphology, using keys of Nilson, (1964); Ingold (1975) and Descals and Webster (pers comm), was assessed for each disc. Relative importance values of aquatic hyphomycetes on leaf material exposed to copper in laboratory tests (Section 5.3. 2. b) was also assessed, in the same way, after one and six weeks exposure to copper.

5.3. 4. Food preferences.

Techniques used previously in the laboratory to determine *Gammarus* food preferences (Chapter 2, Section 2.3. 7) were not suitable in determining the food preferences of *G.pulex* for naturally conditioned material deployed in the artificial streams as dry weighing of leaf discs before feeding would kill any fungi that were present on the leaves. Given that it may be these fungi that were responsible for differences in *Gammarus* food preferences, experimental procedures were developed which limited effects on the fungal assemblage.

As *G.pulex* feeds by chewing through leaf material thereby reducing leaf area (Fig. 49), image analysis techniques could be used for the measurement of the proportion of each disc eaten. this approach removes the necessity to dry leaves in order to calculate weight loss.

The preferences of 30 individual *G.pulex* for control or copper contaminated food was examined with both previously conditioned and sterile leaves. 16 mm diameter leaf discs were cut from leaves which had been exposed to copper in the artificial streams for one week. Animals were placed in a 250 ml vessel containing 200 ml of artificial pond water and were maintained at 15^{o}C with a 12 hour alternating light-dark photoperiod. Discs from each of the six treatments were labelled with coloured pins and were offered to individual *G.pulex* (Chapter 2, Fig. 4). The animals were thus offered a choice of six leaf discs, each incubated in a different level of copper. The area of each disc eaten in 38.5 hours was determined by image analysis. The difference between leaf area measured before and after feeding being taken as the amount consumed.

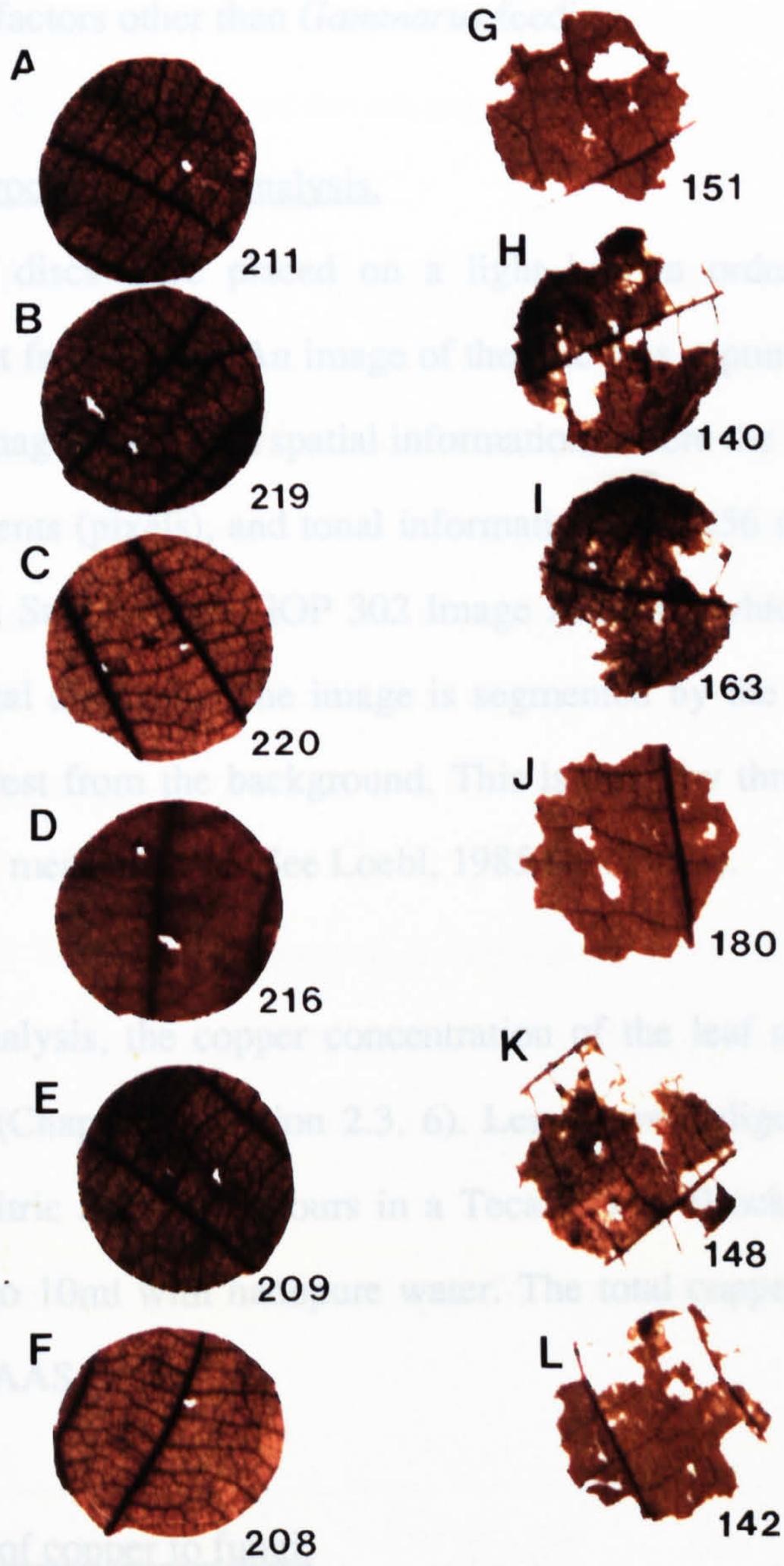


Fig. 49. Typical decrease in area of leaf discs prior to and after feeding by *G.pulex*. A to F Leaf discs prior to feeding. G to L Leaf discs after 44 hours of exposure to *G.pulex*. Area of leaf disc determined by image analysis shown in mm^2

Five control leaf sets were set up containing leaf discs but no animal to control for area changes due to factors other than *Gammarus* feeding.

Leaf imaging procedures and analysis.

Individual leaf discs were placed on a light box in order to illuminate them with transmitted light from below. An image of the disc was captured using a Pul Mix camera. The captured image holds both spatial information, where the image is divided into a grid of picture elements (pixels), and tonal information with 256 intensity levels. The camera was linked to a StruerVision GOP 302 Image Analyser which used built-in software to measure the total disc area. The image is segmented by the image analyser to separate regions of interest from the background. This is done by thresholding, object detection, elimination and measurement (See Loebl, 1985 for details).

After image analysis, the copper concentration of the leaf material was determined by acid digestion (Chapter 2, section 2.3. 6). Leaves were digested in 2 ml of 70 percent primar grade nitric acid for 8 hours in a Tecam DG1 Block Digester, the solution was then made up to 10ml with nanopure water. The total copper content of the leaves was determined by AAS.

5.3 5. Toxicity of copper to fungi.

From results of experiments on the effects of copper on the structure of aquatic hyphomycete communities exposed to copper in artificial streams, two species of aquatic hyphomycetes were selected in order to examine the mechanisms by which copper affected them. Effects of copper on the growth, biomass production, sporulation, and germination of the fungi were examined.

Tetracladium marchalianum was selected as it appeared to be tolerant of high concentrations of copper, being consistently found on leaf discs incubated in even the

highest dosed streams. This species was the dominant member of the aquatic hyphomycete community, based on RIV data (Appendix 8), in non-contaminated streams and was the dominant member of the community throughout all copper concentrations.

Anguillospora longissima was selected as the second species as it was relatively important in the aquatic hyphomycete assemblage on leaves in the control stream and was one of the more common species found on preconditioned leaf material. However, it was not found on any sterile leaves at concentrations of 24.14 µgCu/l or over. Hence, copper appeared to prevent *A.longissima* from colonising leaf material in the streams.

a. Culturing techniques.

Pure cultures of *T.marchalianum* and *A.longissima* were obtained from S.Bermingham (Sheffield University). Fungi were maintained in two percent malt extract agar (MEA) (Table 28) and were subcultured every two to three weeks.

Table 28: 2 percent malt extract agar for maintenance of aquatic hyphomycete cultures.

compound	supplier	Quantity (g)
Agar No. 2	Lab M	15
Peptone	Lab M	5
Malt Extract	Lab M	30

The above compounds were mixed together and made up to one litre with distilled water. The medium was then autoclaved at 121^{o}C, 15 PSI, for 20 minutes, before pouring onto 9 cm sterile petri dishes, under sterile conditions. Dishes containing medium were incubated for 48 hours at 25^{o}C to ensure sterility, after which they were inoculated with

0.5 cm diameter discs of fungi, using sterile techniques. All cultures were maintained at 15°C and were allowed to at least double their diameter before use of cultures in toxicity experiments.

b. Growth.

The effect of copper on the growth rate of the two fungi was investigated by determining radial growth of fungi grown on solid media. The growth medium used was adapted by S. Bermingham (Unpublished PhD) from studies by Thornton (1963), Duddridge and Wainwright (1980) and Abel and Barlocher (1984). The growth medium contained both micronutrients (Table 29) and macronutrients (Table 30).

Table 29: Micronutrients stock solution for growth medium.

Compound	Supplier	Quantity (g)
$ZnSO_4.6H_2O$	BDH	0.287
$NaMoO_4.2H_2O$	Fisons	0.48
$CoCl_2.6H_2O$	Fisons	0.17
H_3BO_3	Fisons	2.81
$MnCl_2.4H_2O$	Fisons	1.78
$FeCl_2.6H_2O$	Fisons	0.76

The micronutrients (Table 29) were mixed together and made up to one litre with distilled water in a volumetric flask. To aid mixing of the solution, each compound was first dissolved in 5 to 10 ml of dissolved water prior to adding to the flask.

Table 30. Macronutrients for growth medium.

Compound	Supplier	Quantity (g)
D-Glucose	Fisons	5.0
Vitamin-free acid hydrolysed caesin	Lab M	2.0
$MgSO_4.7H_2O$	Fisons	0.2
KH_2PO_4	Fisons	0.34
K_2HPO_4	BDH	0.57
$CaCl_2.2H_2O$ (Stock)	Fisons	58.8

The calcium stock was made by dissolving the 58.8g of $CaCl_2.2H_2O$ in one litre of distilled water. 5 ml of this stock solution was then added to the other macronutrients.

The above macronutrients (Table 30) were placed in a one litre volumetric along with 1 ml of micronutrient solution (Table 29). The medium was amended with copper sulphate to obtain nominal copper concentrations of 0, 2.5, 11.5, 24, 60, 300 µg/l. The medium was then made up to one litre with distilled water and poured into a Schott bottle. The concentration of copper in the medium was determined using FAAS or AAS. 20g/l of agar No. 2 (Lab M) was then added to the macronutrient plus micronutrient solution and the media was autoclaved at 121^{o}C, 15 PSI, for 20 minutes.

After autoclaving twenty, 20 ml aliquots of medium per concentration were immediately transferred with a sterile pipette into heat sterilized glass petri dishes in a laminar flow cabinet. The medium was allowed to cool and set before a 1 cm diameter plug of fungus taken from the growing edge of the cultured colony was placed in the centre of each

plate. There were ten plates per fungal species. Two strips of mm Scalefix tape (Scalefix Company Ltd, UK) were placed at right angles across the bottom of each petri dish and used to measure radial growth of the fungal colony.

The cultures were incubated at 15^{o}C for 32 days and radial growth measured after six days and every three days thereafter.

c. Biomass.

The effect of copper on the biomass production of the two fungal species was determined in liquid culture. Because aquatic hyphomycetes are able to sporulate under water, biomass production would include the production of spores and any secondary colonies arising from them in the culture. The medium used was the same as used in the growth experiment (Tables 29 and 30) except that the agar was omitted.

The medium was prepared and amended with copper to give nominal values as above (Section 2.3. 5. b). Twenty, 20 ml aliquots of medium per concentration were removed with a measuring cylinder and placed in 100 ml glass conical flasks. The flasks were plugged with cotton wool and sealed with foil and tape. The medium was autoclaved as above and allowed to cool. Actual copper concentrations of the media were determined by FAAS or AAS.

A 1 cm diameter plug of fungus was added to each flask under laminar flow conditions using sterile techniques and the flasks were resealed. Ten flasks per concentration were inoculated with *T.marchalianum* and ten with *A.longissima*. Flasks were shaken to submerge the plug and then incubated at 15^{o}C for 21 days. Flasks were shaken by hand daily to encourage growing hyphae to clump together and not stick to the sides of the vessel from which they are difficult to remove.

After three weeks the fungus growing in the flasks was filtered onto pre-weighed ash-free filter papers. The fungal mycelia were washed three times with 20 ml of nanopure water. The filters were folded placed in a 60^{o}C oven for two to three days after which they were reweighed.

d. Sporulation.

The aim here was to determine subsequent effects on the sporulation of fungi grown in the presence of copper. After the fungal cultures from the growth experiment (section 5.5. 3. b) had more than doubled their original diameter, a section of mycelium was removed and placed in conditions to encourage sporulation.

Four, 0.5 cm diameter plugs were cut from the growing edge of colonies from four plates of each concentration using sterile techniques. As aquatic hyphomycetes only sporulate when submerged in well aerated water, individual plugs were randomly allocated to sterile centrifuge tubes containing 10 ml of sterilized distilled water. A hypodermic needle was inserted through the base of each centrifuge tube and attached to a supply of compressed air via a distributor allowing 20 tubes per air line.

The aerated centrifuge tubes containing fungal plugs were covered with foil to reduce evaporation and the level of sterile distilled water topped up to 10 ml daily. The fungi were incubated at 15^{o}C for six days to allow sporulation, after which the plug was removed and the water decanted out of the centrifuge tubes. The tubes were rinsed thoroughly with sterile distilled water and the sides of the tubes scraped to remove any spores attached to the sides.

The decanted water was centrifuged, in a Centaur 2 desk top centrifuge, at 2000 rpm for five minutes. The top 9 ml of water was then removed and the spore suspension resuspended in the remaining 1 ml of water by sucking up and releasing the suspension from a Gilson pipette a number of times. 0.1 ml of this suspension was removed with a

Gilson pipette and the number of spores in the sample were counted with the use of a Fuchs Rosenthal haemocytometer and Wild compound microscope.

e. Germination.

Three 0.01 ml aliquots of each of the concentrated spore suspensions generated in the sporulation experiment (Section 5.5. 3. d) were removed with a Gilson pipette and dropped onto plates of malt extract agar (Table 28). This agar had previously been filtered through Whatman No.1 filter paper to improve its clarity under the microscope. The agar plate was tilted in order to allow the three drops to run down producing smears of spore suspension down the plate. The location of the three smears was marked on the underside of the plate to facilitate the location of the spores once the plate had dried.

The plates were incubated at 15^{o}C for 20 hours and were then examined under a dissecting microscope, at 35 x magnification, for germinating spores. Spores which had clearly produced primary hyphae were considered to have germinated. Three replicates of 100 spores per plate were counted and the proportion that had germinated recorded.

f. Mycelial copper concentration.

The accumulation of copper by the fungal hyphae was determined using mycelia produced in the fungal biomass experiment described in Section 5.3. 5. c. Three filter papers per concentration were ashed in porcelain crucibles in a muffle furnace at 500^{o}C for 24 hours. 0.5 ml of 50 percent primar grade nitric acid was added to each crucible and the residue was heated gently until it had dissolved. The solution was then transferred to a sample tube and the crucible washed out with nanopure water. The sample was made up to 10ml with nanopure water and the copper content of the mycelia determined by AAS.

5.4. Statistical analyses.

All data was tested for normality using n-scores techniques prior to any further analysis. The correction factor (C_1) for the effects of leaching during the initial weeks conditioning of leaves in the supply stream was determined using eqn 5.6

$$C_1 = \Sigma\ (L_{C2} / L_{C1})\ /\ 10 \qquad \text{eqn 5.6}$$

Where L_{C1} represents the weight of control leaves before conditioning and L_{C2} the weight of control leaves after conditioning in the supply stream.

Percentage weight loss of leaf material exposed to copper in the artificial streams was compared using one way analysis of variance and the Tukey multiple range test after arcsine transformation of the data. Determination significant differences between groups was determined using the twosample t-tests.

Correlation between leaf weight loss and the density of *Gammarus* trapped on the leaves was performed using least squares regression techniques.

Leaf weight loss in laboratory tests was best described by an exponential relationship. Hence weight loss was expressed as a single exponential decay function (-k), derived from the model:

$$W = e^{-kt}$$

where W = proportion of initial mass remaining (W_t / W_0; W_t = leaf weight at time t , W_0 = initial leaf weight) at time t.

Log-linear plots of W against t are described by equation 5.7 and were used to derive values for -k:

$$\text{Ln}\,(W_t/W_0) = -kt \qquad \text{Eqn 5.7}$$

Effects of copper on exponential decay functions (-k) were then assessed using analysis of covariance. Two sample t-tests were employed to determine the time at which leaf processing rates of contaminated leaf material differed from that of control leaf material.

Differences in the food preferences of *G.pulex* were determined using one-way analysis of variance and the Tukey multiple range test, employing the twosample t-test to determine where significance lay. Differential accumulation of copper by conditioned and non-conditioned leaf material in each artificial stream was compared using two-sample t-tests.

Growth rates of aquatic hyphomycetes exposed to copper were compared using analysis of covariance while biomass production and accumulation of copper by fungal mycelia was compared using one-way analysis of variance.

Due to the non-normality of haemocytometer data, effects of copper on sporulation was determined using the Mann-Whitney and Kruskall-Wallis tests. Effects of copper on the germination of aquatic hyphomycetes was determined using one way analysis of variance after arcsine transformation of the data.

All analysis was performed using Statgraphics, Minitab or Fig P computer software.

5.5 Results.

5.5. 1. Leaf weight loss due to combined effects of microbes, invertebrates and physical processes.

Initial leaching and conditioning in the supply stream prior to deployment in the artificial streams accounted for 23.47 percent of the weight loss of the leaves.

For both pool and riffle deployed leaves there was a decrease in leaf weight loss with increasing copper concentration although the effect was less marked in the riffle section (Fig. 50). There was a significant reduction in the weight loss of leaf material exposed to 309 µgCu/l in the pool sections ($F = 23.722$, $df = 5, 58$, $p < 0.001$) and at 25 µgCu/l and above in the riffle sections ($F = 25.455$, $df = 5, 58$, $p < 0.001$) (Fig. 50).

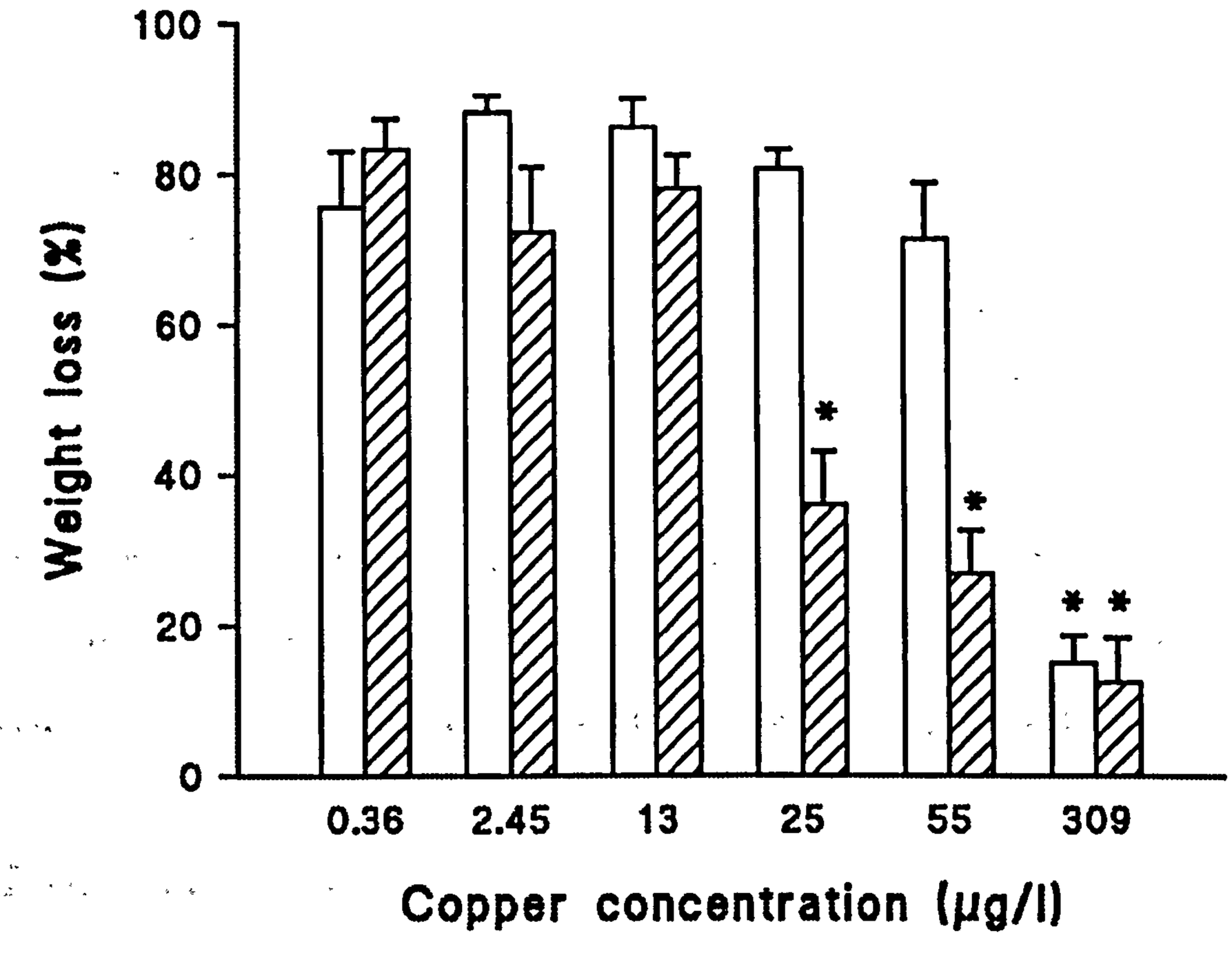


Fig. 50. Percentage of conditioned leaf material processed in the pool (open bars) and riffle (hatched bars) section of the artificial streams. Error bars represent 1 standard error, * denotes data significantly different from the control.

The macroinvertebrates trapped on the leaves were all *Gammarus* and there was a strong correlation between the number of *Gammarus* trapped and processing rates of the leaves in both the pool ($r^2 = 90.3\%$, df = 4, $p < 0.05$) and in the riffle sections ($r^2 = 69.2\%$, df = 4, $p < 0.05$) (Fig. 51).

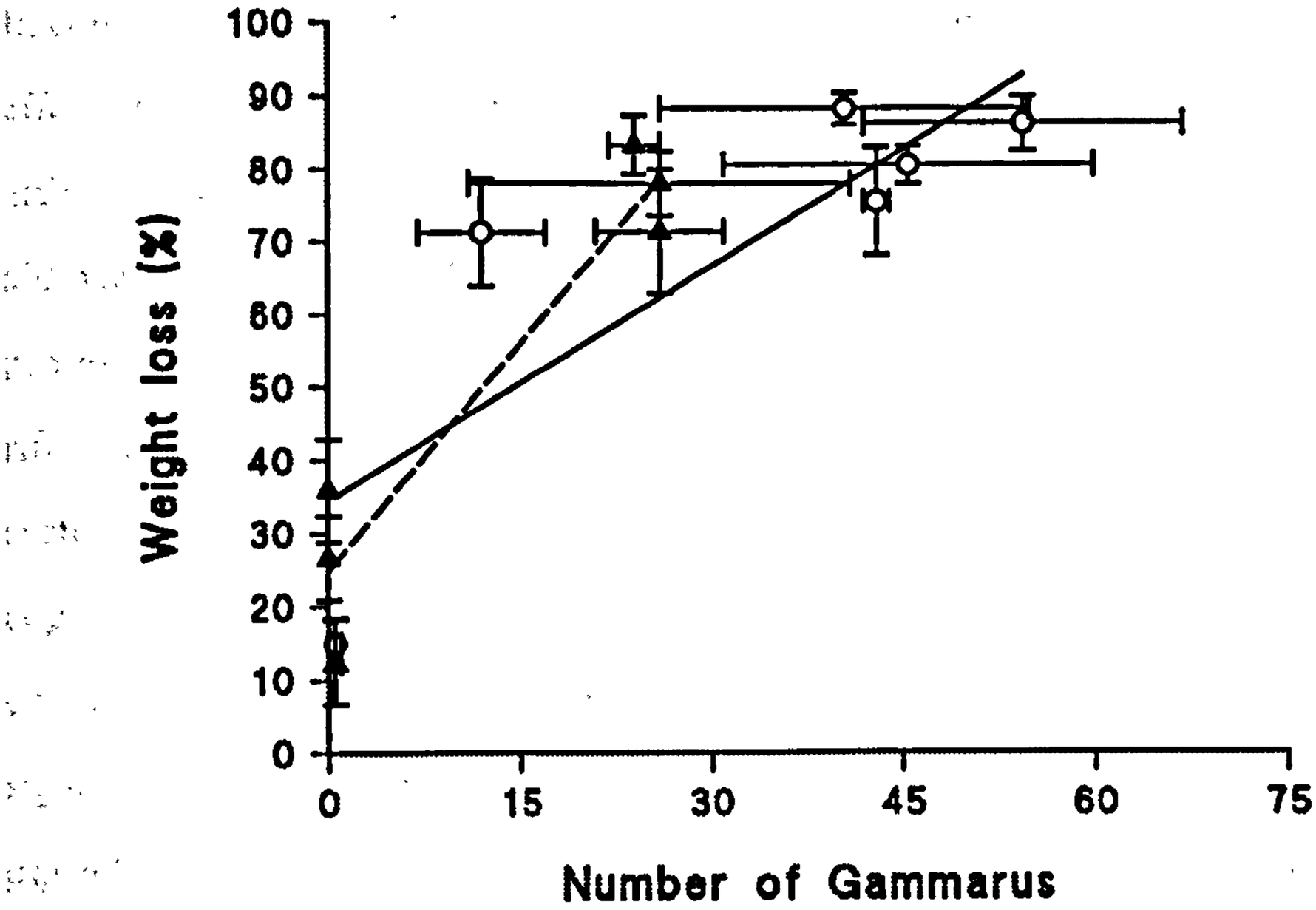


Fig. 51. Correlation between percentage leaf weight loss and numbers of *Gammarus* trapped on leaves in the pool (open circles; solid line) and riffle (solid triangles; dashed line) sections of the artificial streams. Error bars represent 1 standard error.

5.5. 2. Leaf weight loss due to microorganisms.

a. Weight loss of leaves deployed in the artificial streams.

There was a significant effect of both leaf type (conditioned pool, conditioned riffle or sterile pool) (Anova: $F = 33.827$, df = 2, 89, $p < 0.001$) and copper concentration (Anova: $F = 6.22$, df = 5, 89, $p < 0.001$) on the mass loss of leaf material (Fig. 52). However, there

was no significant interaction between them ($F = 1.152$, $df = 10, 89$, $p > 0.05$). The average weight loss of conditioned leaf material in the pool and riffle sections was 11.9 and 14.2 percent respectively while significantly more (19.7 percent) of sterile leaves was lost during the week-long deployment in the pool section of the artificial streams. The greater weight loss of sterile leaves may be explained due to the effects of leaching.

In contrast to the results discussed in the previous section (Section 5.5. 1), copper had no effect on the weight loss of conditioned leaf material deployed in the pool sections of the artificial streams (Anova: $F = 2.591$, $df = 5, 28$, $p > 0.05$) (Fig. 52) indicating that although copper affected invertebrate leaf processing there was no effect on the processing of leaf litter by the microbial community. Both conditioned leaf material in the riffle sections (Anova: $F = 2.9$, $df = 5, 28$, $p < 0.05$) and sterile material in the pool sections (Anova: $F = 3.038$, $df = 5, 28$, $p < 0.05$), however, were significantly affected by copper although not in a linear dose-response manner (Fig. 52). Weight loss of conditioned leaves deployed in the artificial stream riffle dosed with 83.98 µgCu/l lost significantly more weight than leaves in the riffles of streams dosed with 2.33 or 339.17 µgCu/l ($t > 2.6$, $df = 7$, $p > 0.05$) (Fig. 52). The only significant effect of copper on the weight loss of sterile leaves placed in the pool sections of the artificial streams was between leaves exposed to 25.13 and 339.17 µgCu/l ($t = 4.25$, $df = 7$, $p < 0.01$).

Effects of copper on the weight loss of leaf material caused by the action of microbes was studied over the period of one week while that due to combined actions of microbes, invertebrates and physical factors were studied over 27 hours. Hence over these time periods the copper concentrations in the artificial streams were slightly different (Figs. 50 and 52) for reasons discussed in Chapter 4 (Section 4.3. 1). Weight loss of leaf material due to combined action of microbes, invertebrates and physical factors under control conditions caused approximately an 80 percent weight loss as opposed to only about 10 to 15 percent caused by microbes (Figs 50 and 52). This enhanced weight loss was likely to be caused primarily by the action of *Gammarus*, with a significant correlation between

the numbers of *Gammarus* trapped on the leaf material and the percentage leaf weight loss (Fig. 51). Further, microbially mediated leaf weight loss appeared rather insensitive to copper (Fig. 52) while leaf processing predominantly due to invertebrates was sensitive to copper in a dose related manner (Fig. 50).

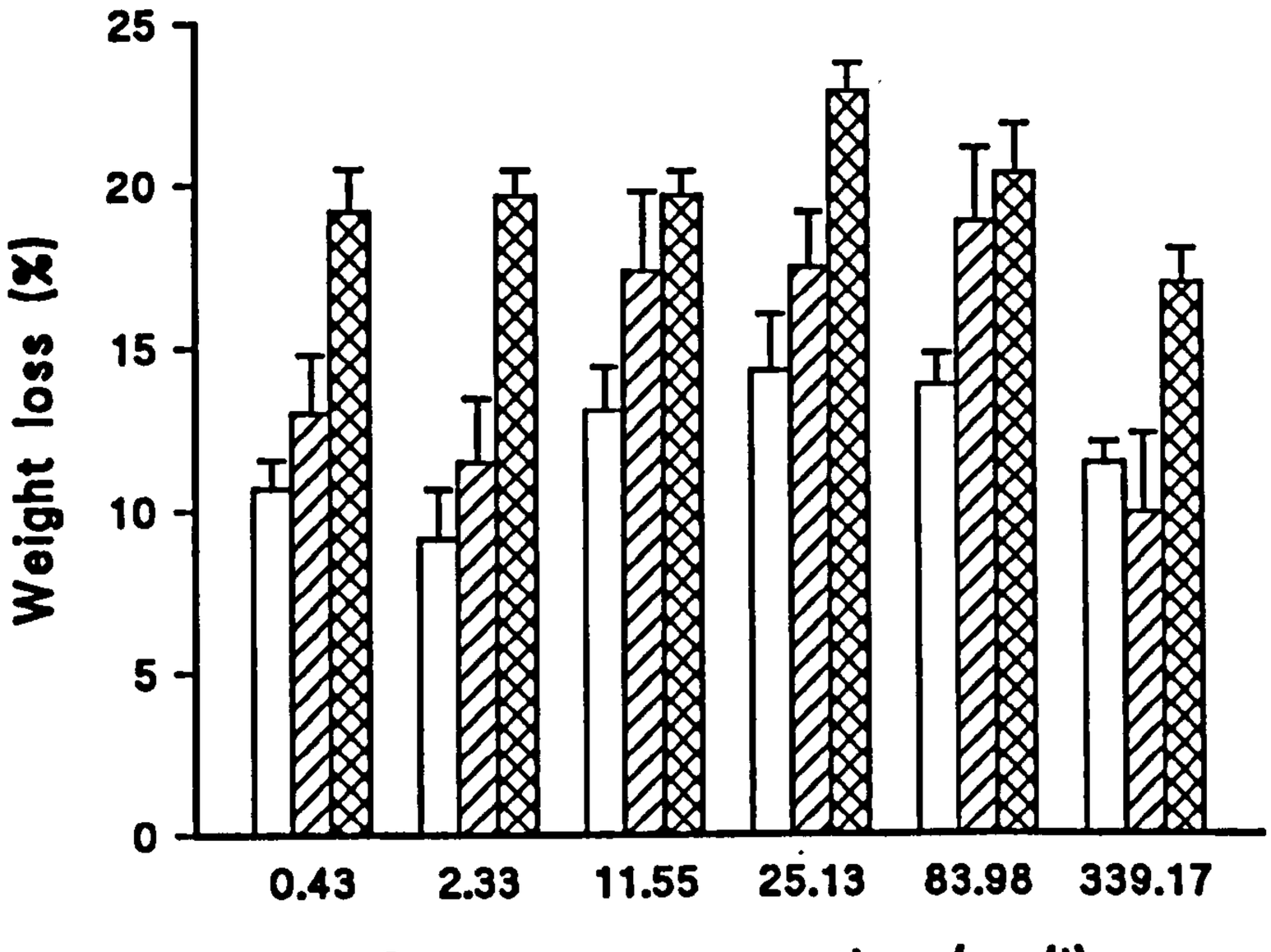


Fig. 52. Effect of copper on the percentage of conditioned leaf material processed microbially in the pool (open bars) and riffle (hatched bars) section and of sterile leaf material processed in the pool section (cross-hatched bars) of artificial streams. Error bars represent 1 standard error.

<u>b. Weight loss of leaves deployed in laboratory tests.</u>

The lack of effect of copper on microbially mediated weight loss could either be due to: insensitivity of microorganisms to copper contamination, or processes such as leaching causing large mass losses during the week-long deployment masking effects of copper on microbially-mediated leaf processing. Figures 53 and 54 illustrate the effects of copper on

microbially mediated weight loss of conditioned and sterile leaves respectively, over a six to seven week period in laboratory tests. Results indicate the lack of an effect of copper on the microbially mediated leaf weight loss may be due to the second possibility (i.e. the masking of effects due to weight loss caused by leaching).

Regression lines of leaf processing rates with time were significant for both conditioned ($r^2 > 83.8\%$, df = 4, $p < 0.05$) and sterile ($r^2 > 86.2\%$, df = 4, $p < 0.05$) leaves.

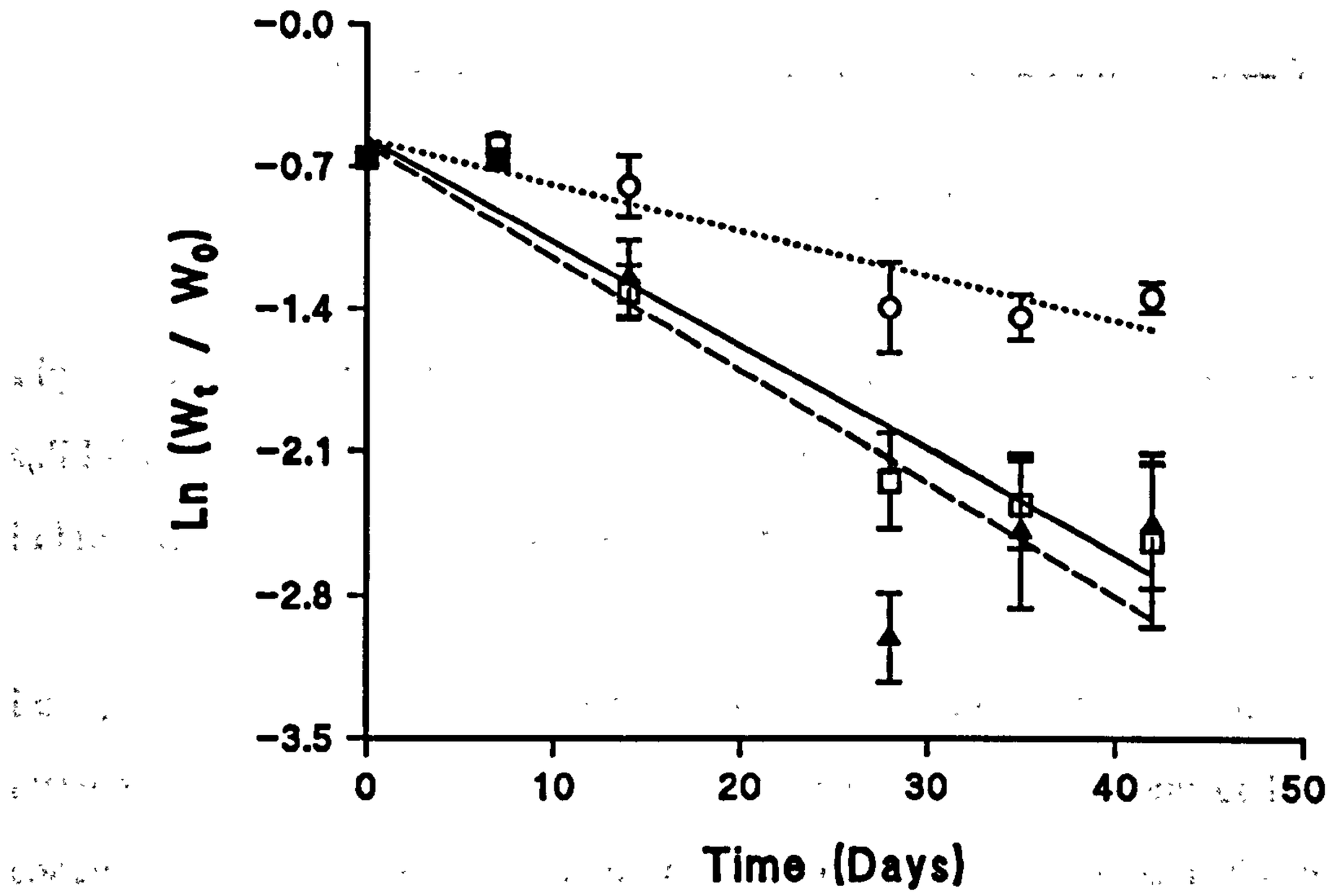


Fig 53. Leaf processing rates of conditioned leaf material exposed to 0 (open squares; solid lines), 65 (solid triangles; dashed line), and 132 (open circles; dotted line) µgCu/l in laboratory tests. Error bars represent 1 standard error.

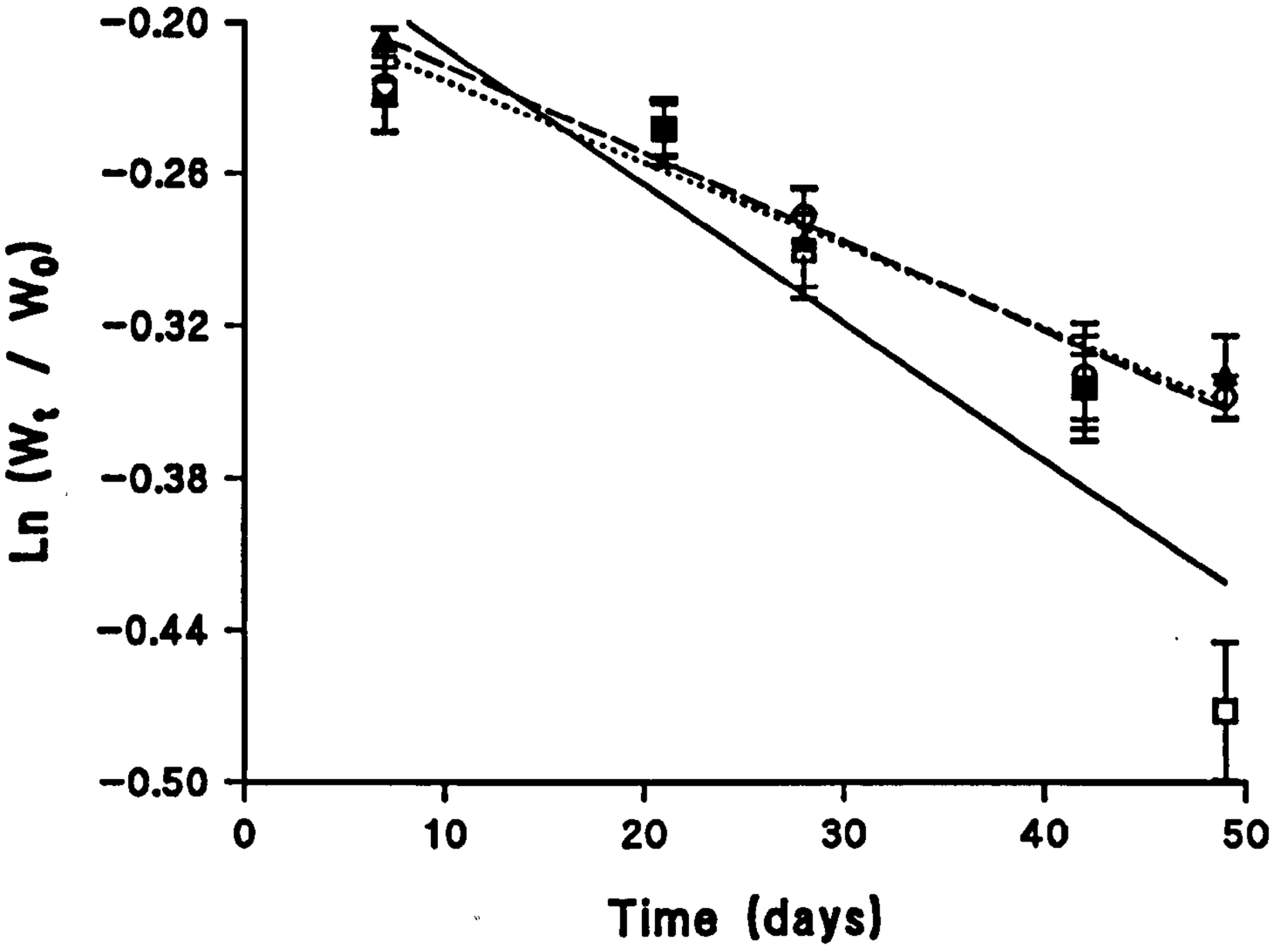


Fig. 54. Leaf processing rates of sterile leaf material exposed to 0 (open squares; solid lines), 67 (solid triangles; dashed line) or 113 (open circles; dotted line) µgCu/l in laboratory tests. Error bars represent 1 standard error.

Leaf processing rates of conditioned leaf material were much greater than that of sterile material (Fig. 55). The low -k values of sterile leaf material compared to those of conditioned leaf material may have been due to the low levels of inocula that would have been received in these tests compared to that which would be available in natural stream water.

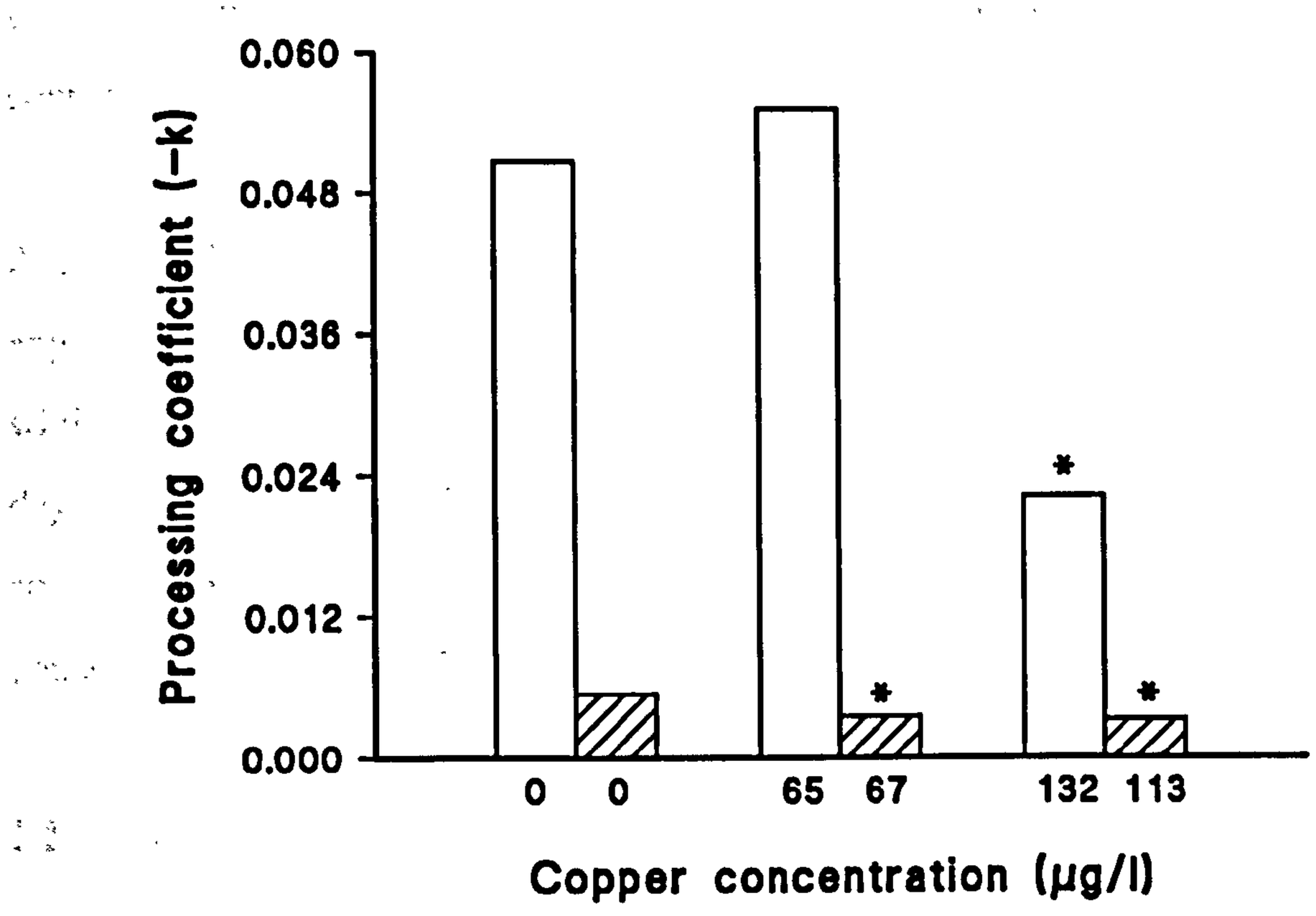


Fig. 55. Processing coefficient (-k) for conditioned (open bars) and sterile (hatched bars) leaf material exposed to copper in laboratory tests. * indicated data significantly different from control ($p < 0.05$)

There was no effect on rates of weight loss (-k) of leaves exposed to 65 µgCu/l (Ancova: $F < 0.07$, df = 1, 58, $p > 0.05$), although concentrations of 132 µgCu/l did significantly reduce the decomposition rates of preconditioned leaves (Ancova: $F = 8.82$, df = 1, 58, $p < 0.01$) (Fig. 55). The effect of copper on microbially-mediated leaf weight loss was greater for sterile leaf material with the processing rate (-k) significantly reduced at concentrations of 67 µgCu/l and over ($F = 5.26$, df = 1, 58, $p < 0.05$) (Fig. 55). In contrast, copper had no effect on the weight loss of leaf material exposed in the artificial streams to concentrations as high as 339.17 µgCu/l after 7 days.

The difference in decomposition rates between the preconditioned control leaves and preconditioned leaves exposed to 132 µg/l copper only became significant after 14 days of exposure ($t > 2.78$, df = 7, $p < 0.032$). In the artificial streams no effects of copper on

microbially mediated weight loss were detected after one week exposure even up to copper concentrations upto 339.17 µg/l.

A period of at least 14 days exposure was required before effects on sterile leaves exposed to 113 µgCu/l differed significantly from the control ($t > 2.78$, $df = 7$, $p < 0.05$), while those exposed to 67 µg/l copper differed significantly from control leaves after 28 days exposure ($t > 2.7$, $df = 7$, $p < 0.05$). Again one week exposure in the artificial streams was insufficient for detection of any effects even when exposed to copper concentrations upto 339.17 µgCu/l.

5.5. 3. Effects of copper on structure of the microbial community.

a. Scanning electron microscopy.

The percentage cover of fungi on conditioned and sterile leaf material exposed to copper in the artificial streams is given in Table 31. Sterile leaves were examined from the control stream (0.43 µgCu/l) and the middle and top dosed streams (25.13 and 339.17 µgCu/l). There was 100 percent hyphal cover on sterile leaves incubated in the control stream (Table 31) with extensive colonisation by fungi producing a thick mat of mycelial strands with a mucus-like substance apparent. Algal cells and other debris were also visible entrapped in this mat (Fig. 56 a).

However, no hyphal cover was detected on leaves from the two other streams studied (Table 31, Fig. 56, b and c,). The cellular architecture of the leaves is clearly visible. The surface structure of the epidermal cells and stomata are easily identified and bacterial cells become very evident on these leaves.

Scanning electron micrographs of conditioned leaf material from the control stream and those exposed to copper concentrations upto 25.13 µgCu/l show extensive colonisation by

fungi with particularly dense colonisation around the veins; percentage cover ranging from 68 to 84 percent (Table 31, Figs. 57 a - c). Aquatic hyphomycete spores, especially those of *T.marchalianum*, are clearly visible on the leaf surface along with algal cells and other debris. The fungal mycelia is dense and appears matted in many areas by a mucus like substance but cover is rather patchy.

Percentage hyphal cover on leaves exposed to 83.98 µgCu/l was reduced to less than 50 percent although there was still patchy colonisation by fungi and a great number of aquatic hyphomycete spores evident over the leaf surface. Once again there were a large number of *Tetracladium marchalianum* spores particularly evident (Table 31, Fig. 57, d).

Fungal hyphae on conditioned leaves incubated in the 339.17 µg/l copper stream covered only 2 percent of the leaf surface. Extensive areas of the bare under-surface of the leaves were seen showing clearly the morphology of the leaf. A few mycelial strands may be observed along with a few algal cells and other debris (Table 31, Fig. 57, e).

Table 31: Estimated percentage fungal cover on conditioned and sterile leaf material exposed to copper in the artificial streams. (/ denotes material not examined).

Copper Concentration (µg/l)	Percentage cover	
	Conditioned	Sterile
0.43	69.3	100
2.33	84.8	/
25.13	68.1	0
83.98	44.3	/
339.17	2.0	0

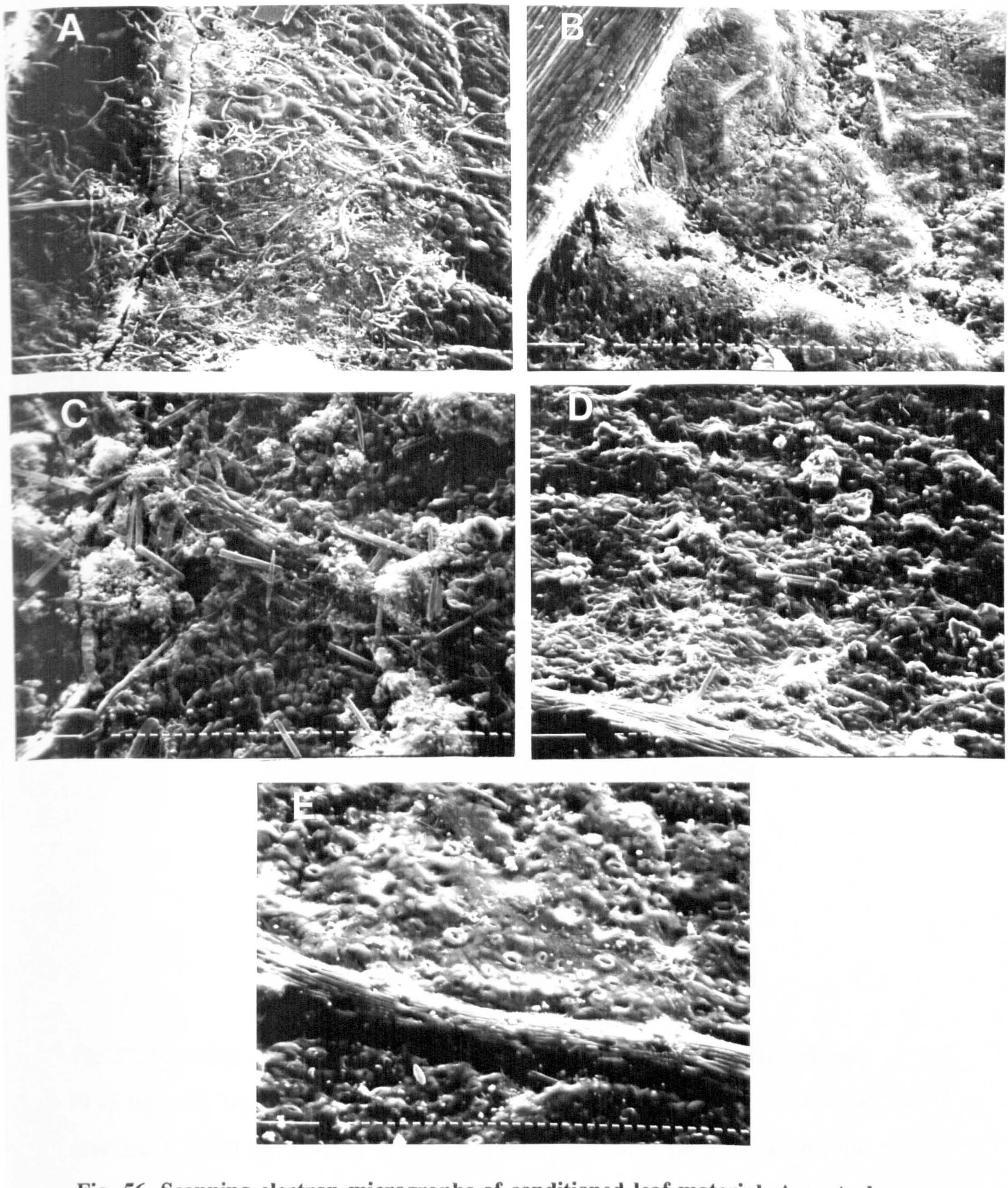
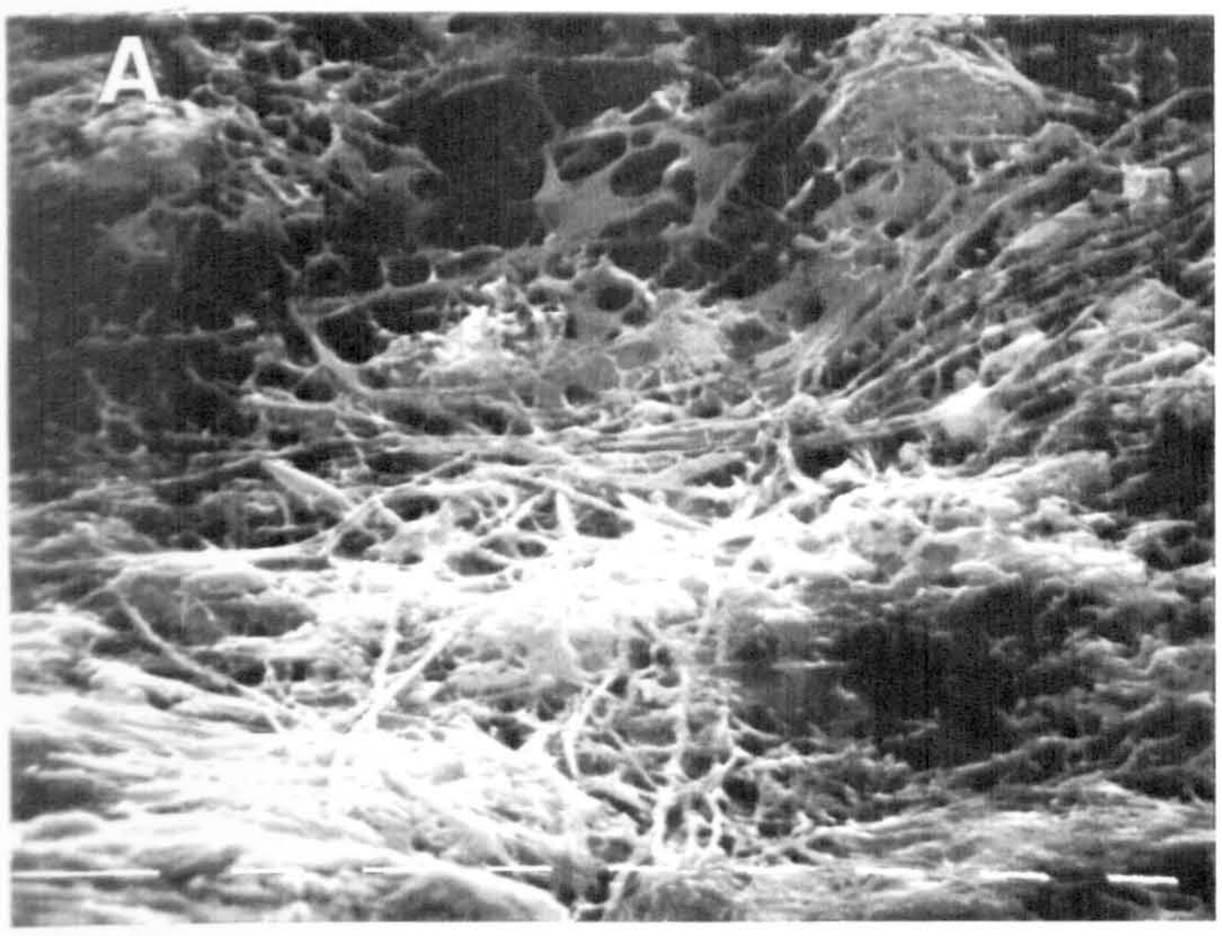
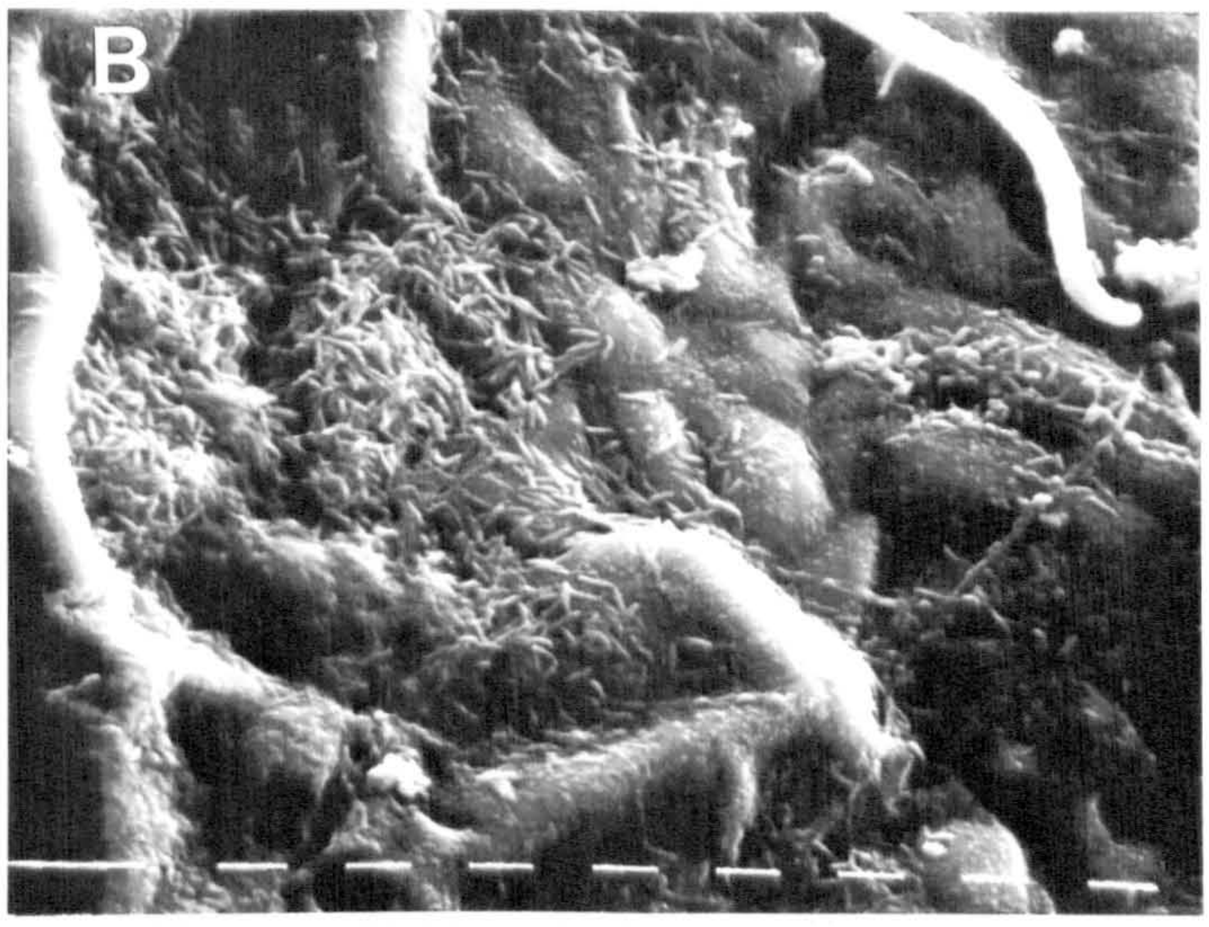


Fig. 56. Scanning electron micrographs of conditioned leaf material. A control stream (0.43 μgCu/l), B 2.33 μgCu/l and C 25.13 μgCu/l extensive fungal cover with aquatic hyphomycete spores, algal cells and other debris apparent. D 83.98 μgCu/l and E 339.17 μgCu/l reduced fungal cover revealing cellular architecture of the leaf. Dashed bar = 10 μm.





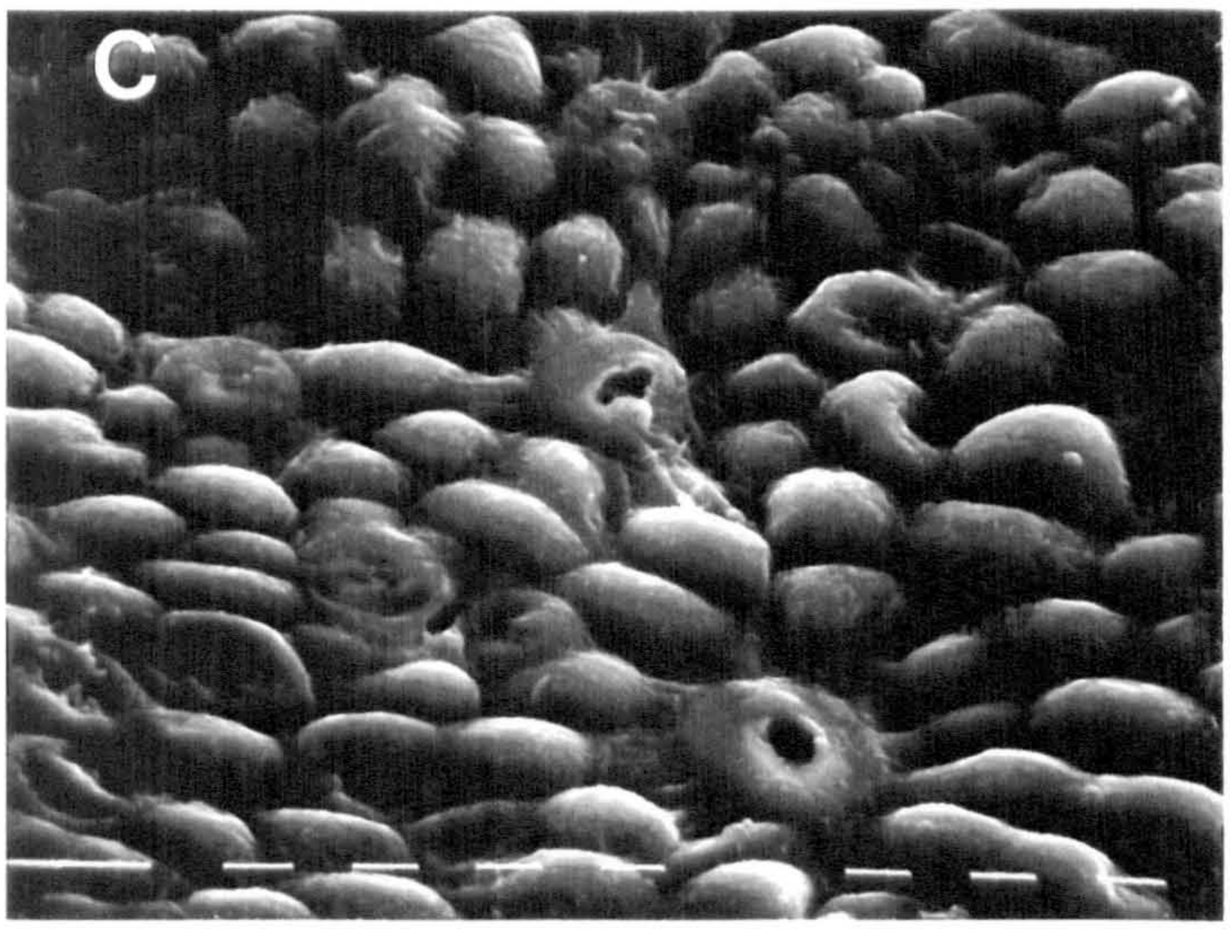


Fig. 57. Scanning electron micrographs of sterile leaf material. A control stream (0.43 μgCu/l). Dense fungal cover with mucilage appearing to bind a mat of mycelia. B 25.13 μgCu/l and C 339.17 μgCu/l. No fungal cover apparent, but increased abundance of bacterial cells and cellular architecture of the leaf clearly visible. Dashed bar = 10 μm.

b. Relative Importance Values.

i. RIVs of leaves in the artificial Streams.

In total 21 aquatic hyphomycete species were found on conditioned leaves deployed in the pool section, 14 on conditioned leaves deployed in the riffle section and 12 on sterile leaves deployed in the pool section (Fig. 58).

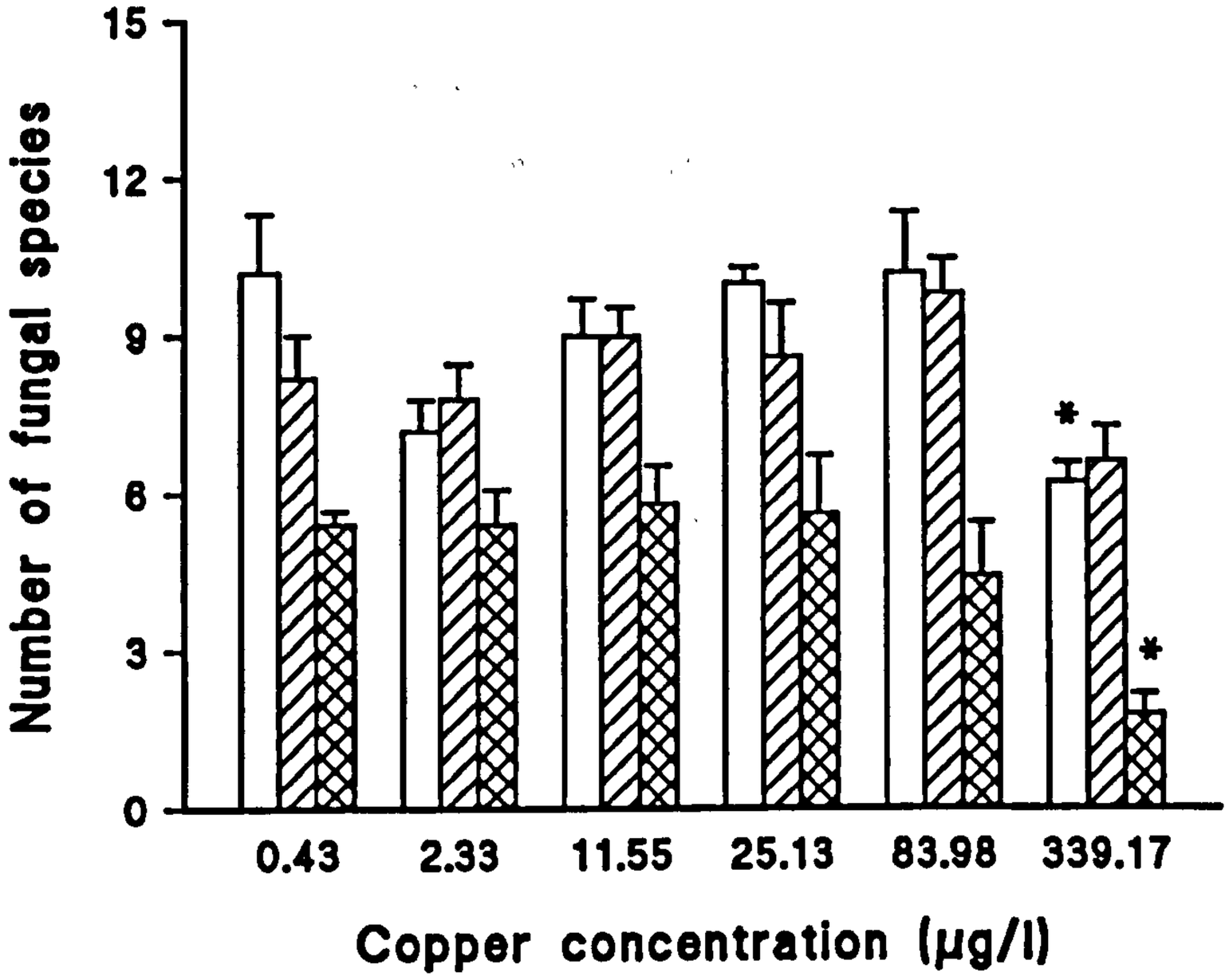


Fig. 58. Mean number of aquatic hyphomycete species found on leaf material exposed to copper in artificial streams. Preconditioned pool (open bars), preconditioned riffle (hatched bars), non-conditioned pool (cross-hatched bars). Error bars represent 1 standard error, * denotes data significantly different from the control.

Two-way analysis of variance showed that although both copper concentration ($F = 8.75$, $df = 5, 89$, $p < 0.001$) and leaf type ($F = 50.84$, $df = 2, 89$, $p < 0.001$) had a significant effect on the mean number of aquatic hyphomycete species found on the leaf material the

effects were independent ($F = 1.27$, $df = 10, 89$, $p > 0.05$). The mean number of species found per leaf on conditioned and sterile leaves incubated in the pool section was significantly lower on leaf material exposed to 339.17 µgCu/l than leaf material incubated in the control stream ($F = 3.918$, $df = 5, 59$, $p < 0.01$) (Fig. 58). However, fungal numbers were not significantly affected by copper on leaf material in the riffle section ($F = 2.146$, $df = 5, 59$, $p > 0.05$).

The RIVs indicate a changing community of aquatic hyphomycetes with copper concentration (Tables 32 to 34). Conditioned leaves were dominated by T*etracladium marchalianum, Anguillospora crassa, Anguillospora longissima* and *Articulospora tetracladia* in both the pool and riffle section of the control stream (Tables 32 and 33). The relative importance of the first three species increases with increasing copper concentration indicating a decrease in the variability of fungal species on the leaves. The relative importance of *Centrospora aquatica* and *Articulospora tetracladia*, diminishes in the riffle and pool section along with *Tetracladium setigerum* in the pool section only. Many species (approximately 50 percent) appear to maintain their RIVs across copper concentrations (Tables 32 and 33). There is an indication of an increase in the patchiness of the distribution of some fungi on the leaves with increasing copper concentration. This is indicated by an increase in the ratio of A values (i.e an index of the distribution of aquatic hyphomycete between leaves) to B values (i.e. an indication of the distribution of aquatic hyphomycetes on a leaf) (Section 5.3. 3. b) (see Appendix 8). The increase in A values relative to B values occurred in eight out of twelve species and five out of ten species remaining between the control and top dosed stream in the riffle and pool sections respectively. The A:B ratio of *Heliscus lugdunensis* in the riffle, for example, is 2.3 : 1 in the control stream but increases to 8.3 : 1 in the top dosed stream.

Table 32. Relative importance values of aquatic hyphomycete fungi found on conditioned leaves exposed to copper in the pool section of artificial streams for 1 week. (- denotes species not detected).

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.5	299.66
Tetracladium marchalianum	0.080	0.109	0.006	0.079	0.075	0.139
Anguillospora crassa	0.008	0.071	0.078	0.076	0.068	0.088
Articulospora tetracladia	0.063	0.047	0.049	0.055	0.062	0.076
Anguillospora longissima	0.053	0.073	0.065	0.060	0.051	0.053
Centrospora aquatica	0.042	0.026	0.024	0.019	0.027	0.022
Tetrachaetum elegans	0.038	0.033	0.046	0.046	0.034	0.042
Tetracladium setigerum	0.038	0.041	0.028	0.028	0.021	0.011
Heliscus lugdunensis	0.033	0.033	0.044	0.034	0.033	0.039
Clavatospora stellata	0.018	0.018	-	0.016	0.018	0.011
Clavariopsis aquatica	0.016	0.013	0.044	0.042	0.040	-
Tetracladium angulata	0.014	0.009	0.011	-	0.014	0.004
Dactylella aquatica	0.011	-	-	-	-	-
Lemoniera aquatica	0.008	0.000	0.008	0.006	0.009	-
Flabellospora acuminata	0.008	0.016	0.008	-	0.015	-
Scorpiosporium minutum	0.006	0.011	0.007	-	0.009	-
Campylospora parvula	0.006	0.000	0.002	-	0.006	-
Lemoniera terrestris	-	-	-	0.017	0.006	-
Alatospora acuminata	-	-	-	0.006	-	-
Dendrospora sp.	-	-	-	0.006	-	-
Clavatospora longibrachiata	-	-	0.007	-	0.007	-
Tetracladium furcatum	-	-	-	-	0.006	-

Table 33. Relative importance values of aquatic hyphomycete fungi found on conditioned leaves exposed to copper in the riffle section of artificial streams for 1 week. (- denotes species not detected).

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.55	299.66
Tetracladium marchalianum	0.095	0.097	0.080	0.087	0.082	0.150
Anguillospora longissima	0.081	0.060	0.072	0.080	0.062	0.083
Articulospora tetracladia	0.074	0.070	0.061	0.055	0.045	0.034
Anguillospora crassa	0.065	0.084	0.077	0.085	0.068	0.069
Centrospora aquatica	0.042	0.054	0.053	0.028	0.036	0.024
Heliscus lugdunensis	0.033	0.008	0.014	0.025	0.038	0.024
Clavariopsis aquatica	0.028	0.031	0.052	0.046	0.032	0.031
Tetrachaetum elegans	0.025	0.012	0.026	0.024	0.052	0.021
Tetracladium setigerum	0.016	0.043	0.025	0.018	0.031	-
Vargamyces aquatica	0.011	0.008	-	0.008	-	0.010
Clavatospora stellata	0.009	0.018	0.008	0.024	0.008	0.010
Scorpiosporium minutum	0.008	0.016	0.028	0.014	0.021	0.034
Campylospora parvula	0.008	-	0.007	0.008	-	0.010
Tricladium angulatum	0.008	-	-	-	0.021	-

The sterile leaves which had not been previously conditioned in the natural stream were found to be dominated by the same four species that were also found dominant on conditioned leaves (Tables 32 to 34). However, the effects of copper on the structure of the fungal community on these leaves was far more marked (Table 34). *Tetracladium marchalianum* was the only species to show an increase in its RIV, appearing to do well at the expense of other species. This effect could, however, be an artifact of the method as all values are relative and hence the loss of other species would cause an increase in the relative importance of remaining species.

Anguillospora crassa diminished in relative importance on exposure to increased copper concentrations but was not excluded at high concentrations. *Articulospora tetracladia* showed a decrease in RIV with increasing copper concentration and was not detected at the top concentration of 339.17 µgCu/l, while *Anguillospora longissima* appeared to be even less copper tolerant showing a decreasing RIV with copper concentration and total

exclusion from the top three concentrations of 339.17 µgCu/l and above. All but five species were excluded from the top copper concentration of 339.17 µgCu/l. (One species, *Tetracladium setigerum* was not detected on leaf material from the control streams or the lowest two dosed streams but was detected in the three highest dosed streams on leaf material exposed to 25.13 µg/l copper and above (Table 34)). As with conditioned leaves, an increase in the ratio of A:B values indicates an increase in the patchiness of the distribution of fungi on the leaves (see Appendix 8). Although comparison of A:B ratios of species on leaves from the control and top dosed streams are difficult due to the high species loss in the top dosed stream, the A:B ratio increased in five out of eight species in the next from top dosed stream.

Table 31. Relative importance values of aquatic hyphomycete fungi found on sterile leaves exposed to copper in the pool section of artificial streams for 1 week. (- denotes species not detected).

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.55	299.66
Tetracladium marchalianum	0.152	0.123	0.153	0.168	0.222	0.330
Anguillospora crassa	0.097	0.089	0.106	0.086	0.045	0.043
Articulospora tetracladia	0.074	0.065	0.037	0.053	0.053	-
Anguillospora longissima	0.055	0.037	0.028	-	-	-
Tetrachaetum elegans	0.040	0.059	0.040	0.025	0.016	0.043
Heliscus lugdunensis	0.040	0.033	0.047	0.041	0.032	0.043
Scorpiosporum minutum	0.017	0.033	0.028	0.063	0.020	-
Centrospora aquatica	0.013	0.040	0.037	0.013	0.020	-
Tricladium angulatum	0.013	-	0.013	0.013	0.016	-
Clavariopsis aquatica	-	-	-	-	0.016	-
Clavatospora stellata	-	0.023	0.013	0.013	0.008	-
Tetracladium setigerum	-	-	-	0.028	0.044	0.043

Therefore, there appears to be a general shift in the aquatic hyphomycete community in response to copper contamination. The general pattern of this shift appears to be from a community consisting of many species with approximately four to eight dominant species plus a number of less important species to one with fewer total species present, dominated

by just one species. The effect of copper on sterile leaves was more marked than on conditioned leaves. The main effect of copper on the community of conditioned leaves is a loss of less important species (i.e. those with low RIVs). The community on sterile leaves shifts from one dominated by approximately four species plus approximately five less important species to, one dominated by just one species (i.e. *Tetracladium marchalianum*) and just a few less important species. The greater effect of copper on the community detected on sterile leaves indicates copper may inhibit colonisation of the leaf material.

ii. RIVs of leaves in laboratory tests.

As aquatic hyphomycetes are considered to be important in the breakdown of leaf litter, a change in the structure of the community may be associated with a change in the functioning of that community. Hence the RIVs of aquatic hyphomycetes detected on leaf material exposed to copper in the laboratory for six to seven weeks were also determined to see if the observed decrease in leaf weight loss (section 5.5. 2. b) was accompanied by a change in the aquatic hyphomycete community structure. The RIVs of aquatic hyphomycetes on conditioned and sterile control leaf material and material exposed to a nominal copper concentration of 150 µgCu/l were determined after one week and after six weeks of exposure to copper (Tables 35 to 38).

In total 17 aquatic hyphomycete species were observed on conditioned leaf material incubated in clean water (Table 35). After six weeks a diverse community of fungi was apparent consisting of ten common and four less common species (Table 36). The A:B ratio increased only in one (*Tetrachaetum elegans*) of the ten species present at week one and week six, indicating the distribution of fungi on the leaf became more regular with time (Appendix 9). However, after six weeks of exposure of conditioned leaf material to 132 µgCu/l a total of only eleven species of aquatic hyphomycetes were detected on the leaf material (Table 36). The relative importance of *Anguillospora longissima*, the

dominant species on control leaf material, was significantly reduced by exposure to copper being ranked only fourth most important on contaminated leaf material. The community structure on the leaf material is seen to have changed from one dominated by *Anguillospora longissima*, *Tetrachaetum elegans* and *Anguillospora crassa* to one dominated by *Tetracladium marchalianum*, *Tetracladium angulata* and *Tetrachaetum elegans*. As in the artificial streams an increase in the ratio of A values (i.e an index of the distribution of aquatic hyphomycete between leaves) to B values (i.e. an indication of the distribution of aquatic hyphomycetes on a leaf) (section 5.3. 3. b) indicates an increase in the patchiness of the distribution of fungi on the leaves with the A:B ratio of nine out of the ten species common to control and copper contaminated leaves increased when exposed to copper for six weeks (see Appendix 9).

Table 35. Relative importance values of aquatic hyphomycetes found on conditioned leaf material incubated in control water for 6 weeks in laboratory tests. (- denotes species not detected).

Species	Week 1	Week 6
Anguillospora longissima	0.111	0.249
Tetrachaetum elegans	0.111	0.234
Anguillospora crassa	0.111	0.226
Alatospora acuminata	0.067	0.206
Tetracladium angulata	0.067	0.188
Tetracladium marchalianum	0.067	0.159
Centrospora aquatica	0.067	0.159
Clavariopsis stellata	-	0.122
Scorpiosporum minutum	-	0.110
Clavariopsis aquatica	0.089	0.106
Lemoniera aquatica	0.067	0.085
Heliscus lugdunensis	0.111	0.044
Tetracladium setigerum	0.067	0.044
Margaritispora aquatica	-	0.044
Lunulospora curvula	-	0.022
Dactyella aquatica	0.044	-
Flabellospora sp	0.022	-

Table 36. Relative importance values of aquatic hyphomycetes found on conditioned leaf material exposed to 132 µgCu/l for 6 weeks in laboratory tests. (- denotes species not detected).

Species	Week 1	Week 6
Tetracladium marchalianum	0.071	0.514
Tetracladium angulata	0.036	0.460
Tetrachaetum elegans	0.179	0.290
Anguillospora longissima	0.143	0.214
Lemoniera aquatica	0.107	0.129
Alatospora acuminata	0.107	0.114
Anguillospora crassa	0.107	0.084
Tetracladium setigerum	-	0.068
Clavariopsis aquatica	-	0.053
Centrospora aquatica	0.036	0.037
Dactyella aquatica	-	0.037
Heliscus lugdunensis	0.071	-
Picularia sp.	0.071	-
Articullospora tetracladia	0.071	-

A total of 16 aquatic hyphomycete species were detected on sterile control leaf material (Table 37). After six weeks a diverse community was present consisting of nine common and six less common species (Table 38). After six weeks of exposure to 113 µgCu/l only twelve species of aquatic hyphomycete were detected on sterile leaf material (Table 38). Hence the colonisation of three species (*Margaritispora aquatica, Tetracladium setigerum, Heliscus lugdunensis*) was completely inhibited by exposure to copper. However, these three species were not common on control leaves. The relative importance of *Tetracladium angulata* appeared to be most affected by copper. This species was the most dominant on control leaf material but was ranked only fifth on leaf material exposed to copper. This may be the result of a weakening of the competitive ability of this species by exposure to copper. Exposure of leaf material to copper caused colonisation of seven out of twelve species to be more patchy (as indicated by an increased A:B ratio) than on control leaf material after six weeks of exposure (see Appendix 9).

A more diverse flora than was expected is seen on sterile leaf material exposed to copper, this may be due to a loss of copper from the water in the tanks between the weekly changes.

Table 37. Relative importance values of aquatic hyphomycetes found on sterile leaf material incubated in control water for 6 weeks in laboratory tests. (- denotes species not detected).

species	Week 1	Week 6
Tetracladium angulata	-	0.270
Alatospora acuminata	-	0.249
Clavariopsis aquatica	-	0.229
Anguillospora longissima	0.472	0.219
Tetracladium marchalianum	-	0.172
Tetrachaetum elegans	1.181	0.162
Clavariopsis stellata	-	0.152
Anguillospora crassa	-	0.137
Scorpiosporum minutum	-	0.117
Centrospora aquatica	-	0.086
Dactyella aquatica	-	0.064
Lemoniera aquatica	-	0.055
Heliscus lugdunensis	-	0.055
Tetracladium setigerum	-	0.025
Margaritispora aquatica	-	0.025
Articullospora tetracladia	0.347	-

Table 38. Relative importance values of aquatic hyphomycetes found on sterile leaf material exposed to 113 µg Cu/l for 6 weeks in laboratory tests. (- denotes species not detected).

species	Week 1	Week 6
Clavariopsis aquatica	-	0.357
Alatospora acuminata	-	0.263
Tetracladium marchalianum	-	0.246
Anguillospora longissima	-	0.246
Centrospora aquatica	-	0.232
Tetracladium angulata	-	0.160
Lemoniera aquatica	-	0.160
Tetrachaetum elegans	2.000	0.158
Tetracladium setigerum	-	0.044
Scorpiosporum minutum	-	0.044
Clavariopsis stellata	-	0.044
Dactyella aquatica	-	0.043

In general, the trend was for a decrease in species diversity and an increase in patchiness of aquatic hyphomycetes on leaf material exposed to copper. This was more marked for sterile than conditioned leaf material, suggesting copper inhibits initial colonisation of leaf material by aquatic hyphomycete fungi.

5.5. 4. Food preferences

There was a significant dose related preference by *G.pulex* for sterile leaves incubated in the control stream (ANOVA: $F = 13.584$, $df = 5, 141$, $p < 0.001$) (Fig. 59). Leaves incubated in the control stream were preferred to those incubated in 2.33 µg/l copper and above ($t > 2.67$, $df > 40$, $p < 0.01$).

Copper also had a significant effect on the preferences of *G.pulex* for conditioned leaves (ANOVA: $F = 2.67$, $df = 5, 142$, $p < 0.05$) however the pattern of preference was quite different (Fig. 59). There was a curvilinear relationship between preference and copper concentration such that animals exhibited a statistically significant preference for

conditioned leaves incubated in 11.55 and 25.13 µg/l copper over leaves incubated in the control stream ($t > 2.44$, $df > 40$, $p < 0.019$).

Food preferences of *G.pulex* could be driven by the copper concentration of the leaf material or a change in the microbial community as previously detected in this study (Section 5.5. 3). In order to address this the copper concentration of the leaf material offered to *G.pulex* during food preference experiments was determined. Figure 60 shows differential accumulation of copper by sterile and conditioned leaves with conditioned leaf material accumulating a significantly greater concentration of copper than non-conditioned (ANCOVA: $F = 14.18$, $df = 1, 118$, $p < 0.001$).

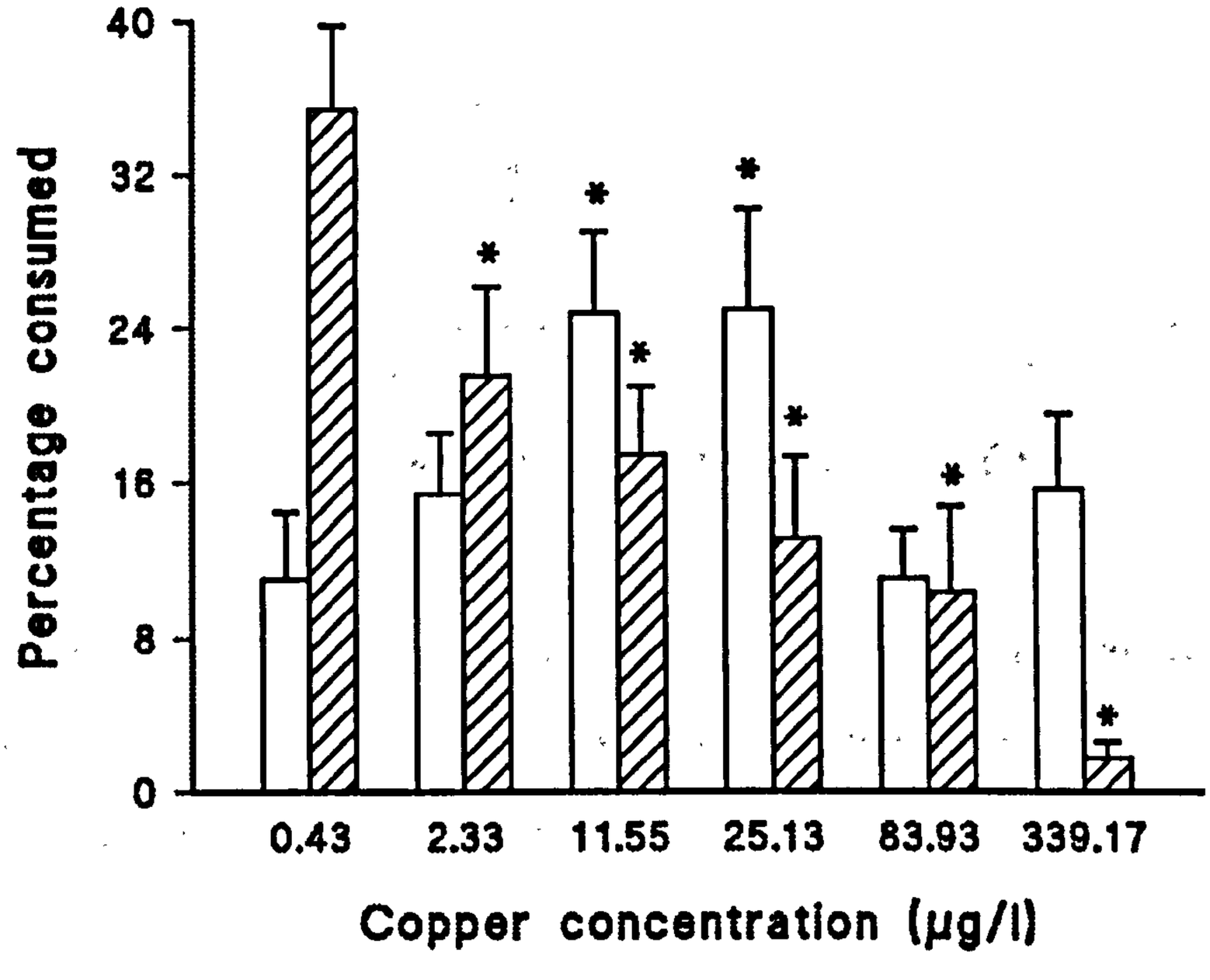


Fig. 59. Food preferences of *G.pulex* for conditioned (open bars) and sterile (hatched bars) leaf material exposed to copper in artificial streams. Error bars represent 1 standard error, * denotes data significantly different from the control.

There was no significant difference between the amount of copper accumulated by conditioned or sterile leaves at copper concentrations of 11.55 µg/l and below ($t < 0.93$, $df > 11$, $p > 0.05$). However, at concentrations of 25.13 µgCu/l and above the amount of copper accumulated by conditioned leaves was significantly greater than that accumulated by sterile leaves ($t > 2.2$, $df > 11$, $p < 0.05$) (Fig. 60).

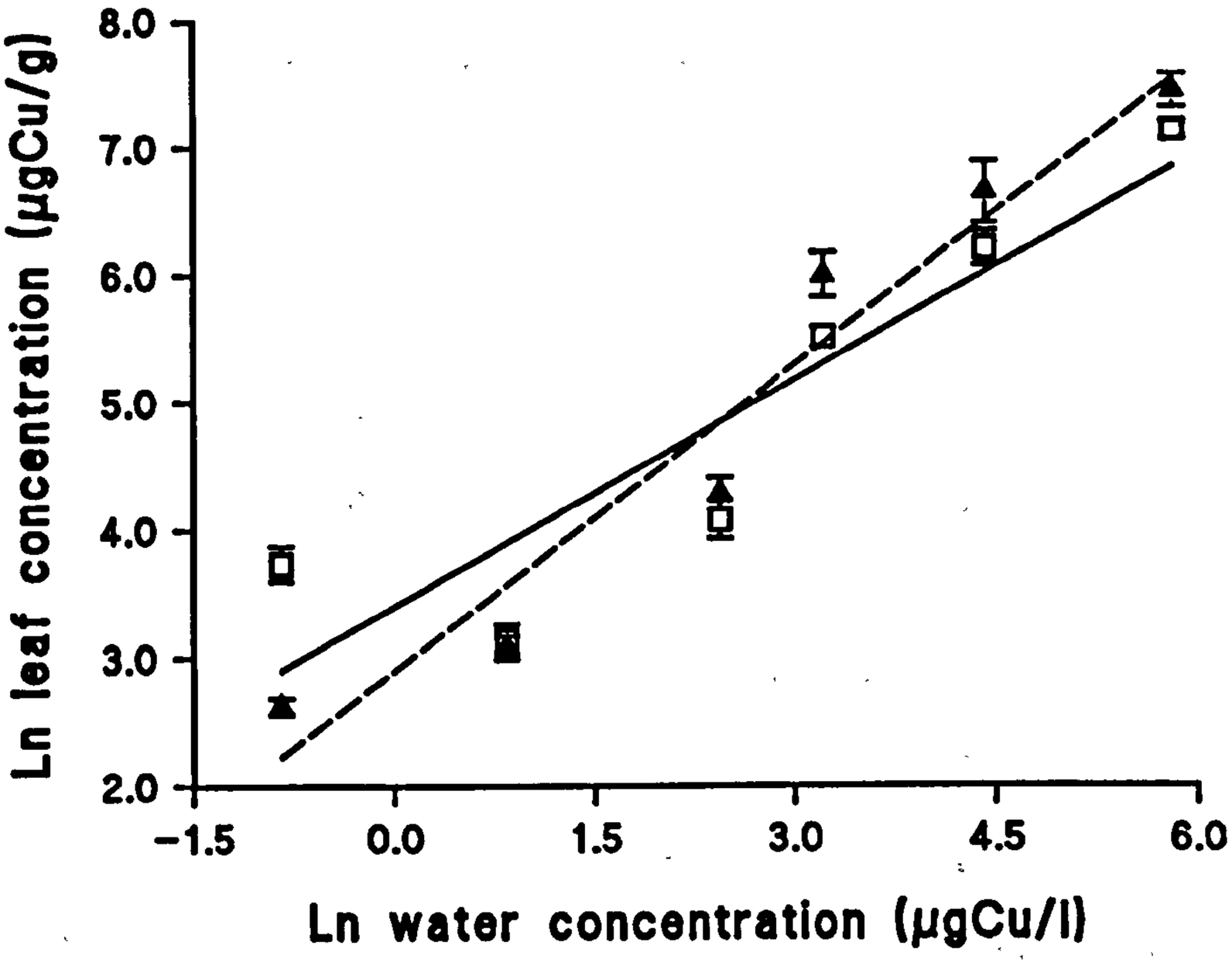


Fig. 60. Copper concentration of conditioned (solid triangles; dashed line) and sterile (open squares; solid line) leaf material offered to *G.pulex* in food choice experiments. Error bars represent 1 standard error.

Conditioned leaves therefore accumulated copper to a greater degree than did sterile leaves. This enhanced copper uptake is likely to be due to the greater microflora of conditioned leaf material. Animals did not reject conditioned leaf material which had accumulated the highest concentrations of copper but sterile leaf material was rejected. Hence food preferences of *G.pulex* appear to be driven by the extent of fungal

colonisation (Section 5.5. 3) rather than the copper concentration of leaf material (see also laboratory food preference experiments: Chapter 2, Section 2.5. 7).

5.5. 5. Toxicity of copper to fungi.

Tetracladium marchalianum and *Anguillospora longissima* were exposed to copper concentrations of 0, 6.3, 9.0, 25.3, 46.8, 175 µg/l and effects on growth rate, biomass production, sporulation and germination were determined using pure culture techniques.

a. Growth.

Growth rates of both *Tetracladium marchalianum* and *Anguillospora longissima* conformed to a linear model at all concentrations ($r^2 > 98.06\%$, df = 8, $p < 0.001$). Growth rates of *T.marchalianum* ranged from 1.75 to 1.84 mm/d but they were not significantly affected by copper concentration (Ancova: $F = 1.67$, df = 5, 535, $p > 0.05$) (Fig 61). The growth rate of *A.longissima* ranged between 1.73 and 1.85 mm/d and in contrast to *T.marchalianum*, growth rates were enhanced at copper concentrations of 9.0 and 46.8 µg/l (Ancova: $F > 4.8$, df = 1, 176, $p < 0.05$) although there were no significant effects at 6.3, 25.3 or 175.0 µgCu/l (Ancova: $F > 2.76$, df = 1, 176, $p > 0.05$). Hence growth rate of *A.longissima* did not vary in a dose response manner (Fig 61).

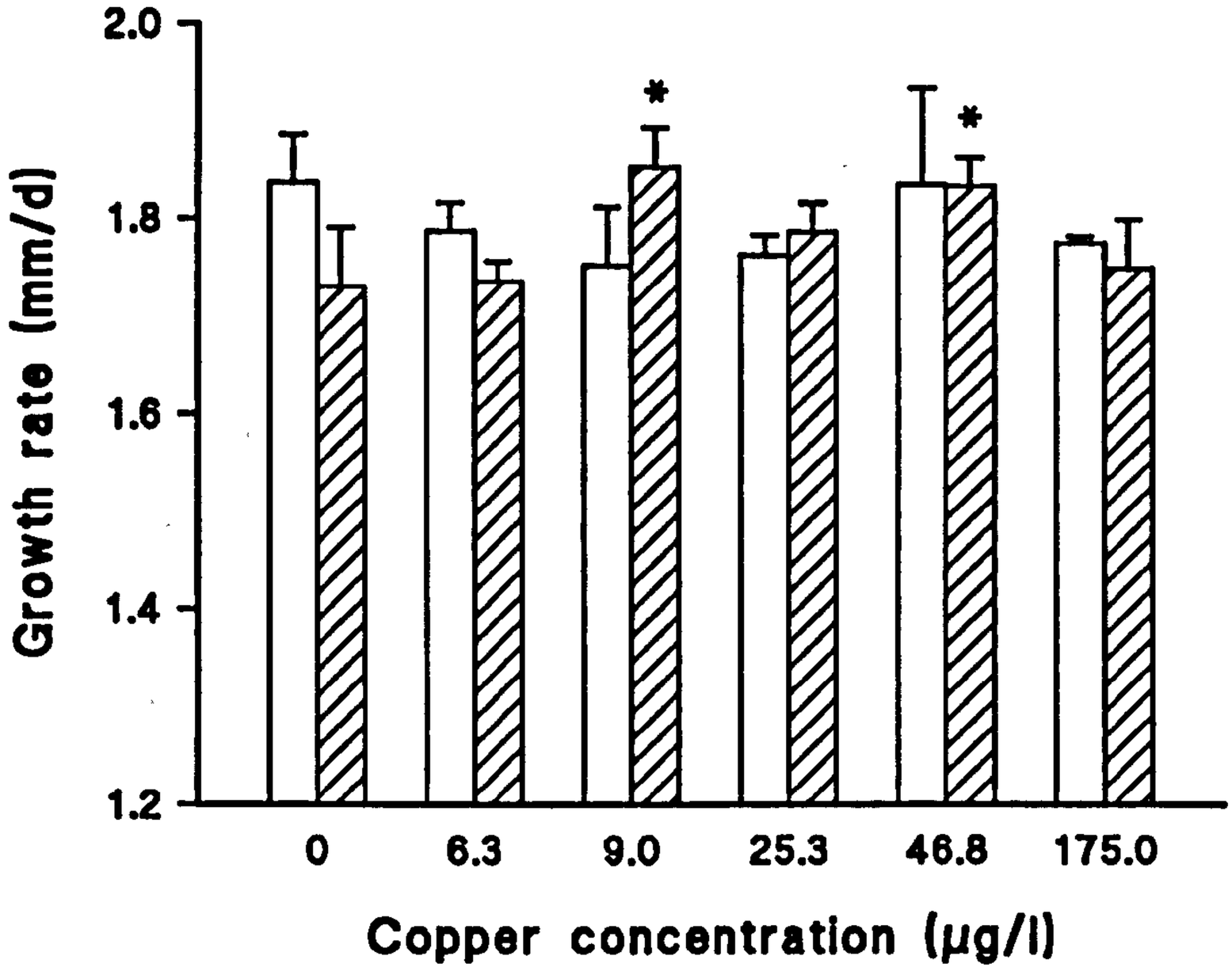


Fig. 61. Mean growth rates (mm/d) of *T.marchalianum* (open bars) and *A.longissima* (hatched bars) exposed to copper. Error bars represent 1 standard error, * denotes data significantly different from the control.

b. Biomass.

Despite similar linear growth rates *T.marchalianum* produced a significantly greater amount of biomass than *A.longissima* at all copper concentrations (Fig. 62) ($t > 2.65$, $df > 10$, $p < 0.05$). There was no significant effect of copper on the amount of biomass produced by *A.longissima* (Fig. 62) ($F = 2.215$, $df = 5, 58$, $p > 0.05$). Biomass production by *T.marchalianum* was not significantly different from the control at any copper concentration ($t < 1.95$, $df = 18$, $p > 0.05$) although mycelia exposed to 25.3 µgCu/l produced significantly more biomass than that exposed to 6.3 or 9.0 µgCu/l ($t > 61$, $df = 18$, $p < 0.05$).

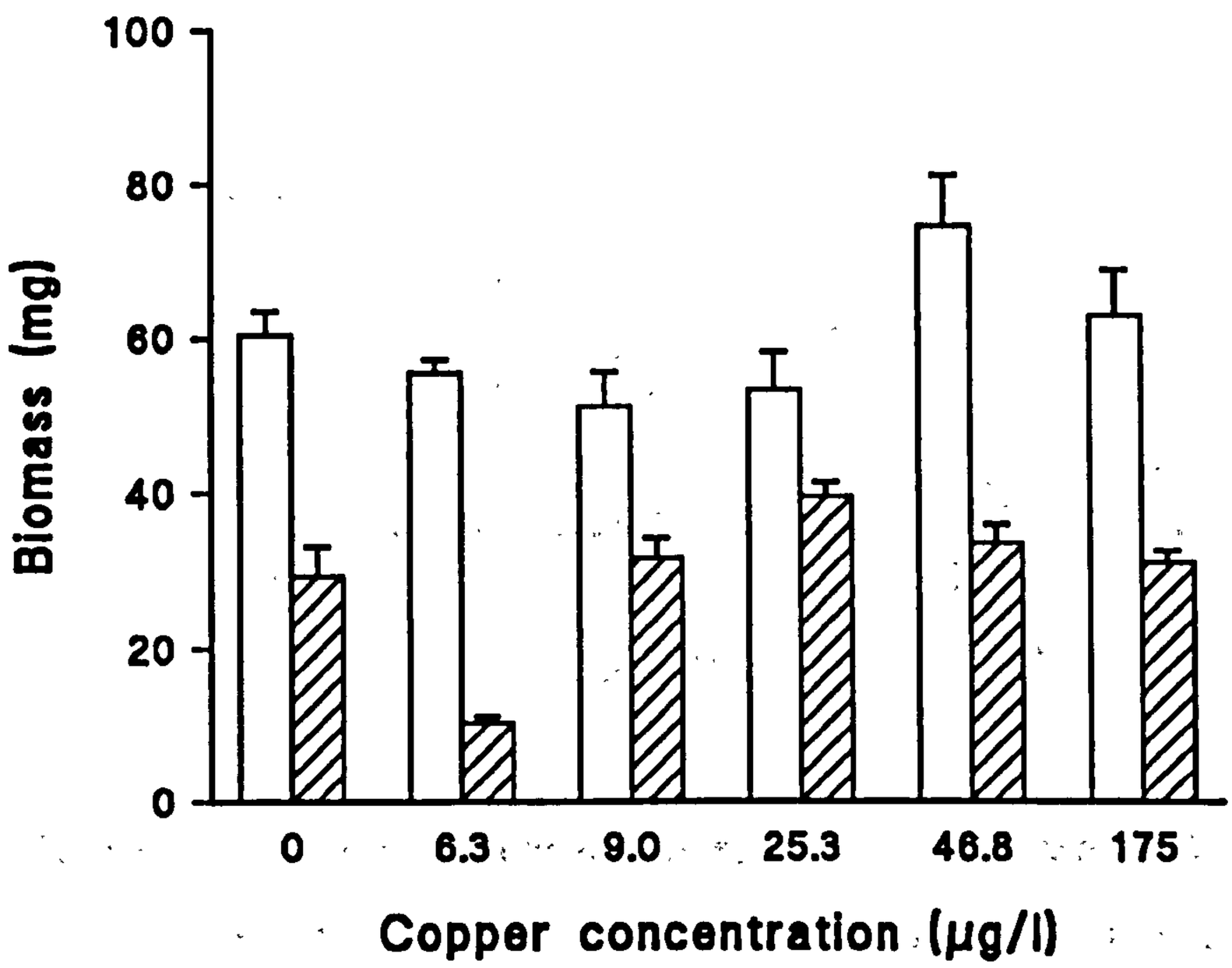


Fig. 62. Biomass production of *T.marchalianum* (open bars) and *A.longissima* (hatched bars) exposed to copper. Error bars represent 1 standard error.

c. Sporulation.

There was no significant effect of copper on the sporulation of *T.marchalianum* (Fig 64) ($H = 8.8$, $df = 5, 34$, $p > 0.05$). However. there was a significant effect of copper on the sporulation of *A.longissima* ($H = 14.66$, $df = 5, 34$, $p < 0.05$) with a significant reduction in the number of spores produced, compared to the control, when exposed to the top copper concentration of 175 µgCu/l ($W = 25.5$, $df = 4$, $p < 0.05$) (Fig. 63).

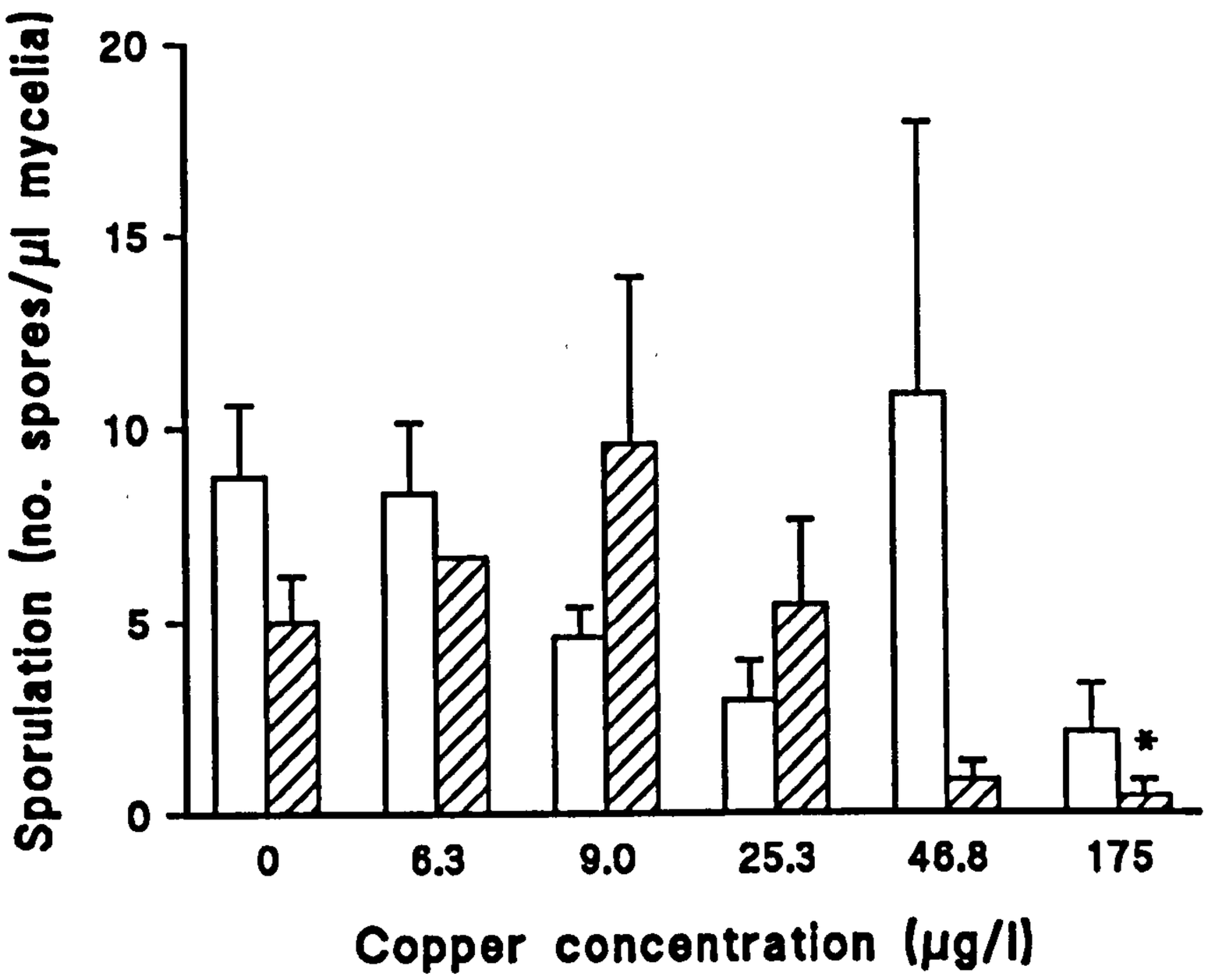


Fig. 63. Effects of exposure to copper on the sporulation of *T.marchalianum* (open bars) and *A.longissima* (hatched bars). Error bars represent 1 standard error, * denotes data significantly different from the control.

d. Germination.

There was no significant effect of prior exposure to copper on the percentage spore germination of *T.marchalianum* (Fig. 64) ($H = 3.68$, $df = 5, 34$ $p > 0.05$). However, copper caused a significant reduction in percentage germination of *A.longissima* spores ($H = 21.27$, $df = 5, 34$, $p < 0.001$) with a significant reduction observed at concentrations of 25.3 µg/l and over ($W > 26$, $df = 4$, $p < 0.05$) (Fig. 64).

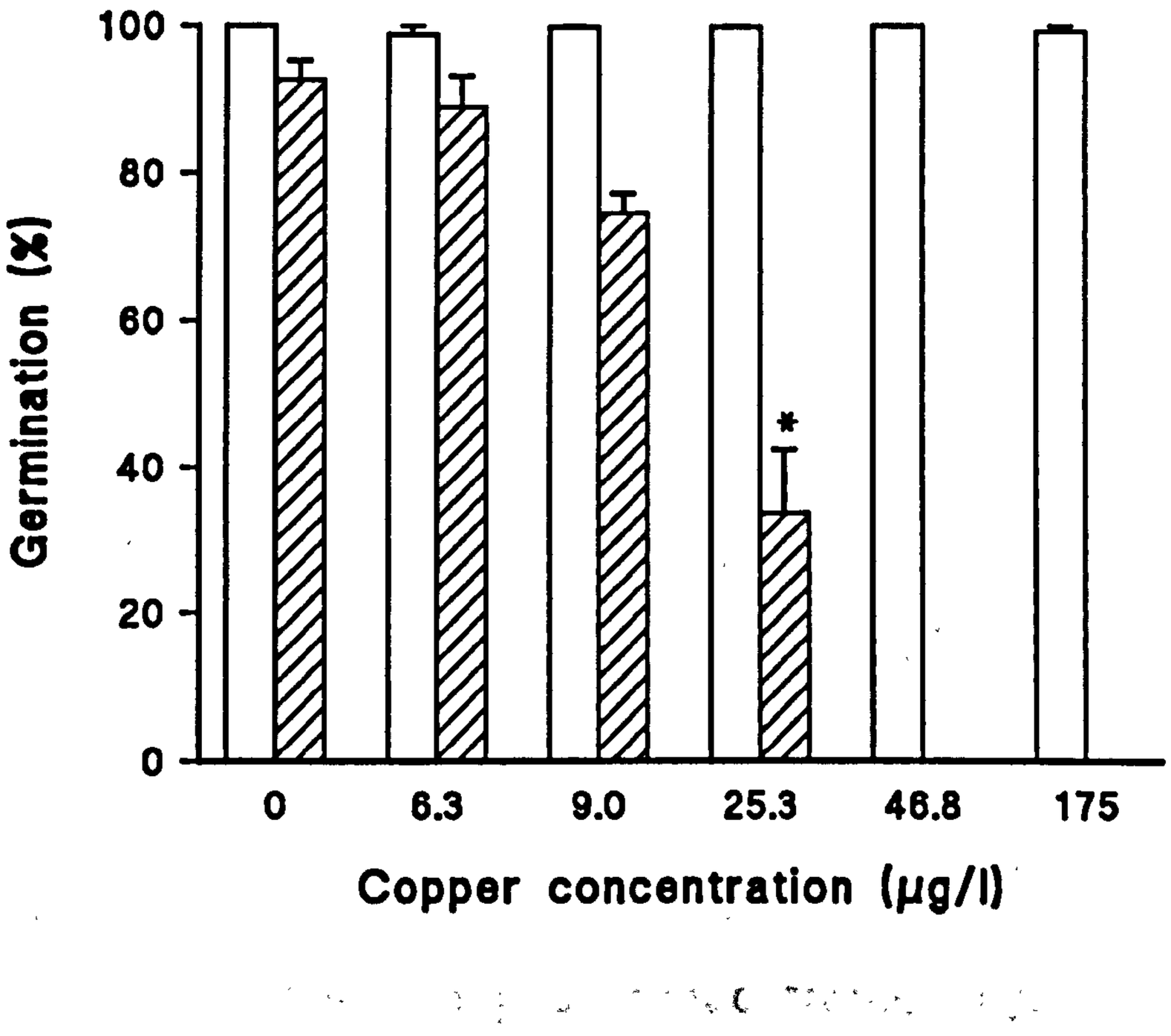


Fig. 64. Effect of exposure to copper on the germination of spores of *T.marchalianum* (open bars) and *A.longissima* (hatched bars). Error bars represent 1 standard error. * denotes data significantly different from the control.

d. Fungal copper accumulation.

Two-way analysis of variance indicated that media copper concentration had a significant effect on the accumulation of copper by both fungal species ($F = 8.825$, $df = 5, 35$, $p < 0.001$), the pattern and magnitude of which was not significantly different (Fig. 65) ($F = 1.81$, $df > 1, 35$, $p > 0.05$). A significant increase in the accumulation of copper over control levels was detected in both species when exposed to 175 µgCu/l ($t > 4.11$, $df = 2$, $p < 0.05$).

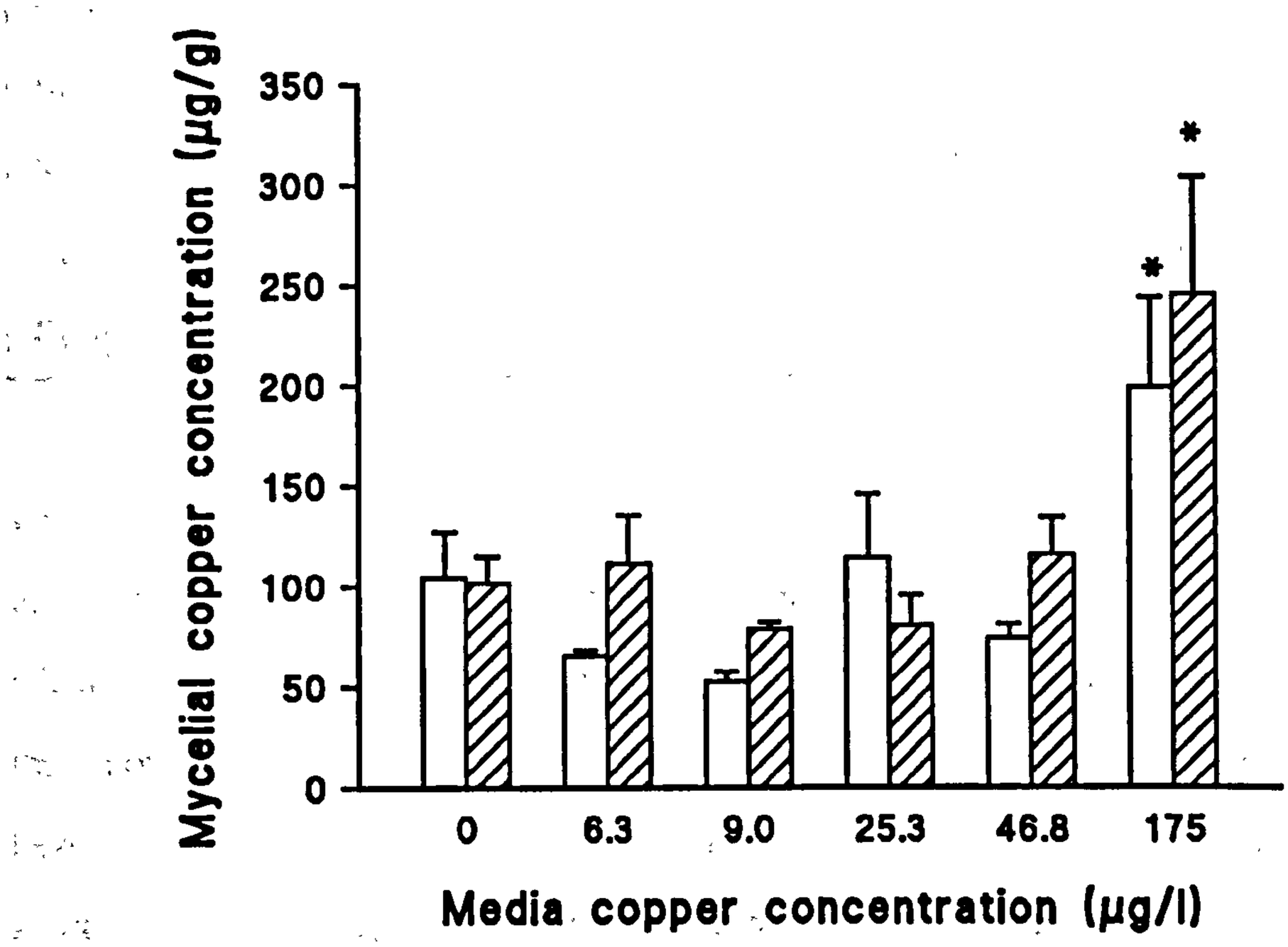


Fig. 65. Accumulation of copper in the mycelia of *T.marchalianum* (open bars) and *A.longissima* (hatched bars) exposed to copper. Error bars represent 1 standard error, * denotes data significantly different from the control.

While the accumulation of copper by the two species was very similar their physiological responses were quite different. *T.marchalianum* was largely insensitive to copper

contamination, while germination, and to a lesser extent sporulation, of *A.longissima* was significantly inhibited by copper (Table 39).

Table 39 Summary of effects of copper on the physiology of *T.marchalianum* and *A.longissima*.

Parameter	*T.marchalianum*	*A.longissima*	Copper concentration
Linear growth rate	NS	NS	/
Biomass	NS	NS	/
Sporulation	NS	$p < 0.01$	175.0 µgCu/l
Germination	NS	$p < 0.001$	25.3 µgCu/l
Copper accumulation	$p < 0.05$,	$p < 0.05$	175.0 µgCu/l

T.marchalianum appeared to be relatively insensitive to copper contamination with none of the physiological parameters measured significantly affected despite copper being taken up by the mycelia. In contrast, although there was no significant difference between the amount of copper taken up by *A.longissima* relative to *T.marchalianum*, copper did have a significant effect on its physiology. Germination, and to a lesser extent sporulation, in *A.longissima* were significantly decreased indicating that this species would be unable to colonise leaf material when exposed to copper.

5.6 Discussion.

Mean leaf weight losses due to leaching of alder leaves (23.47 percent) compare well with values of between 12 and 33 percent reported by other workers (Nykvist, 1959; Gray and Ward, 1983; Webster and Benfield, 1986; Gessner and Schwoerbel, 1989). Leaching losses of alder leaves are generally quite high indicating a large amount of soluble

material in the leaf. However the drying of leaf material prior to use in experiments may enhance mass loss due to leaching. The drying of leaf material before experimental leaf decomposition investigations has been criticized by some workers, as drying may fracture membranes and alter the cuticle causing increased leaching rates and rendering the leaf more susceptible to attack by microorganisms and invertebrates (Stout et al, 1985; Gessner and Schoerbel, 1989; Boulton and Boon, 1991; Gessner, 1991). These workers argue that fresh leaves exhibit a more constant rate of processing and do not exhibit the initial high mass loss due to leaching which is observed in dried leaves (Stout et al, 1985; Gessner, 1991). However, Barlocher (1991) found no effect of drying on surface morphology of leaves when air dried leaves were examined using SEM. Further, terrestrially derived leaf litter is likely to dry naturally before entering the streams (Boulton and Boon, 1991) and the use of dried litter is convenient as a tool for assessing the comparative effects of a pollutant (Gessner, 1991).

Leaf bags were used in this study in order to determine leaf weight losses primarily due to the action of microbes. However, the use of leaf bags in the study of litter breakdown does have some limitations including:

1. loss of FPOM through the mesh resulting in an overestimate of leaf decomposition.
2. accumulation of sediment in and around bags causing a reduction in exchange rates possibly resulting in anaerobic conditions and an underestimate of microbial processing.
3. difficulties in washing off accumulated sediment and in standardising washing procedures. Washing also results in a loss of FPOM.

(Whitkamp and Olson, 1963; Petersen and Cummins, 1974; Boulton and Boon, 1991).

The small mesh size of bags used in this study (i.e 350 μm) would have limited the loss of FPOM. Despite the small mesh size little sediment appeared to collect around the bags and hence reduced processing due to anaerobic conditions was unlikely. While some FPOM was undoubtedly lost during washing, the standard washing procedure resulted in any fine sediment which did accumulate being removed.

Processing rates of conditioned leaves in laboratory tests were somewhat greater than processing rates of alder leaves reported by other workers. The processing rates of sterile leaves were similar to lower estimates of other workers (Table 40).

Many factors influence leaf processing and the high -k values for conditioned leaves found in this study may be due to higher temperatures than those of other studies and the short period of time over which decomposition was studied. The low -k values of non-conditioned leaf material may be due the low levels of inocula that would be received in these tests compared to that which would be available in natural stream water.

Copper had no effect on the microbially mediated weight loss of leaves in the one week artificial stream tests. This could have been due to effects of copper being undetectable over this time period or to effects not being present. This was examined by investigating effects of copper on microbially mediated weight loss of leaves over a six to seven week period in the laboratory. These experiments illustrated that copper did indeed cause a decrease in the microbial processing of leaf litter. Copper induced decrease in leaf weight loss was most apparent with sterile leaf material. Processing of sterile and conditioned leaf material was significantly reduced after 14 days exposure to 113 and 132 µgCu/l respectively and processing of sterile leaf material was also significantly reduced after 28 days of exposure to 67 µgCu/l. These levels are far higher than those observed by Leland and Carter (1985) who detected effects on the microbial community at copper concentrations of 2.5 µg/l and over. However, they used different response criteria i.e. microbial respiration and N:C ratios, effects were observed over a longer time period (i.e. 90 days) and water temperature was 5 to 10^{o}C higher than in this study which may have accounted for some of the differences. Reduction in micobially mediated leaf weight loss due to the effects of various pollutants has also been demonstrated by various workers (Geisy and Drawer, 1978; Carpenter et al, 1983; Leland and Carter, 1985; Charmier, 1987; Palumbo et al, 1987)

Table 40. Reported leaf processing rates (-k) of alder leaves. (cond and sterile refers to conditioned and sterile leaves respectively).

Processing	treatment	-k	ref
Combined	none	0.011	1
Combined	none	0.0147	2
Combined	Acid mine	0.0032	2
Combined	none	0.0269	3
Combined	none	0.0417	3
Microbial	none	0.004	1
Microbial	none	0.0166	3
Microbial	none	0.0213	3
Microbial	none (dry)	0.0146	4
Microbial	none (fresh)	0.0137	4
Microbial	pH 6.8	0.0164-0.0119	5
Microbial	pH 5.5 - 4.9	0.00311-0.0029	5
Microbial	control (cond)	0.0507	6
Microbial	65 µgCu/l(cond)	0.0552	6
Microbial	132 µgCu/l(cond)	0.0223	6
Microbial	control (sterile)	0.0054	6
Microbial	67 µgCu/l(sterile)	0.0035	6
Microbial	132 µgCu/l(sterile)	0.0032	6

1. Chergui and Pattee 1990, 2. Gray and Ward 1983, 3. Barnes et al, 1986, 4. Barlocher 1991, 5. Charmier 1987, 6. This study.

Effects of copper on the weight loss of leaf material caused by the combined effects of microbes, invertebrates and physical factors were detected at lower concentrations and within a much shorter time period than effects of microbes alone. Leaf weight loss was significantly reduced after 27 hours exposure to 25 µgCu/l in the riffle section and 309 µgCu/l in the pool section. The major role of leaf shredding invertebrates in this process was indicated by the significant correlation between invertebrate abundance on leaf material and leaf weight (Fig. 51). A reduction in leaf processing due to the effects of various pollutants on the stream macroinvertebrates has also been demonstrated by other workers (Stout and Cooper, 1983; Burton et al, 1985; Wallace et al, 1986; Newman et al, 1987; Stout and Coburn, 1989). For example, Newman et al (1987) examined the effects of chloride on litter breakdown in outdoor artificial streams and found breakdown was reduced at high chloride concentrations primarily due to a reduction in the colonisation of leaf litter by amphipod shredders.

Given that leaf weight losses in the artificial streams appear to be primarily the result of *Gammarus* feeding, then effects of copper on the processing of leaf litter should be predictable from a knowledge of the effects of copper on *Gammarus* consumption rates and population density. In the previous chapter (Chapter 4) effects of copper on these parameters were addressed, and a summary of results are presented in Table 41. Results show very good agreement between effects of copper on the consumption rate and population density of *G.pulex* and effects on leaf weight loss in the artificial streams (Table 41). The concentration of copper at which leaf weight loss in the riffle section of the artificial streams was affected is accurately predicted from effects on consumption rates and population density. In the pool section of the artificial streams the effects of copper on leaf weight loss was not as great as expected. However predictions based on consumption rates and population density would be conservative of effects on leaf weight loss in pool sections.

Table 41: Comparative effects of copper (µg/l) on consumption rates and population density of *G.pulex* and of leaf processing rates in the artificial streams.

Parameter	Time period	LOEC (µg/l)
Consumption rates	6 days	23.83
Population density (Riffle)	14 days	20.47
Population density (Riffle)	28 days	20.47
Population density (Pool)	28 days	48.36
Leaf weight loss (Pool)	27 hours	309.0
Leaf weight loss (Riffle)	27 hours	25.0

Gray and Ward (1983) observed a decrease in leaf processing as a result of extensive coatings of ferric hydroxide from acid mine drainage inhibiting microbial colonisation and shredder feeding. Boyne et al (1982) found a decrease in shredder density downstream of the same mine investigated by Gray and Ward (1983) but heavy metal concentrations were well below toxic levels for most species of shredder. Hence the decrease in leaf processing observed was due to the ferric hydroxide inhibiting microbial colonisation, hence decreasing food quality and restricting shredders from the site. The possibility that copper could also affect consumption rates of *G.pulex* indirectly due to changes in food quality was investigated via food preference experiments (Fig 60).

In this study, no preference for control leaves over contaminated leaves was shown for preconditioned leaves, but a strong dose response relationship was observed when animals were offered sterile leaves, animals preferring leaves incubated in the control stream over those exposed to concentrations as low as 2.33 µg/l copper and over.

These food preferences may be due to a number of factors including detection of high levels of copper in the leaf material and / or detection of copper-induced changes in the microbial community.

Data presented in Figure 60 show that leaf material accumulated high concentrations of copper whilst exposed in the artificial streams, and that preconditioned leaves accumulated copper to a greater extent than did sterile leaves. This was probably due to the accumulation of copper by the greater cover of microorganisms. If differences in the form and availability of copper occurred between conditioned or sterile leaves, preferences of the animals could be based on a detection of such. However, total copper concentration of the leaf material could not explain the observed food preferences. This was in agreement with laboratory tests indicating *G.pulex* was unable to select non-contaminated food on the basis of total copper concentration (Chapter 2, section 2.5. 7).

The preference for sterile control leaves over leaves exposed to copper was therefore likely to be due to the greater microbial conditioning of control leaves. A change in the structure of the fungal community due to the effects of copper on leaf material deployed in the artificial streams was evident. The change in the structure of the microbial community appeared to be more sensitive to copper than a change in its functioning as a reduction in leaf processing was detected after the change in fungal community structure was observed. This was probably due to a masking of effects of copper on microbial leaf processing due to large mass losses caused by leaching and the requirement of the establishment of a microbial community on leaf material before processing of leaf material by the action of microbial enzymes occurs.

The RIVs of fungi found on leaf material in both laboratory and artificial stream tests indicated a community dominated by four to six species of aquatic hyphomycete plus a number of rarer species. This pattern of distribution is similar to that observed by other workers (Charmier and Dixon, 1982a; Barlocher, 1982a, 1985; Shearer and Lane, 1983;

Shearer and Webster, 1985). In general, the dominant species arrive early and persist through the substrate's lifetime; later colonists have lower quantities of nutritionally inferior substrate available. Barlocher (1982a), found that sterile leaves inoculated with a single fungal species and then exposed in streams were remarkably resistant to invasion by other fungi for up to 6 months. Fungal succession is rare due to the short lifetime of the substrate (Barlocher, 1982a; Charmier and Dixon, 1982). Hence the community observed in this study was probably representative of the community which would have persisted throughout the leafs decay.

The fewer species observed on sterile leaf material could have been due to the shorter incubation time. Conditioned leaves had an extra week in the supply stream in addition to the week of deployment in the artificial streams. Charmier and Dixon (1982), found time to peak colonisation was dependent on the level of inoculum in the water and on the extent of colonisation. However, the mean number of species on leaf material after one week in the supply stream was 11.3 (SE = 0.75) hence the lower species numbers on sterile leaf material must have been caused by some other factor (e.g. lower inocula in the water).

The RIVs of the dominant fungi on conditioned leaves increased with increasing copper concentration at the expense of a number of less common species. Less common species are often later colonists as mentioned above and hence it would appear that while copper may not affect fungi which have already colonised leaf material it does inhibit colonisation by new species. This conclusion is further substantiated by the dramatic effects of copper on the structure of the aquatic hyphomycete community on sterile leaves. *Tetracladium marchalianum* appears to be the only species able to colonise copper contaminated leaves to any great degree and hence increase its relative importance when exposed to copper at the expense of other less tolerant species.

An increase in patchiness of the distribution of fungi on a leaf is also indicated by the changing ratio of A (indicating distribution of fungi between leaves) to B (indication of distribution of fungi on a leaf) values observed in the RIV data (Appendix 8). At low copper concentrations A and B values are similar but as copper concentration increases A values remain relatively high while B values become relatively low indicating an increase in the patchiness of the distribution of fungi on a leaf (see appendix 8).

The scanning electron photo-micrographs clearly showed copper caused a decrease in the colonisation of leaf material by fungi and may even limit the fungal biomass on conditioned leaves. Copper was found to severely limit colonisation of sterile leaf material by fungi at concentrations of at least 83.98 µgCu/l and above (Table 31).

Sterile leaf material exposed to concentrations of copper causing an inhibition of fungal colonisation was found to be densely covered with bacteria. This may either have been due to bacteria present becoming visible due to fungal hyphae not masking them or due to an invasion of bacteria on the leaf material that would otherwise have been inhibited by fungal growth. Other workers have previously found that the development of other microbial elements was rapidly suppressed by fungal hyphal development, (Kaushik and Hynes, 1968, Harley, 1971). Therefore, it would appear probable that bacteria have been able to invade and colonise the leaf material due to the inhibition of the fungal element of the microflora by copper.

Despite various problems and sources of error involving RIV and SEM techniques used in the study of the effects of copper on the structure of the microbial community (Appendix 10), it is evident that copper causes a decrease in the cover and species diversity of aquatic hyphomycetes.

The difference in response to copper of two aquatic hyphomycete species, *T.marchalianum* (relatively tolerant species) and *A.longissima* (relatively sensitive

species) gave some insight into the mode of toxic action of copper on the fungal community. However responses of these two species may not be representative of all other species in the community and different degrees of sensitivity and modes of action may occur in other species.

The lack of response of the growth rate and biomass production of the two species was expected due to the lack of apparent effect of copper on conditioned leaf material. However these laboratory tests should be viewed with some caution due to various experimental factors which may affect the observed results. For example, the medium on which the fungi are grown may affect the bioavailability of copper, as many compounds are known to complex with copper. Cas-amino acids were added to the media as a nitrogen source but are known to bind metals, and glucose was added as a carbon source which may also effect the availability. Further, as copper is taken up from the media by the fungi the copper concentration in the media will decrease through time. Hence the fungus is exposed to an ever decreasing concentration of copper in the media with time resulting in conservative estimates of toxicity.

The parameters measured may also influence the relevance of observed results. Radial growth accounts only for the linear growth rate of a fungus across a plate taking no account of growth in height or depth in the media hence is not necessarily a good measure of the amount of fungus present (Ainsworth and Sussman, 1965). As aquatic hyphomycetes are able to sporulate when submerged (Webster 1959) the measure of fungal biomass includes any spores produced as well as any secondary colonies arising from those spores. This may explain the high variability in the biomass data.

A reduction in the sporulation of *A.longissima* was observed when hyphae had been exposed to copper concentrations above 46.8 µgCu/l but this was only significant at 175 µgCu/l. *T.marchalianum* was unaffected at any concentration. Abel and Barlocher (1984),

studied the effects of cadmium on the growth and sporulation of five aquatic hyphomycete species and also found sporulation to be more sensitive than growth rates.

The differential effects of copper on the germination of *T.marchalianum* and *A.longissima* were most striking. Germination of *A.longissima* was significantly reduced when hyphae had been exposed to copper concentrations of 25.3 µg/l and over while germination of *T.marchalianum* was unaffected at any concentration. The mechanism of toxic action of copper on *A.longissima* is therefore an inhibition of germination and to a lesser degree an inhibition of sporulation.

The effects of copper primarily on the germination of *A.longissima* are in agreement with the action of copper observed on other fungi by numerous other workers (M^{c}Callan, 1949; Ruhling and Tyler, 1973; Ross 1975). Copper has been known for its fungicidal properties for over a century (Ruhling and Tyler, 1973) and it has long been used as a fungicide (e.g. Bordeaux mixture). Copper is however essential for fungal growth and is chiefly associated with enzymes; it being a common component of metalloenzymes (Ross, 1975).

T.marchalianum appeared remarkably resistant to copper. Copper resistance and even stimulation of growth by copper has been found in several other fungal species. Kendrick (1962) found several species of soil fungi in a copper swamp were found exclusively in samples with high copper concentrations while other species were unaffected by high or low copper concentrations. These adaptations to copper were thought to have occurred over about fifty years.

Fungal resistance to copper could result from various mechanisms. Resistant could be due to a physiological response, e.g. precipitation of copper by sulphide (Ashida et al 1963), or oxalate which is produced by some fungi as a self inhibitor and hence complexation with copper can even stimulate growth (Englander and Corden, 1971; Murphy and Levy,

1983). Alternatively, resistance could be a result of localisation within the cell (Williams and Pugh, 1975) or a decreased uptake or permeability (Gadd and Griffiths 1980).

It is unlikely that the resistance of *T.marchalianum* to copper was due to a reduction in uptake or permeability as the amount of copper accumulated by *T.marchalianum* was significantly increased at 175 µg/l while no effect on its physiology was observed and the pattern of accumulation of copper was very similar in both species despite the differences in sensitivity. Hence the mechanism of toxic action at the cellular level would require additional information from transmission electron microscopy, energy dispersive x-ray analysis, or biochemical techniques to determine the location of copper in the cell or the production of chemical providing protection.

The observed preferences of *G.pulex* for sterile leaves from the control stream are therefore likely due to the effects of copper on the degree of conditioning, species composition of the fungi and microbial modification of the leaf material. Further, effects of copper on the microbial flora of leaf material may be elicited at concentrations far below those detected in laboratory techniques as *G.pulex* consumed leaves from the control stream in preference even to leaves exposed to copper concentrations as low as 2.33 µg/l. It may be that this concentration of copper selectively inhibited colonisation of, or elicited very subtle changes in activity of particular important fungi. No rejection of preconditioned leaves exposed to various copper concentrations was shown by *G.pulex* as all the leaves may have received an acceptable inoculum of fungi prior to exposure to copper and hence did not invoke any behavioural discrimination, by *G.pulex*, between leaves.

The effects of fungi on the food preferences of detritivorous invertebrates have been widely studied by other workers. Conditioned leaf material is always preferred to sterile (Hargrave, 1970; Kaushik and Hynes, 1971; Kostalos and Seymour, 1976; Willoughby and Sutcliffe, 1976; Sutcliffe et al, 1981; Fano et al, 1982). Invertebrates have also been

found to prefer particular leaf types to others (McDiffett, 1970; Wallace et al, 1970; Kaushik and Hynes, 1971) and, although certain leaves e.g. Alder, have relatively high nitrogen or protein content and can be palatable without prior conditioning, (Iversen, 1974; Anderson and Graffius, 1975), most differences are undoubtedly mediated through the microflora of the leaves (Cummins et al, 1973).

Barlocher and Kendrick (1982) were able to reverse the leaf choice of two caddis species by inoculating the leaves with different species of fungi. Different fungal species also elicit a preferential feeding response and survival and growth of animals fed pure fungal mycelium range from very high to very low or may even cause death (Cummins et al, 1973; Willoughby and Sutcliffe, 1976; Suberkropp et al, 1983).

The idea that feeding by invertebrates is a function of microbial colonisation is supported by the existence of a lag phase between leaves entering the water and invertebrate colonisation (Petersen and Cummins, 1974). For a given leaf-fungus combination, optimal palatability follows the period of optimal fungal growth, hence conditioning time can profoundly effect palatablilty (Arsuffi and Suberkropp, 1984; Suberkropp and Arsuffi, 1984).

It has generally been found that more palatable, conditioned leaves are generally; used more efficiently, assimilated to a higher degree (Barlocher, 1982b; Grafius and Anderson, 1980; Nilsson, 1974), allow better growth (Anderson and Graffius, 1975; Rossi and Fano, 1979; Sutcliffe et al, 1981; Willoughby and Sutcliffe, 1976) and better survival (Kostalos and Seymour, 1976; Rossi and Fano, 1979; Rossi et al, 1983) of invertebrates.

The increased palatability of leaf material to invertebrates caused by micro-organisms may be due to two important mechanisms (Barlocher, 1982b):

1. Microbial production: Enrichment of the leaf substrate with microbial cells and secretions. Barlocher (1985) found microbially enriched food contained two to four times as much nutrient per unit weight as the average unconditioned leaf.

2. Microbial catalysis: Breakdown of leaf material into subunits which are more easily digestible by invertebrates.

1. Microbial Production.

Arsuffi and Suberkropp, (1988) found that the fungal mycelia on leaf material played a greater role in food selection and was a better food source to invertebrates than a combination of extracellular enzymes, modified leaf material and associated mycelia. Iversen (1974) found preferences and growth rates of a trichopteran were positively correlated with nitrogen content of the conditioned leaves which closely follows microbial biomass. However, based in part on the low estimates of fungal biomass associated with leaves (2 to 10 percent), several workers have concluded that microbial production can only support a small proportion of the growth and energy requirements of detritivores (Iversen, 1974; Nilsson, 1974; Cummins and Klug, 1979; Barlocher and Kendrick, 1981; Lawson et al, 1984; Findley et al, 1986 a, b). The determination of microbial biomass is, however, hampered by poor methodology and estimates vary depending on techniques used. Further, Barlocher and Kendrick (1975) found that assimilation of fungal mycelium by *Gammarus pseodolimnaeus* was considerably higher than on leaf substrates and therefore the contribution of fungi to the diet may be considerably greater than that suggested by estimates of fungal biomass.

2. Microbial Catalysis.

Evidence for the role of microbial catalysis in food preferences of detritivores comes from work of Barlocher and Kendrick (1975) who offered *G.pseudolimnaeus* the choice between: untreated leaf discs and leaf discs exposed to fungal secretions and excretions and between fungal discs and filter paper exposed to a hydrolysing agent (hot HCl). They

found that changes in the leaf substrate caused by exposure to fungal secretions and excretions or hydrolysing agent caused an increase in the leaf palatability. Therefore, the leaf substrate itself can become more palatable as a result of microbial attack.

The increase in leaf palatability due to microbial catalysis may be due to partial decomposition of the leaf material into subunits digestible by the invertebrates own enzymes and / or microbial exoenzymes remaining active when ingested and continuing to breakdown leaf components in the invertebrates gut (Barlocher, 1982b).

Arsuffi and Suberkropp (1984) characterized various aspects of the fungal modification of leaf substrate including weight loss, degree of softening, ATP, nitrogen, protease activity and the activity of ten polysaccharide degrading enzymes and examined their effect on the palatability of leaf material to two species of caddis. However they were unable to determine any relationship between the measured parameters and detritivore preferences.

A number of studies have investigated the digestive attributes (e.g. gut size and morphology, gut microflora, pH, digestive enzymes etc) of representatives of the major groups of invertebrate detritovores (Cummins and Klug, 1979; Monk, 1976, 1977; Martin et al, 1980; Martin, Martin and Kukor, 1981; Martin et al, 1981; Barlocher, 1982b, 1983; Sinsabaugh et al, 1985). Much of the energy in leaf material cannot be used directly by most detritivores while microorganisms with their superior enzymatic capability are able to decompose most leaf substances.

All aquatic and terrestrial detritivores so far studied appear to have the ability to degrade proteins, cellobiose (intermediate of cellulose digestion) and soluble cellulose (CMC) to some degree but only very few (with the exception of snails (Calow and Calow, 1975)) show even weak activity towards native crystalline cellulose (Nielsen, 1962; Bjarnov, 1972; Kristensen, 1972; Monk, 1976, 1977). The enzymatic capability of *G.pulex* has

been studied by many workers (Nielsen, 1962; Bjarnov, 1972; Monk, 1977; Charmier and Willoughby, 1986) and although *G.pulex* is able to degrade CMC and cellulose it is not able to do so with great vigour; eg. Monk found *G.pulex* only degraded 1.6 percent of cellulose powder to glucose. The gut of *G.pulex* appears to be divided into anterior-acid and posterior-neutral sections, suggesting protein and carbohydrate digestion may occur in different parts of the gut. Charmier and Willoughby (1986) however concluded that *G.pulex* could produce enzymes active on native cellulose. However, an incubation period of 44 hours at 37^{o}C was required to demonstrate the action of *G.pulex* enzymes on native cellulose and a shorter incubation period and lower temperatures did not yield results with the methods used. Therefore it is highly questionable whether the gut enzyme activity observed in these experiments would be observed under a more natural temperature regime (5 to 15^{o}C) and within the period of more usual gut passage times (four to seven hours).

In contrast to invertebrates, all five species of aquatic hyphomycete studied by Suberkropp and Klug (1980) degraded cellulose present in autumn leaves. Acquired enzymes from the microflora, which remain active in the gut of many species have been demonstrated: Fungal carbohydrases ingested with leaf material remained active in the guts of *Gammarus tigrinus* and *Hydropsyche bettini* allowing them to degrade polysaccharides of unconditioned leaf powder after ingesting leaf discs on which *T.marchlianum* had been growing (Barlocher and Porter, 1986). Acquired enzymes have also been demonstrated in a stonefly and a caddis which were observed to lose cellulase activity completely after a few weeks when nymphs were fed filter paper (Sinsabaugh et al, 1985). Acquired enzymes were offered by Barlocher and Porter (1986) as an explanation for the weak and sporadic occurrence of cellulase activity observed in several stream invertebrates (Bjarnov, 1972; Monk, 1976).

Monk (1977) found both fungi and bacteria were present in the gut of *G.pulex* but removal of microbes by filtering or by toluene inactivation did not cause any decrease in

cellulase activity of the gut enzymes. However microbial enzymes may already have been released and hence removal of microbial cells might not necessarily mean microbial enzymes were not involved. The reliance of *G.pulex* on the modification of leaf material by fungi rather than the fungal biomass itself is demonstrated by Sutcliffe et al (1981) and Willoughby and Sutcliffe (1976), who found survival and growth of *G.pulex* was higher on conditioned leaves than on sterile leaves or two pure fungal cultures.

Hence microbial enzymes may be required to supplement the incomplete set of enzymes possessed by most leaf eating invertebrates (Barlocher, 1985). The reliance of various invertebrates on fungi for the digestion of cellulose may offer an explanation for the strong food preferences of *G.pulex* observed in this study. Diet breadth studies have shown that *Gammarus* sp are much more selective than other shredders (Arsuffi and Suberkropp, 1989) and relationships between degree of mobility and digestive specialisations suggested that exploitation may be affected by phylogenetic constraints.

5.7. Summary.

1. Copper caused a significant reduction in the processing of leaf material by the combined action of invertebrates, microorganisms and physical factors in artificial streams. The processing rate of leaf material was significantly correlated with the number of *Gammarus* associated with leaf material in both the riffle and pool section.

2. Copper had no effect on the processing rates of leaf material by microorganisms in the artificial streams but this was probably due to the short period of exposure. In longer term laboratory experiments copper caused a significant reduction in microbial processing particularly of sterile leaf material.

3. As well as affecting the functioning of the microbial community, copper caused a change in the community structure of aquatic hyphomycetes on leaf material. Copper

caused a reduction in species numbers, a shift in the relative importance of species, a reduction in microbial cover and increase in patchiness of fungi on leaf material. Effects of copper were greater with sterile leaf material than with conditioned leaves indicating the primary effect of copper was to inhibit microbial colonisation.

4. Laboratory toxicity experiments with pure fungal cultures indicated that the primary effect of copper on a sensitive species, *Anguillospora longissima*, was to inhibit germination and to a lesser degree sporulation. In contrast, at concentrations in the range of 0 to 175 µgCu/l, there was no effect of copper on a more tolerant species, *T.marchalianum*. Hence the effect of copper on the fungal component of the community appeared to be a reduction in the colonisation of leaf material due to an inhibition of germination.

5. While food preferences of *G.pulex* for conditioned leaf material did not vary in a dose response manner, *G.pulex* showed a strong dose-response relationship for selection of sterile leaves from the control stream over sterile leaves exposed to copper. As the copper concentration of conditioned leaves was higher than that of sterile leaves, preferences were unlikely to be due to rejection of copper contaminated food. A more probable explanation is that sterile leaves exposed to copper were rejected due to the reduction in microbial colonisation of the leaf material caused by copper. Hence copper can affect the consumption of leaf material by *G.pulex* indirectly via a reduction in the quality of its food.

6. The effect of copper on processing rates of leaf material in the riffle section of the artificial streams was accurately predicted from results based on consumption rates and population density of *G.pulex*. Effects of copper on leaf processing in the pool section were not as great as expected but predictions from effects of copper on consumption rates and population density would have been protective.

CHAPTER 6.

DISCUSSION.

The aim of this study was to investigate the validity and ecological relevance of laboratory toxicity tests, based on energy budget parameters, using copper as a reference toxicant. For a laboratory test to be valid, it should mirror the pattern of response and sensitivity which is observed under field conditions. To be ecologically relevant a test should be indicative of effects at higher levels of organization which are important for the structure and / or for the functioning of the ecosystem.

The ecological relevance of any laboratory test may be enhanced by careful selection of the test species to be used. Selection of an organism which is important for the structure and function of freshwater ecosystems would allow predictions to be made concerning effects of a pollutant at levels of organisation higher than that of the organism by inference from effects on the species chosen. The leaf shredding amphipod, *Gammarus pulex* (L), was selected as the test species for this study. *G.pulex* is widely distributed throughout Europe and plays an important role in detritus processing. By feeding on coarse particulate organic matter (CPOM) *G.pulex* plays a key role in the detritus food web (Chapter 1; Fig. 1). It transfers energy to higher trophic levels such as predatory invertebrates and fish, and to other invertebrates that feed on fine particulate organic matter (FPOM), generated by its feeding activities (Cummins et al, 1973; Cummins, 1973, 1974; Anderson and Sedell, 1979; Cummins and Klug, 1979). Hence any adverse effects of a pollutant on this species could result in deleterious changes in both the structure and the function of such systems.

Selection of response criteria indicative of effects at higher levels of organisation would also enhance the potential relevance of laboratory tests. The measurement of energy budget parameters allows the determination of the energy status of an organism. From a

measure of energy acquired via consumption (C), and that lost via faeces (F) and metabolic processes (standard respiration Rs; active and feeding respiration, respectively Ra and Rf) a measure of the amount of energy available for production (P) or 'scope for growth' (SfG) can be estimated (Chapter 2). This energy may be used for growth and / or reproduction (Chapter 3) and since both are important for population dynamics, effects of a pollutant on the energy available for production should be indicative of effects on population processes.

This study was designed to investigate the effects of a reference pollutant (copper) on the energy budget of *G.pulex* in laboratory tests. The validity and ecological relevance of these tests was then determined with the use of outdoor artificial streams.

In laboratory tests, copper caused a significant reduction in the energy acquired via consumption and that lost via faecal production (Section 2.5. 2. a). However, there was no effect on the amount of energy lost via standard respiration (Section 2.5. 2. c). Despite a linear relationship between consumption rates and faecal production, significant effects of copper on absorption and scope for growth were detected at higher concentrations than effects on consumption rates (Section 2.5. 2. d). The relative sensitivity of consumption rates and insensitivity of respiration rates has been demonstrated in other studies concerning the effects of various pollutants on the energy budget of *G.pulex* (Naylor et al, 1989; Maltby et al, 1990a). Hence it would appear that the measurement of consumption rates alone would be adequate for determining the sensitivity of *G.pulex* to a pollutant. However, caution must be exercised in ignoring respiratory costs, as animals require metabolic energy in addition to standard metabolism for a wide range of additional functions. The effects of two of the most important of these additional functions (activity and feeding) on the respiration rate of *G.pulex* were therefore also investigated.

Copper caused an increase in the activity of *G.pulex* and an associated increase in respiratory rate (Section 2.5. 3. a). Active metabolism was equal to approximately three

times the standard respiration rate; this compares well with estimates of other workers for *Gammarus sp.* ranging from two to four times standard respiration rates (Culver and Poulson, 1971; Bulnheim, 1972 in Maltby, 1991; Maltby, 1991). However, this increase in activity was likely to be transitory; animals exposed to copper in six day tests appeared to be no more active than control animals. Respiration rate increased due to feeding but was not related to the amount of food consumed (2.5. 3. b). This was in agreement with Nelson et al, (1977) and Barber et al, (1990) who also failed to find any relationship between consumption and respiration rate although a significant positive relationship between consumption and respiration has been found by other workers (Beamish 1974; Bohrer and Lampert, 1988; Carefoot, 1990a, b, c). It may have been that the short period of feeding allowed in these tests (i.e. 1 hour) did not result in a large enough difference in the amount consumed by animals for a significant relationship to be detected. Hence from these tests it would appear that consumption rates alone can be used to indicate the sensitivity of *G.pulex* to copper as relatively little information is gained from a measure of standard, active or feeding respiration rate.

A measure of production, or scope for growth, gives an estimate of the energy available for growth and reproduction. Therefore, as copper caused a decrease in the energy available for scope for growth it was expected that growth and reproduction would also be adversely affected by copper. Indeed, copper exposure caused a reduction in the growth rates of juvenile *G.pulex* held in groups, although this reduction was not significant for animals maintained individually (Section 3.5. 1.). As well as the differences between results obtained from animals held in groups or individually, there was also a high degree of mortality during growth experiments and measurements had to be conducted over a long time period (four to six weeks). Hence, in agreement with Ringwood (1992), growth rates are considered too tedious and time consuming to be used routinely especially as the sensitivity of growth as a response criterion was comparable to that of consumption rates.

The main effect of copper on reproduction in *G.pulex* was an increase in the percentage of females aborting their broods; females appearing to sacrifice their brood in order to enhance their own survival (Section 3.5. 2.). This result was in common with the effects of zinc and of ammonia on reproduction of *G.pulex* (Maltby and Naylor, 1990; Maltby, 1991). The abortion of young by the female is thought to be due to the effects of copper on the energy status of the female. This parameter was slightly more sensitive than consumption rates but took a much longer time to measure (3 to 4 weeks as opposed to six days).

Because both growth and reproduction were time consuming and laborious to measure, it would be advantageous to be able to predict effects of a pollutant on these parameters using energy budget determinations. Attempts to predict the effects of copper on growth and reproduction from a knowledge of the energy budget of *G.pulex* were fairly accurate in indicating the trends but were unsuccessful in predicting actual growth rates and reproductive output.

Hence, consumption rate was identified as an easily quantifiable and sensitive response criterion to copper stress in *Gammarus pulex*, with a significant reduction detected at 23 µgCu/l (Section 2.5. 2. a. Fig. 5). The determination of consumption rates as the most sensitive of the energy budget parameters measured was in agreement with the findings of many other workers concerning the effects of a range of both metal and organic toxicants on the energy budget parameters of a variety of aquatic species (Stickle et al, 1984; Widdows, 1985; Widdows et al, 1987a, b; Widdows and Johnson, 1988; Widdows et al, 1990; Maltby et al 1990, a, b). The copper-induced reduction in consumption rates of *G.pulex* in this study was a result of an increase in their body copper concentration via uptake of copper from the medium (Section 2.5. 6. and 2.5. 7. Figs. 14 and 15). As faecal production reflected consumption rates and respiration rates were unaffected by exposure to copper, consumption rates alone were sufficient to reflect effects of copper on the energy status of an individual rendering additional energy budget parameter

measurements unnecessary. Effects of copper on consumption rates are therefore considered sufficient for determining the effects of copper on an individual. However, this statement would only be true if tests based on consumption rates were valid in the natural environment and were protective of the freshwater ecosystem.

The primary aim of laboratory tests is to predict effects of toxicants in natural systems, in order to protect the structure and / or the functioning of the ecosystem. Therefore, for laboratory tests to have any real meaning they should be both valid and ecologically relevant in natural systems. Hence, artificial streams were used to investigate the validity and ecological relevance of laboratory tests (Chapters 4 and 5). These streams were set up to imitate a natural freshwater detritivorous ecosystem while allowing a degree of control over many of the variables.

The extent to which laboratory tests were able to predict effects in the artificial streams was found to vary depending on the response criterion used (Table 26). Under these more natural conditions, as in laboratory tests, consumption rates were identified as the response criterion most sensitive to copper stress. Further, there was no significant difference between the response of *G.pulex* to copper, in terms of consumption rates, in laboratory or field tests (Fig. 36). Hence laboratory tests based on consumption rates were valid in more natural systems.

This demonstrates that laboratory results can predict effects in the natural environment as long as they are based on carefully selected response criteria. These results call into question the use of arbitrary application factors as used by workers such as Giddings and Franco (1985) for application of laboratory results in the natural environment. Application factors are rendered unnecessary when both sensitive and relevant response criteria are used and experiments are conducted under conditions allowing direct extrapolation to natural environments.

Potential additional effects on the consumption rates of *G.pulex* due to copper-induced reductions in food quality were also indicated in this study (Section 5.5. 4). Copper caused a decrease in the abundance and a change in the community structure of aquatic hyphomycete fungi growing on leaf material (Section 5.5. 3). These changes in the structure of the aquatic hyphomycete community were likely to be due to copper-induced reductions in germination and sporulation (Section 5.5. 5). As palatability of leaf material is strongly influenced by the fungal component of leaf material (e.g. Sedell et al, 1975; Barlocher, 1980, 1985; Findley et al 1986a and b), indirect effects of copper on consumption rates of *G.pulex* may also be important. The potential impact of these indirect effects warrants further study. One situation where this could be especially important might be a pulsed input of a toxicant. Abel and Barlocher, (1988) found three species of aquatic hyphomycetes accumulated cadmium from media very rapidly and postulated that if the duration of a pulse was too short to have a significant direct effect on stream invertebrates the ingestion of contaminated food over a long time period might have a more adverse effect. In this study there was no evidence for significant accumulation of copper from the food source at least over a six day experimental period (Section 2.5. 6.) however the invertebrate community might suffer more from a copper-induced reduction in food quality as a result of restricted fungal abundance and / or a change in the fungal species composition caused by a pulse of toxicant.

It has therefore been shown that tests based on consumption rates as a response criterion were both sensitive and valid in a more natural environment. However, for laboratory tests to have any real meaning their ecological relevance should also be demonstrated. As discussed above, consumption rates were indicative of the energy status of the individual and were therefore indicative of effects on growth and reproduction and hence potential effects of a pollutant on population processes could be inferred. Further, because of the importance of consumption by *G.pulex* for the functioning of the detritus food web, effects of copper on consumption rates should be indicative of potential adverse effects on detritus processing in the system.

Population density of *G.pulex* and the processing of leaf material in the system were selected as response criteria indicative of effects on the structure and functioning of the freshwater ecosystem respectively. Table 41 (Section 5.6) indicates that effects of copper on consumption rates were indeed implicit of subsequent effects on population density with the lowest concentration at which effects on population density were observed being 20.47 µgCu/l. In addition to the relevance of consumption rates as a response criterion at the population level of organisation, Table 41 (Section 5.6) indicates that it was also relevant in terms of the functioning of the detritivorous system. The lowest concentration at which copper significantly reduced overall leaf processing in the artificial streams was 25.0 µgCu/l.

The importance of *G.pulex* in the processing of leaf litter was indicated by the significant correlation between leaf weight loss and *G.pulex* numbers (Fig. 51). Other studies have demonstrated the importance of shredders in leaf processing; decreases in shredder abundance caused a decrease in leaf litter decomposition rates (Petersen and Cummins, 1974; Sedell et al, 1975; Cuffney et al, 1984; Wallace et al, 1986, 1989).

Although shredders exploit coarse particulate organic matter (CPOM) as their food source, their assimilation efficiencies are generally low (i.e. approximately 30 percent) indicating that they can potentially generate large amounts of fine particulate organic matter (FPOM) and dissolved organic matter (DOM) (Grafius and Anderson, 1980). Indeed, more than 30 percent of the processing of CPOM to FPOM in streams has been attributed to the action of shredder invertebrates (Petersen and Cummins, 1974; Cuffney et al, 1990). The production of FPOM may represent an important source of energy and nutrients for the downstream fauna (Anderson and Sedell, 1979; Short and Maslin, 1977; Wallace et al, 1986; Cummins et al, 1973). Calculations of consumption rates of detritivores, based on ingestion rates and production data show that, while they only assimilate a small portion of the energy consumed (Fisher and Likens, 1973) they may

ingest 32 to 50 percent of the annual input of leaf litter and consequently have a great effect on the rates and pathways of energy flow in streams, (Cummins et al, 1973; Petersen and Cummins, 1974; Webster and Patten, 1979; Grafius and Anderson, 1980; Webster, 1983).

Short and Maslin (1977), clearly demonstrated the reliance of collector invertebrates on the production of FPOM due to the action of shredders. They found that the incorporation of radio-labelled alder leaf material by two species of collectors was significantly increased when a shredder species was also present.

On the basis of the trophic linkages between functional feeding groups (Chapter 1; Fig. 1) the potential consequences of the effects of copper on *G.pulex* to the structure and functioning of the whole community can be predicted. Copper caused a decrease in the consumption rates and population density of *G.pulex* in the artificial streams and, because of the importance of this species in the breakdown of CPOM, there was a decrease in the processing of leaf material in the system. Because shredder species are important in the breakdown of CPOM and the production of FPOM a copper induced reduction in leaf processing by *G.pulex* would reduce the amount of FPOM generated. This would result in a reduction in the food supply for collector invertebrates downstream, potentially leading to a reduction in their population density. The reduction in the population density of *G.pulex* and collector invertebrates would result in a reduction in the food supply for predatory invertebrates and fish (Chapter 1. Fig. 1).

Hence, this study has demonstrated the usefulness of consumption rates as a response criterion for determining the effects of a pollutant in the freshwater detritivorous ecosystem. Laboratory tests based on consumption rates of *G.pulex* were sensitive, valid and ecologically relevant being indicative of potential effects of a toxicant on both structure and function at the population and community levels of organisation.

Appendix 1.

Recipes for the experimental media for the maintenance of *Gammarus pulex* in the laboratory

a. Filtered stream water (FSW).

Table 42. Stream water analysis (Mean and standard error for samples taken at beginning and end of test).

Anion / metal	Concentration (mg/l)	SE
Cl	64.750	1.50
NO_2	0.320	0.09
NO_3	48.320	4.60
Br	0.155	0.03
SO_4	124.330	5.19
Cu	0.005	0.00
Zn	0.011	0.01
Cd	0.007	0.00
Pb	0.000	0.00
Fe	0.015	0.00
Mg	44.530	0.02
Mn	0.010	0.00
Ni	0.000	0.00
Ca	98.485	0.51
Al	0.000	0.00

b. Artificial pond water medium (APW).

Table 43. APW stock solution.

	Stock solution	Quantity (g/l)
1.	$CaCl_2.2H_2O$	58.80
2.	$MgSO_4.7H_2O$	24.65
3.	$NaHCO_3$	12.95
4.	KCl	1.15

Mix 50 ml of each stock solution and make up to ten litres with distilled water.

c. Low chloride artificial pond water (LowClAPW)

Table 44. LowClAPW stock solution.

	Stock solution	Quantity (g/l)
1.	$CaCl_2.2H_2O$	58.8
2.	$CaSO_4.7H_2O$	2.4
3.	$MgSO_4.7H_2O$	24.65
4.	$NaHCO_3$	12.95
5.	KCl	1.15

Mix 17 ml of stock solution 1, 190 ml of stock solution 2 and 50 ml of stock solutions 3 to 5 and make up to ten litres with distilled water.

d. M4 media (M4).

This media was a modification of the Elendt *Daphnia* culturing media and required 4 sets of stock solutions: (i) macro nutrients, (ii) micronutrients, (iii) buffering nutrients and (iv) vitamin nutrients.

i. Macro nutrients: as for APW.

ii.Micro nutrients.

Table 45. M4 micronutrient stock solution.

	Stock solution	Quantity (mg/l)
1.	H_3BO_4	5719
2.	$Na_2EDTA.2H_2O$	5000
3.	$FeSO_4.7H_2O$	1991
4.	$MnCl_2.4H_2O$	721
5.	LiCl	612
6.	$SrCl_2.6H_2O$	304
7.	$RbCl_2$	142
8.	$Na_2MoO_4.2H_2O$	126
9.	NaBr	32
10.	$ZnCl_2$	26
11.	$CoCl_2.6H_2O$	20
12.	KI	6.5
13.	Na_2SeO_3	4.38
14.	NH_4VO_3	1.5

Mix 5 ml per 10 litres of final medium. Micronutrients 2 and 3 were poured together and autoclaved immediately (121^{o}C, 15 PSI, for 20 minutes). The resulting solution was used at 10 ml per 10 litres final medium.

iii. Buffering nutrients.

Table 46. M4 buffering nutrients stock solution.

	stock solution	Quantity (mg/l)
1.	$Na_2SiO.9H_2O$	1000
2.	$NaNO_3$	27.4
3.	K_2HPO_4	18.4
4.	KH_2PO_4	14.3

Mix 100 ml per 10 litres of final medium.

iv. Vitamin nutrients.

Table 47. M4 vitamin stock solution.

	Stock solution	Quantity (g/l)
1.	Thiamine hydrochloride	75
2.	Cyanocobalamine (vit. B12)	1.0
3.	D (+) biotin (vit. H)	0.75

These vitamins were prepared as a single stock solution and stored frozen. 10 ml of each vitamin stock solution was added per 10 litres of final medium.

The final medium was made up with distilled water.

Appendix 2.

Effects of different media (given in Appendix 1) on survival of *G.pulex*.

Surival of *G.pulex* maintained in the four different experimental media (see Appendix 1) was examined over an eight-week period. Percentage survival of animals was normalised by arcsine transformation. There was a significant linear relationship between survival and time for each media ($r^2 > 95.1\%$, df = 3, $p < 0.01$) but no significant effect of media on survivorship (ANCOVA: F = 0.55, df = 3, 22, $p > 0.05$) and no significant interaction (ANCOVA: F = 2.3, df = 3, 22, $p > 0.05$). However, survival of animals maintained in APW was slightly greater than that of animals maintained in the other 3 media although this was not significant:

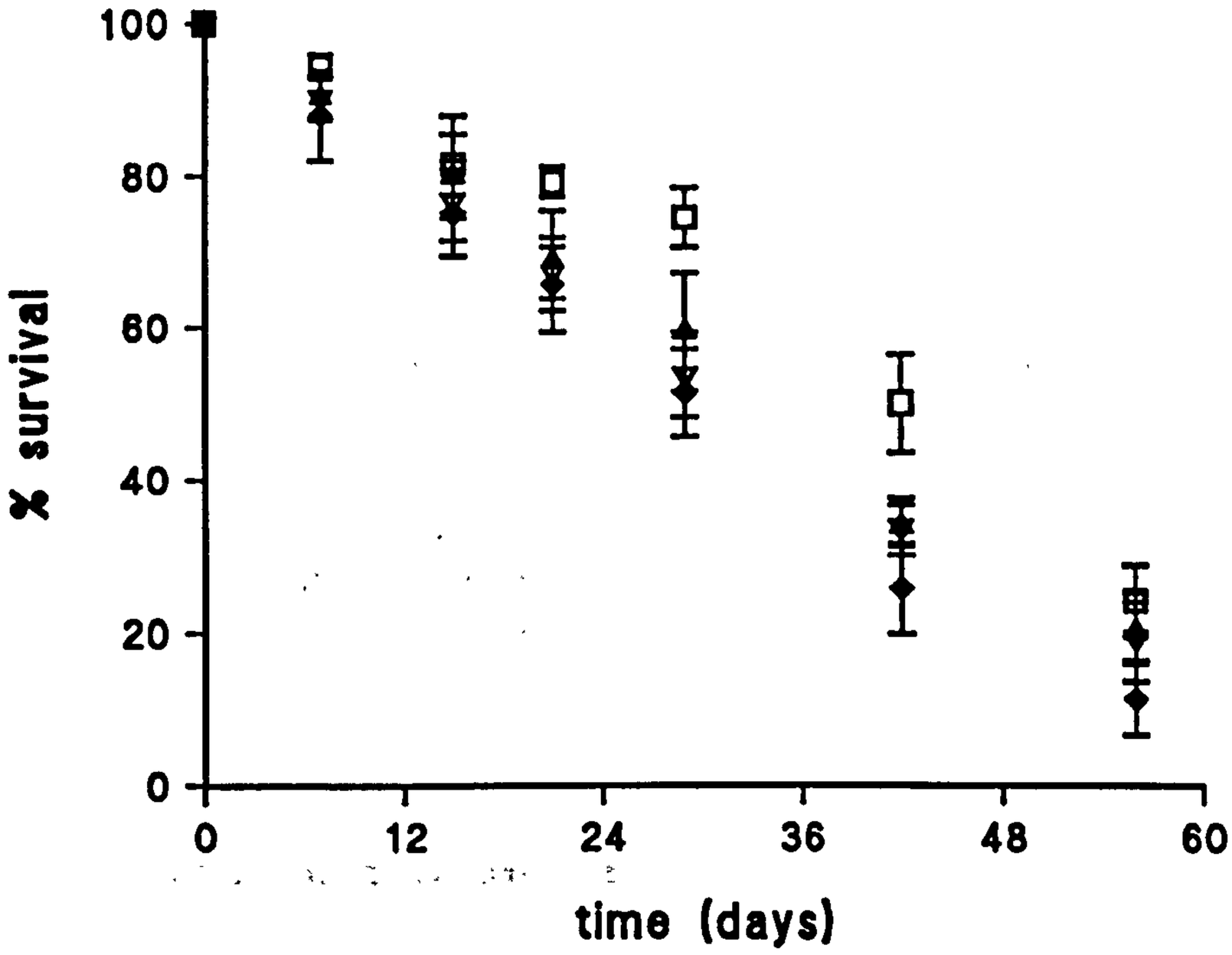


Fig. 66. Percentage survival of *G.pulex* maintained in APW (open squares), Low chloride APW (open triangles), M4 (solid triangles), or filtered stream water (solid diamonds) (see Appendix 1) for eight weeks. Error bars represent 1 standard error.

Appendix 3.

Recipe for enriched distilled water:

Table 48. Stock solution 1.

	Compound	Quantity (g)
1	$(NH_4)_2HPO_4$	66.04
2	KH_2PO_4	68.05
3	K_2HPO_4	87.09

All compounds of stock solution 1 were dissolved in a little distilled water before mixing together and making upto one litre with distilled water.

Table 49. Stock solution 2.

	Compound	Quantity (g)
1	$CaCl_2.2H_2O$	18.38
2	$MgCl_2.6H_2O$	25.42

All components of stock 2 were disolved in a little distilled water before mixing together and making upto one litre with distilled water.

For enriched distilled water add 0.5ml of stock 1 and 0.1ml of stock 2 to one litre of distilled water.

Appendix 4.

Calibration of the semi-micro bomb calorimeter.

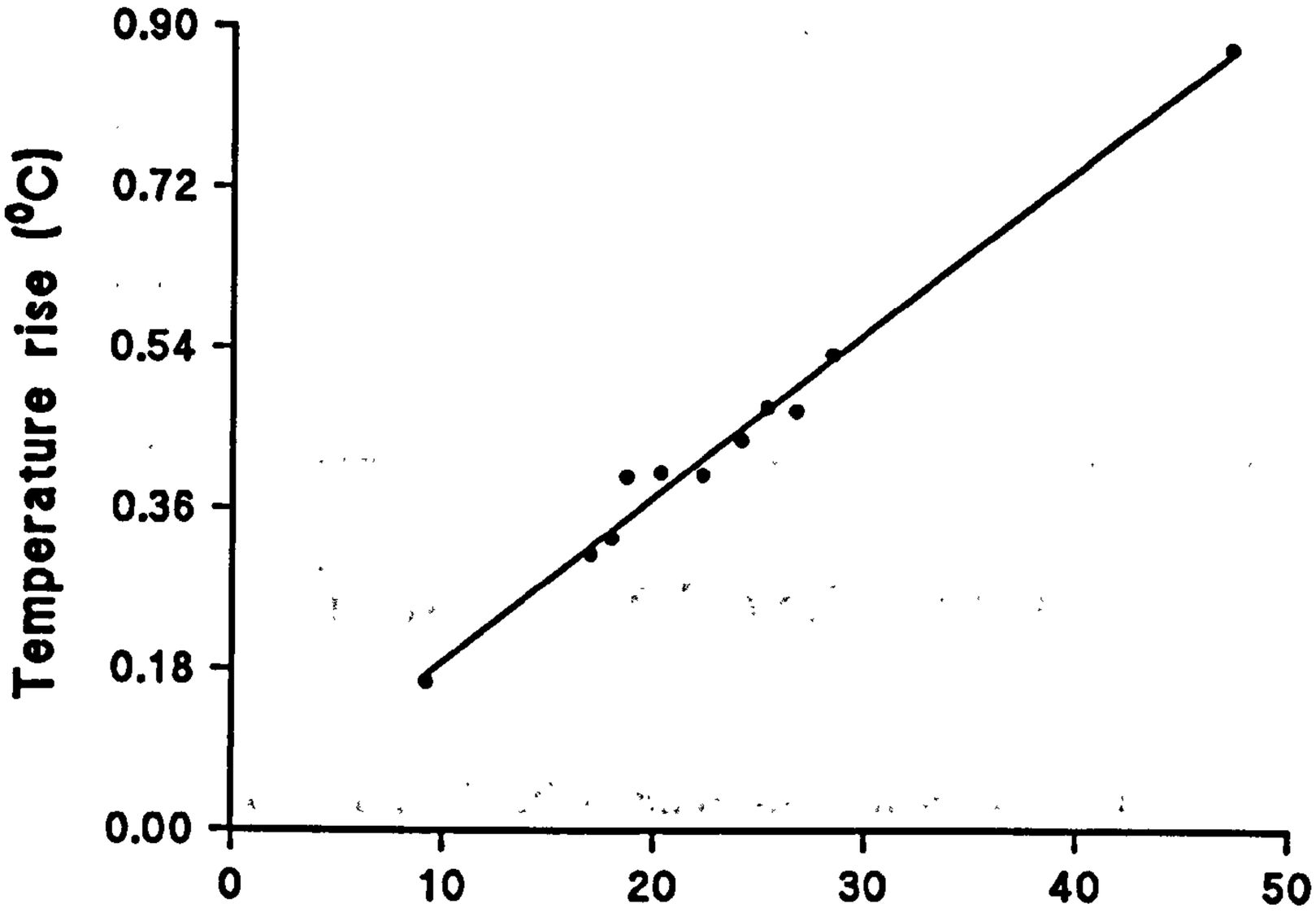


Fig. 67. Relationship between weight of benzoic acid burned and temperature rise detected by the semi-micro bomb calorimeter.

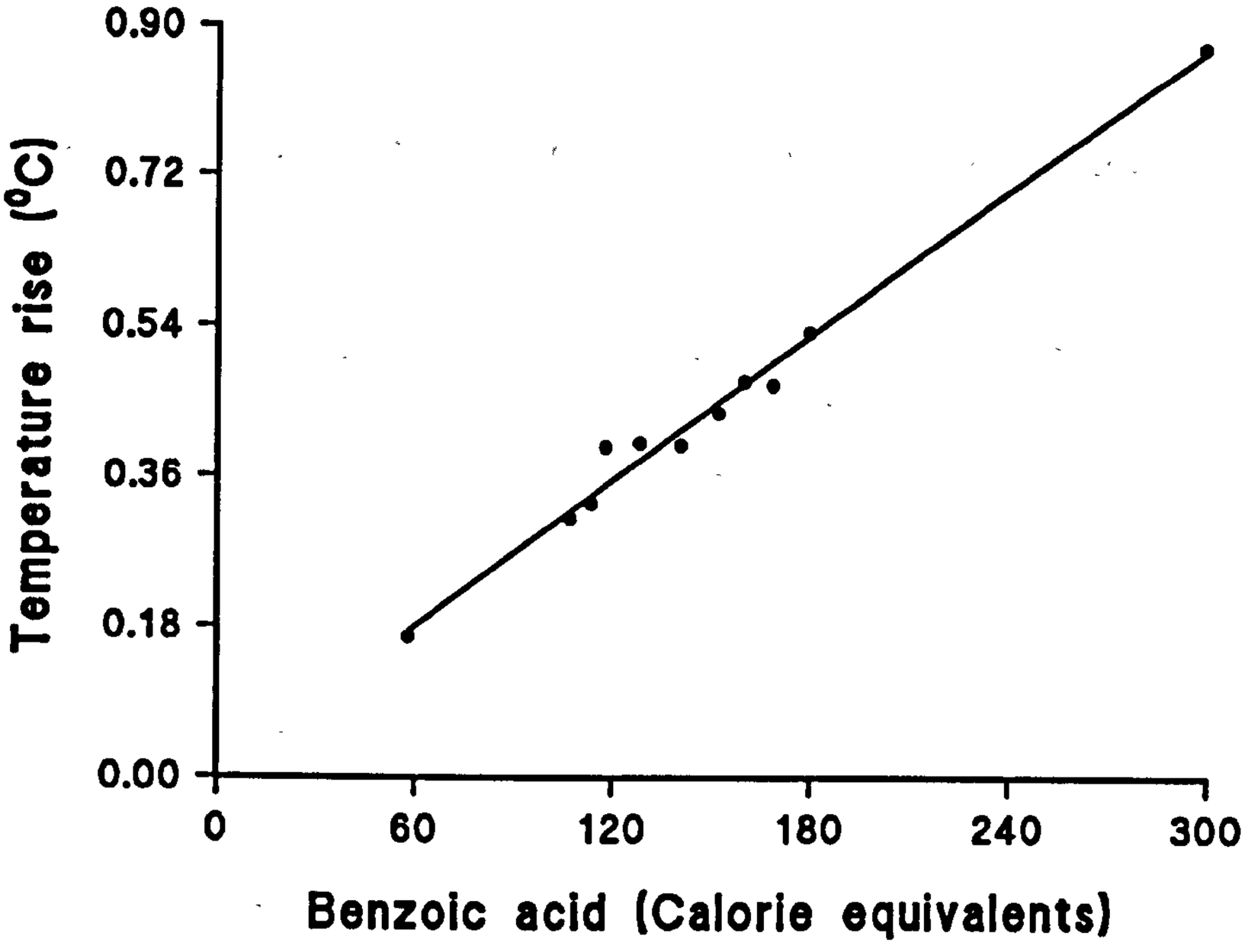


Fig. 68. Bomb calorimeter calibration curve for conversion of temperature rise to energy equivalents.

Appendix 5.

Table 50. Joule equivalents (J/mg) of food and faeces exposed to copper.

Sample	Concentration (µgCu/l)	J/mg	SE	n
Food	0	21.065	0.256	5
	13	21.441	0.442	5
	23	21.276	0.490	5
	27	20.106	0.331	5
	30	20.293	0.325	5
	36	20.438	0.449	5
Faeces	0	19.648	0.443	3
	15	20.125	0.423	3
	30	19.478	0.340	3

Appendix 6.

Effect of periodic wet weighing or anaesthetisation with soda water on the survivorship of *G.pulex*.

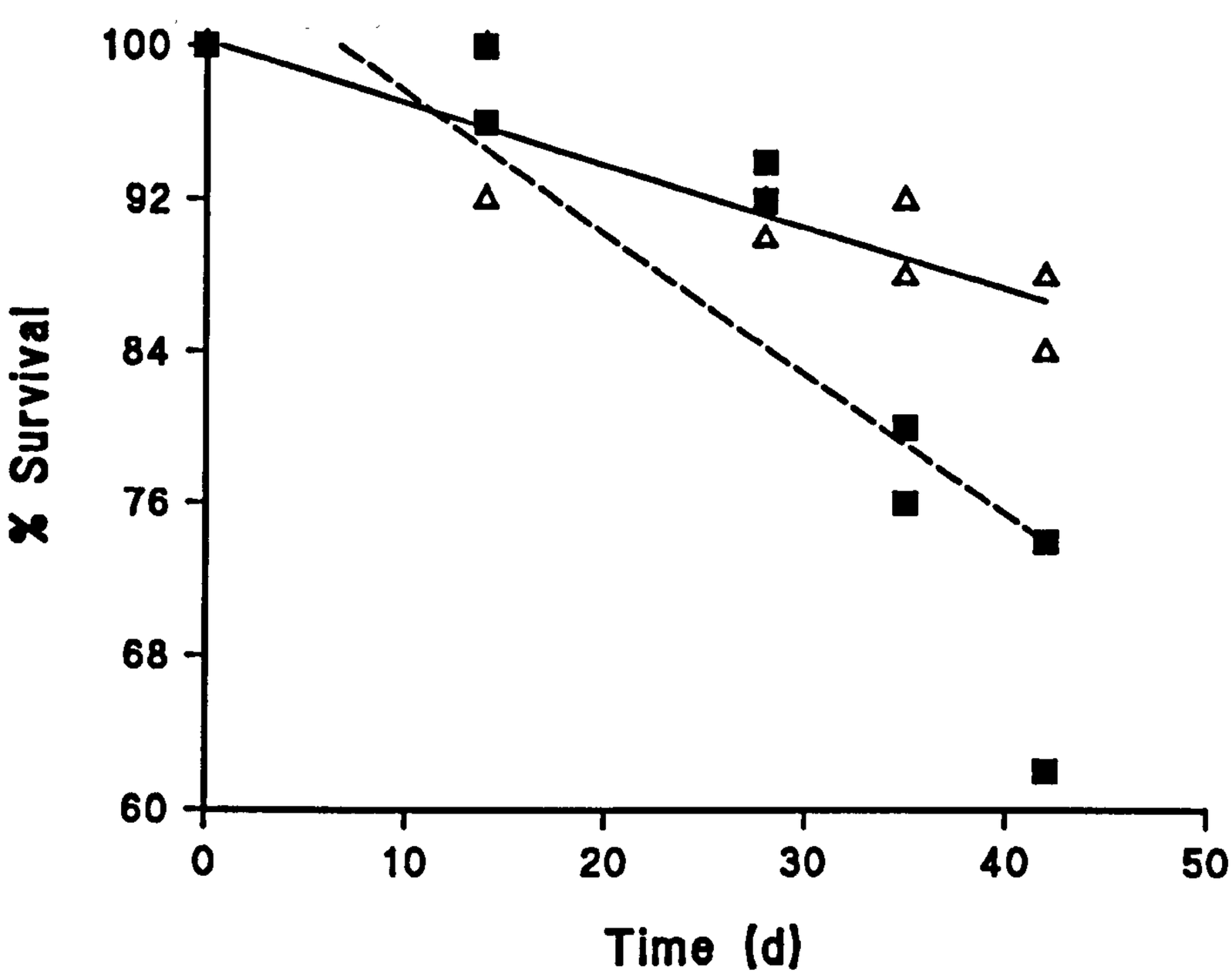


Fig. 69. Percentage survival of *G.pulex* after periodic wet weighing (open triangles; solid line), or anaesthetization (solid squares; dashed line) over a six-week time period.

Percentage survival of animals was normalised by arcsine transformation. Survival of animals subject to wet weighing and to anaethetisation decreased linearly over the period studied ($r^2 > 79.5\%$, df = 8, $p < 0.001$). Analysis of covariance indicated a significant interaction between survival through time and treatment (ANCOVA: F = 4.7, df = 1, 19, $p < 0.05$).

Appendix 7

Table 51. Physicochemical data for the river Don, Penistone.

(NGR SK 216209)

Anion / metal	Concentration (mg/l)	SE
Cl	17.913	6.32
NO_3	48.320	4.60
Br	0.030	0.01
SO_4	51.210	6.32
Cu	0.003	0.00
Zn	0.075	0.03
Cd	0.004	0.00
Pb	0.004	0.00
Fe	0.425	0.26
Mg	7.680	2.02
Mn	0.210	0.11
Ni	0.045	0.03
Ca	18.280	7.76
Al	1.265	0.82
pH	7.4	

Appendix 8

A, B and RIVs of fungi on conditioned leaves exposed to copper in the pool and riffle section of the artificial streams and of sterile leaves exposed to copper in the pool section of the artificial streams.

Table 52. A Values for fungal species on conditioned leaf material exposed to copper in the pool section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.5	299.66
Tetracladium marchalianum	0.102	0.139	0.111	0.102	0.094	0.161
Anguillospora crassa	0.102	0.139	0.111	0.102	0.094	0.161
Articulospora tetracladia	0.102	0.083	0.089	0.102	0.094	0.094
Anguillospora longissima	0.102	0.139	0.111	0.102	0.094	0.129
Centrospora aquatica	0.102	0.056	0.067	0.061	0.075	4.45
Tetrachaetum elegans	0.082	0.083	0.111	0.102	0.075	0.097
Tetracladium setigerum	0.082	0.083	0.067	0.102	0.057	0.032
Heliscus lugdunensis	0.082	0.083	0.111	0.082	0.075	0.097
Clavatospora stellata	0.061	0.056	-	0.041	0.057	0.032
Clavariopsis aquatica	0.041	0.028	0.111	0.102	0.094	-
Tetracladium angulata	0.041	0.028	0.022	-	0.038	0.065
Dactylella aquatica	0.020	-	-	-	-	-
Lemoniera aquatica	0.020	-	0.022	0.020	0.019	-
Flabellospora acuminata	0.020	0.056	0.022	-	0.038	-
Scorpiosporium minutum	0.020	0.028	0.022	-	0.019	-
Campylospora parvula	0.020	-	0.022	-	0.019	-
Lemoniera terrestris	-	-	-	0.041	0.019	-
Alatospora acuminata	-	-	-	0.020	-	-
Dendrospora sp.	-	-	-	0.020	-	-
Clavatospora longibrachiata	-	-	0.022	-	0.019	-
Tetracladium furcatum	-	-	-	-	0.019	-

Table 53. B Values for fungal species on conditioned leaf material exposed to copper in the pool section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.5	299.66
Tetracladium marchalianum	0.216	0.298	0.214	0.213	0.207	0.393
Anguillospora crassa	0.168	0.145	0.200	0.202	0.176	0.190
Articulospora tetracladia	0.151	0.105	0.107	0.117	0.155	0.143
Anguillospora longissima	0.108	0.153	0.150	0.138	0.109	0.083
Centrospora aquatica	0.065	0.048	0.029	0.016	0.031	0.024
Tetrachaetum elegans	0.070	0.048	0.071	0.080	0.062	0.071
Tetracladium setigerum	0.070	0.081	0.043	0.005	0.026	0.012
Heliscus lugdunensis	0.049	0.048	0.064	0.053	0.057	0.060
Clavatospora stellata	0.011	0.016	0.000	0.021	0.016	0.012
Clavariopsis aquatica	0.022	0.024	0.064	0.064	0.067	-
Tetracladium angulata	0.016	0.008	0.021	-	0.016	0.012
Dactylella aquatica	0.022	-	-	-	-	-
Lemoniera aquatica	0.011	-	0.007	0.005	0.016	-
Flabellospora acuminata	0.011	0.008	0.007	-	0.021	-
Scorpiosporium minutum	0.005	0.016	0.007	-	0.016	-
Campylospora parvula	0.005	-	0.007	-	0.005	-
Lemoniera terrestris	-	-	-	0.027	0.005	-
Alatospora acuminata	-	-	-	0.005	-	-
Dendrospora sp.	-	-	-	0.005	-	-
Clavatospora longibrachiata	-	-	0.007	-	0.010	-
Tetracladium furcatum	-	-	-	-	0.005	-

Table 54. RIVs for fungal species on conditioned leaf material exposed to copper in the pool section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.5	299.66
Tetracladium marchalianum	0.080	0.109	0.006	0.079	0.075	0.139
Anguillospora crassa	0.008	0.071	0.078	0.076	0.068	0.088
Articulospora tetracladia	0.063	0.047	0.049	0.055	0.062	0.076
Anguillospora longissima	0.053	0.073	0.065	0.060	0.051	0.053
Centrospora aquatica	0.042	0.026	0.024	0.019	0.027	0.022
Tetrachaetum elegans	0.038	0.033	0.046	0.046	0.034	0.042
Tetracladium setigerum	0.038	0.041	0.028	0.028	0.021	0.011
Heliscus lugdunensis	0.033	0.033	0.044	0.034	0.033	0.039
Clavatospora stellata	0.018	0.018	-	0.016	0.018	0.011
Clavariopsis aquatica	0.016	0.013	0.044	0.042	0.040	-
Tetracladium angulata	0.014	0.009	0.011	-	0.014	0.004
Dactylella aquatica	0.011	0.000	0.000	0.000	0.000	-
Lemoniera aquatica	0.008	0.000	0.008	0.006	0.009	-
Flabellospora acuminata	0.008	0.016	0.008	-	0.015	-
Scorpiosporium minutum	0.006	0.011	0.007	-	0.009	-
Campylospora parvula	0.006	-	0.002	-	0.006	-
Lemoniera terrestris	-	-	-	0.017	0.006	-
Alatospora acuminata	-	-	-	0.006	-	-
Dendrospora sp.	-	-	-	0.006	-	-
Clavatospora longibrachiata	-	-	0.007	-	0.007	-
Tetracladium furcatum	-	-	-	-	0.006	-

Table 55. A Values for fungal species on conditioned leaf material exposed to copper in the riffle section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.55	299.66
Tetracladium marchalianum	0.122	0.128	0.114	0.122	0.102	0.152
Anguillospora longissima	0.122	0.128	0.114	0.122	0.102	0.152
Articulospora tetracladia	0.122	0.128	0.114	0.122	0.102	0.091
Anguillospora crassa	0.122	0.128	0.114	0.122	0.102	0.152
Centrospora aquatica	0.098	0.128	0.114	0.073	0.082	0.061
Heliscus lugdunensis	0.073	0.077	0.114	0.122	0.082	0.091
Clavariopsis aquatica	0.073	0.077	0.114	0.122	0.082	0.091
Tetrachaetum elegans	0.073	0.026	0.068	0.049	0.102	0.061
Tetracladium setigerum	0.049	0.103	0.068	0.049	0.082	-
Vargamyces aquatica	0.024	0.026	0.00	0.024	-	0.030
Clavatospora stellata	0.024	0.051	0.023	0.073	0.020	0.030
Scorpiosporium minutum	0.024	0.051	0.091	0.049	0.061	0.091
Campylospora parvula	0.024	-	0.023	0.024	-	0.030
Tricladium angulatum	0.024	-	0.000	-	0.061	-

Table 56. B Values for fungal species on conditioned leaf material exposed to copper in the riffle section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.55	299.66
Tetracladium marchalianum	0.258	0.260	0.204	0.225	0.226	0.449
Anguillospora longissima	0.200	0.113	0.173	0.197	0.147	0.180
Articulospora tetracladia	0.174	0.153	0.128	0.096	0.096	0.045
Anguillospora crassa	0.135	0.207	0.194	0.219	0.169	0.124
Centrospora aquatica	0.071	0.087	0.097	0.039	0.062	0.034
Heliscus lugdunensis	0.032	0.007	0.010	0.051	0.051	0.011
Clavariopsis aquatica	0.039	0.047	0.092	0.062	0.045	0.034
Tetrachaetum elegans	0.026	0.020	0.036	0.045	0.107	0.022
Tetracladium setigerum	0.013	0.067	0.031	0.022	0.040	-
Vargamyces aquatica	0.019	0.007	-	0.011	-	0.011
Clavatospora stellata	0.013	0.020	0.010	0.022	0.011	0.011
Scorpiosporium minutum	0.006	0.013	0.020	0.006	0.023	0.045
Campylospora parvula	0.006	-	0.005	0.006	-	0.011
Tricladium angulatum	0.006	-	-	-	0.023	-

Table 57. RIVs for fungal species on conditioned leaf material exposed to copper in the riffle section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.55	299.66
Tetracladium marchalianum	0.095	0.097	0.080	0.087	0.082	0.150
Anguillospora longissima	0.081	0.060	0.072	0.080	0.062	0.083
Articulospora tetracladia	0.074	0.070	0.061	0.055	0.045	0.034
Anguillospora crassa	0.065	0.084	0.077	0.085	0.068	0.069
Centrospora aquatica	0.042	0.054	0.053	0.028	0.036	0.024
Heliscus lugdunensis	0.033	0.008	0.014	0.025	0.038	0.024
Clavariopsis aquatica	0.028	0.031	0.052	0.046	0.032	0.031
Tetrachaetum elegans	0.025	0.012	0.026	0.024	0.052	0.021
Tetracladium setigerum	0.016	0.043	0.025	0.018	0.031	-
Vargamyces aquatica	0.011	0.008	-	0.008	-	0.010
Clavatospora stellata	0.009	0.018	0.008	0.024	0.008	0.010
Scorpiosporium minutum	0.008	0.016	0.028	0.014	0.021	0.034
Campylospora parvula	0.008	-	0.007	0.008	-	0.010
Tricladium angulatum	0.008	-	-	-	0.021	-

Table 58. A Values for fungal species on sterile leaf material exposed to copper in the pool section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.55	299.66
Tetracladium marchalianum	0.185	0.179	0.179	0.179	0.238	0.556
Anguillospora crassa	0.185	0.179	0.179	0.179	0.095	0.111
Articulospora tetracladia	0.185	0.143	0.107	0.143	0.143	-
Anguillospora longissima	0.111	0.107	0.071	-	-	-
Tetrachaetum elegans	0.111	0.143	0.107	0.071	0.048	0.111
Heliscus lugdunensis	0.111	0.036	0.107	0.107	0.095	0.111
Scorpiosporum minutum	0.037	0.036	0.071	0.143	0.048	-
Centrospora aquatica	0.037	0.107	0.107	0.036	0.048	-
Tricladium angulatum	0.037	-	0.036	0.036	0.048	-
Clavariopsis aquatica	-	-	-	-	0.048	-
Clavatospora stellata	-	0.071	0.036	0.036	0.048	-
Tetracladium setigerum	-	-	-	0.071	0.143	0.111

Table 59. B Values for fungal species on sterile leaf material exposed to copper in the pool section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	2.45	11.42	24.14	59.55	299.66
Tetracladium marchalianum	0.424	0.313	0.432	0.493	0.650	0.765
Anguillospora crassa	0.202	0.177	0.243	0.164	0.083	0.059
Articulospora tetracladia	0.109	0.115	0.041	0.068	0.067	-
Anguillospora longissima	0.109	0.042	0.041	-	-	-
Tetrachaetum elegans	0.047	0.094	0.054	0.027	0.017	0.059
Heliscus lugdunensis	0.047	0.044	0.081	0.055	0.033	0.059
Scorpiosporum minutum	0.031	0.094	0.041	0.110	0.033	-
Centrospora aquatica	0.016	0.052	0.041	0.014	0.033	-
Tricladium angulatum	0.016	-	0.014	0.014	0.017	-
Clavariopsis aquatica	-	-	-	-	0.017	-
Clavatospora stellata	-	0.021	0.014	0.014	0.017	-
Tetracladium setigerum	-	-	-	0.041	0.033	0.059

Table 60. RIVs for fungal species on sterile leaf material exposed to copper in the pool section of the artificial streams.

Species	Copper concentration (µg/l)					
	0.38	**2.45**	**11.42**	**24.14**	**59.55**	**299.66**
Tetracladium marchalianum	0.152	0.123	0.153	0.168	0.222	0.330
Anguillospora crassa	0.097	0.089	0.106	0.086	0.045	0.043
Articulospora tetracladia	0.074	0.065	0.037	0.053	0.053	-
Anguillospora longissima	0.055	0.037	0.028	-	-	-
Tetrachaetum elegans	0.040	0.059	0.040	0.025	0.016	0.043
Heliscus lugdunensis	0.040	0.033	0.047	0.041	0.032	0.043
Scorpiosporum minutum	0.017	0.033	0.028	0.063	0.020	-
Centrospora aquatica	0.013	0.040	0.037	0.013	0.020	-
Tricladium angulatum	0.013	-	0.013	0.013	0.016	-
Clavariopsis aquatica	-	-	-	-	0.016	-
Clavatospora stellata	-	0.023	0.013	0.013	0.008	-
Tetracladium setigerum	-	-	-	0.028	0.044	0.043

Appendix 9.

A, B and RIV values of fungi on conditioned and sterile leaf material exposed to copper in laboratory tests.

Table 61. A Values for fungal species on conditioned, control leaf material in laboratory tests.

Species	Week1	Week 6
Anguillospora longissima	0.111	0.089
Tetrachaetum elegans	0.111	0.089
Anguillospora crassa	0.111	0.089
Alatospora acuminata	0.067	0.089
Tetracladium angulata	0.067	0.071
Tetracladium marchalianum	0.067	0.089
Centrospora aquatica	0.067	0.089
Clavariopsis stellata	0.000	0.071
Scorpiosporum minutum	0.000	0.071
Clavariopsis aquatica	0.111	0.071
Lemoniera aquatica	0.067	0.054
Heliscus lugdunensis	0.111	0.036
Tetracladium setigerum	0.067	0.036
Margaritispora aquatica	-	0.036
Lunulospora curvula	-	0.018
Dactyella aquatica	0.044	-
Flabellospora sp	0.022	-

Table 62. B Values for fungal species on conditioned, control leaf material in laboratory tests.

Species	Week1	Week 6
Anguillospora longissima	0.168	0.160
Tetrachaetum elegans	0.352	0.145
Anguillospora crassa	0.088	0.137
Alatospora acuminata	0.048	0.117
Tetracladium angulata	0.048	0.117
Tetracladium marchalianum	0.032	0.070
Centrospora aquatica	0.024	0.070
Clavariopsis stellata	-	0.051
Scorpiosporum minutum	-	0.039
Clavariopsis aquatica	0.056	0.106
Lemoniera aquatica	0.040	0.031
Heliscus lugdunensis	0.080	0.008
Tetracladium setigerum	0.040	0.008
Margaritispora aquatica	-	0.008
Lunulospora curvula	0.008	0.004
Dactyella aquatica	0.016	-
Flabellospora sp	0.022	-

Table 63. RIVs for fungal species on conditioned, control leaf material in laboratory tests.

Species	Week1	Week 6
Anguillospora longissima	0.279	0.249
Tetrachaetum elegans	0.463	0.234
Anguillospora crassa	0.199	0.226
Alatospora acuminata	0.115	0.206
Tetracladium angulata	0.115	0.188
Tetracladium marchalianum	0.099	0.159
Centrospora aquatica	0.091	0.159
Clavariopsis stellata	-	0.122
Scorpiosporum minutum	-	0.110
Clavariopsis aquatica	0.089	0.106
Lemoniera aquatica	0.107	0.085
Heliscus lugdunensis	0.191	0.044
Tetracladium setigerum	0.107	0.044
Margaritispora aquatica	-	0.044
Lunulospora curvula	-	0.022
Dactyella aquatica	0.060	-
Flabellospora sp	0.030	-

Table 64. A Values for fungal species on conditioned leaf material exposed to 132 µgCu/l in laboratory tests.

Species	Week1	Week 6
Tetracladium marchalianum	0.071	0.152
Tetracladium angulata	0.036	0.152
Tetrachaetum elegans	0.179	0.152
Anguillospora longissima	0.143	0.152
Lemoniera aquatica	0.107	0.091
Alatospora acuminata	0.107	0.091
Anguillospora crassa	0.107	0.061
Tetracladium setigerum	-	0.061
Clavariopsis aquatica	-	0.030
Centrospora aquatica	0.036	0.030
Dactyella aquatica	-	0.030
Heliscus lugdunensis	0.071	-
Picularia	0.071	-
Articullospora tetracladia	0.071	-

Table 65. B Values for fungal species on conditioned leaf material exposed to 132 µgCu/l in laboratory tests.

Species	Week1	Week 6
Tetracladium marchalianum	0.071	0.362
Tetracladium angulata	0.022	0.308
Tetrachaetum elegans	0.533	0.138
Anguillospora longissima	0.100	0.062
Lemoniera aquatica	0.078	0.038
Alatospora acuminata	0.100	0.023
Anguillospora crassa	0.044	0.023
Tetracladium setigerum	-	0.007
Clavariopsis aquatica	-	0.023
Centrospora aquatica	0.011	0.007
Dactyella aquatica	-	0.007
Heliscus lugdunensis	0.044	-
Picularia	0.022	-
Articullospora tetracladia	0.022	-

Table 66. RIVs for fungal species on conditioned leaf material exposed to 132 µgCu/l in laboratory tests.

Species	Week1	Week 6
Tetracladium marchalianum	0.093	0.514
Tetracladium angulata	0.058	0.460
Tetrachaetum elegans	0.712	0.290
Anguillospora longissima	0.243	0.214
Lemoniera aquatica	0.185	0.129
Alatospora acuminata	0.207	0.114
Anguillospora crassa	0.151	0.084
Tetracladium setigerum	-	0.068
Clavariopsis aquatica	-	0.053
Centrospora aquatica	0.047	0.037
Dactyella aquatica	-	0.037
Heliscus lugdunensis	0.151	-
Picularia	0.093	-
Articullospora tetracladia	0.093	-

Table 67. A Values for fungal species on sterile, control leaf material in laboratory tests.

Species	Week 1	Week 6
Tetracladium angulata	-	0.100
Alatospora acuminata	-	0.100
Clavariopsis aquatica	-	0.100
Anguillospora longissima	0.222	0.100
Tetracladium marchalianum	-	0.100
Tetrachaetum elegans	0.556	0.100
Clavariopsis stellata	-	0.080
Anguillospora crassa	-	0.080
Scorpiosporum minutum	-	0.060
Centrospora aquatica	-	0.040
Dactyella aquatica	-	0.020
Lemoniera aquatica	-	0.040
Heliscus lugdunensis	-	0.040
Tetracladium setigerum	-	0.020
Margaritispora aquatica	-	0.020
Articullospora tetracladia	0.222	-

Table 68. B Values for fungal species on sterile, control leaf material in laboratory tests.

Species	Week 1	Week 6
Tetracladium angulata	-	0.170
Alatospora acuminata	-	0.149
Clavariopsis aquatica	-	0.129
Anguillospora longissima	0.250	0.119
Tetracladium marchalianum	-	0.072
Tetrachaetum elegans	0.625	0.062
Clavariopsis stellata	-	0.072
Anguillospora crassa	-	0.057
Scorpiosporum minutum	-	0.057
Centrospora aquatica	-	0.046
Dactyella aquatica	-	0.026
Lemoniera aquatica	-	0.015
Heliscus lugdunensis	-	0.015
Tetracladium setigerum	-	0.005
Margaritispora aquatica	-	0.005
Articullospora tetracladia	0.125	-

Table 69. RIVs for fungal species on sterile, control leaf material in laboratory tests.

Species	Week 1	Week 6
Tetracladium angulata	-	0.270
Alatospora acuminata	-	0.249
Clavariopsis aquatica	-	0.229
Anguillospora longissima	0.472	0.219
Tetracladium marchalianum	-	0.172
Tetrachaetum elegans	1.181	0.162
Clavariopsis stellata	-	0.152
Anguillospora crassa	-	0.137
Scorpiosporum minutum	-	0.117
Centrospora aquatica	-	0.086
Dactyella aquatica	-	0.064
Lemoniera aquatica	-	0.055
Heliscus lugdunensis	-	0.055
Tetracladium setigerum	-	0.025
Margaritispora aquatica	-	0.025
Articullospora tetracladia	0.347	-

Table 70. A Values for fungal species on sterile leaf material exposed to 113 µgCu/l in laboratory tests.

Species	Week 1	Week 6
Clavariopsis aquatica	-	0.121
Alatospora acuminata	-	0.152
Tetracladium marchalianum	-	0.121
Anguillospora longissima	-	0.121
Centrospora aquatica	-	0.121
Tetracladium angulata	-	0.091
Lemoniera aquatica	-	0.091
Tetrachaetum elegans	1.000	0.061
Tetracladium setigerum	-	0.030
Scorpiosporum minutum	-	0.030
Clavariopsis stellata	-	0.030
Dactyella aquatica	-	0.030

Table 71. B Values for fungal species on sterile leaf material exposed to 113 µgCu/l in laboratory tests.

Species	Week 1	Week 6
Clavariopsis aquatica	-	0.236
Alatospora acuminata	-	0.111
Tetracladium marchalianum	-	0.125
Anguillospora longissima	-	0.125
Centrospora aquatica	-	0.111
Tetracladium angulata	-	0.069
Lemoniera aquatica	-	0.069
Tetrachaetum elegans	1.000	0.097
Tetracladium setigerum	-	0.014
Scorpiosporum minutum	-	0.014
Clavariopsis stellata	-	0.014
Dactyella aquatica	-	0.013

Table 72. RIVs for fungal species on sterile leaf material exposed to 113 µgCu/l in laboratory tests.

Species	Week 1	Week 6
Clavariopsis aquatica	-	0.357
Alatospora acuminata	-	0.263
Tetracladium marchalianum	-	0.246
Anguillospora longissima	-	0.246
Centrospora aquatica	-	0.232
Tetracladium angulata	-	0.160
Lemoniera aquatica	-	0.160
Tetrachaetum elegans	2.000	0.158
Tetracladium setigerum	-	0.044
Scorpiosporum minutum	-	0.044
Clavariopsis stellata	-	0.044
Dactyella aquatica	-	0.043

Appendix 10.

Limitations of indexes of fungal abundance using RIV's and SEM.

There are inherent problems with both relative importance value and scanning electron microscopy techniques for determining fungal cover:

a. Relative importance values.

It is only the fungal mycelia which cause decomposition of the leaf substrate, but the identity of these hyphae can not be directly determined (Barlocher, 1982). Hence by stimulating the hyphae to sporulate an indication of which species are present may be obtained. However, not all the fungi active on the substrate will necessarily sporulate on it (Barlocher and Kendrick 1974), and this is especially so when the mode of action of a pollutant may be to inhibit sporulation. Hence, just because a species of fungus does not sporulate on a leaf does not necessarily mean the hyphae of that fungus are not growing on the leaf. In addition to these problems, it has also been found that the frequency of isolation of a fungus from leaf matter is a poor guide to the importance of that fungus in decomposition (Hering, 1967).

b. Scanning electron microscopy.

Every precaution was taken to try to randomly select leaf areas and examine as many samples of leaf per concentration as was possible. This was done in order to try to compensate for the patchy distribution of fungi on leaf material. Fungi on leaves exposed to higher concentrations of copper were likely to have a more patchy distribution, and the probability of selecting patches of leaf with no fungal hyphae associated with it would therefore increase with increasing copper concentration. Hence SEM estimates of fungal cover are subject to error due to the low number of observations that may be made and the patchy distribution of fungi on a leaf.

Using RIV techniques, species of aquatic hyphomycetes were detected on sterile leaf material exposed to 83.98 and 339.17 µgCu/l. However, there appeared to be an almost complete lack of fungal colonisation at these concentrations when investigated using SEM techniques. The difference in results was likely to be due to a much larger quantity of leaf material examined using RIV techniques and the fact that the presence of only one spore is needed for the presence of that species to be noted. The occurrence of a species however, may not have been widespread or significant on the leaf material as a whole.

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