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TOXICOLOGICAL STUDIES OF THE COMBINED EFFECTS OF ALUMINIUM, COPPER AND MANGANESE ON A FRESHWATER AMPHIPOD IN ACIDIC WATERS

by

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DEDICACE

for Tar-Nzema, Mwan'anza, my parents Delphin & Aline Musibon, and my wife Gilbertine & children THYFACQ Musibon for their constant love and support...

D.E.M

DECLARATION

This thesis supports the results of original research which I have carried out in the Department of Zoology at the University of Cape Town, between 1995 and 1997. About 30,000 individuals of *Paramelita nigroculus* (B.) were used in this work. None of this work has been submitted in whole or in part for any other degree and any technical assistance I have received is fully acknowledged.

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Abstract

This thesis describes the combined toxicity in aqueous solutions of three common metals (Al, Cu and Mn) on the endemic freshwater amphipod *Paramelita nigroculus* (Barnard) from the south-western Cape region of South Africa.

The aims of the work were three-fold:

1. to examine the toxic effects of combinations of the three metals;
2. to investigate such effects on an indigenous aquatic organism; and
3. to investigate the adequacy of the South African "Guidelines for aquatic ecosystems", which are derived from data based on the effects of individual elements.

Because physical and chemical conditions in south-western Cape streams are different from those in Europe and North America, it is necessary to develop testing procedures using indigenous local organisms to allow the verification of national guidelines.

Experiments were done on survival, growth and reproduction of the amphipod, as well as on interactions between Al, Cu and Mn, the process of bioaccumulation, and the active uptake of these elements by the amphipod. *Paramelita nigroculus* is still being developed as a test organism and so not all aspects of the life table are yet available.

Tests were of the static-renewal type. In all cases, experiments were in replicates of three and each replicate tested ten individual amphipods. Each set of experiments was run five times, giving an overall number of 150 individuals per concentration. Juvenile, mature (excluding breeding females) and moulting individuals were used as test organisms. Either stream water or 'artificial' water (which is distilled water + inorganic salts containing 85 mg/L Na, 10 mg/L Ca and 5 mg/L Mg) was used for dilution. Test containers were not cleaned and solutions were not aerated. Amphipods were fed twice a week with *Rapanea melanophloeos* leaves (0.5 g dry mass per container) and test solutions were changed twice a week. Total hardness as $CaCO_3$ was below 40 mg/L; pH values were maintained between 4.5 and 5.8; conductivity was maintained between 120 and 350 µS/cm and temperature was 14±1°C in the aquarium. Solutions of Al + Cu and Al + Cu + Mn were used. Experiments lasted for 21 days except for those on reproduction, which lasted for 45 days.

Anova, Student's-t, Newman-Keuls and Mann-Witney U-tests, as well as regression and risk analyses, were used as appropriate. Models used were simple and well suited for determining interactions between concentrations of metal mixtures, water types and survival, growth and reproduction. These models, simple, were not mechanistic. Cox's proportional hazards model was also used to determine the risk of death at increasing concentrations and over increasing exposure times.

Survival after 21-day exposures was 70% for mature amphipods in solutions of Al + Cu + Mn in stream water (at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn) and 62% at concentrations of 1.388

mg/L Al + 0.0175 mg/L Cu only. Survival after 21 days of exposure was 71% and 60% for adults in solutions of Al + Cu + Mn or Al + Cu in 'artificial' water at the same concentrations as above. Under the same circumstances, survival of juveniles was 69% and 62% in stream water and 68% and 62% in 'artificial' water. Survival of moulting amphipods was poor (73% mortality in controls without toxins), no further tests were performed on moulting animals.

I conclude that the South African guidelines at their highest values (AEV: 10 µg/L for Al, 1.6 µg/L for Cu and 1300 µg/L for Mn) are adequate for protecting *P. nigroculus* with respect to mixtures of Al, Cu and Mn because LT_{50} was >21 days at concentrations at least 10 times higher than the guidelines values. Another fact in favour of the adequacy of South African interim guidelines for aquatic ecosystems is that high concentrations of Al (221 µg/L), Cu (9 µg/L) and Mn (323 µg/L) were recorded in the stream from which amphipods were collected in abundance. The increase in pH and alkalinity, and the presence of organics and suspended solids, in natural conditions will further reduce the bioavailability of these metals in water and thus the toxicity.

In each set of experiments, mixtures of Al + Cu proved to be more toxic than those of Al + Cu + Mn. In other words, Mn reduces the combined toxicity of Al and Cu. Precipitation of Al as $Al(OH)_n$ may also play an important role in the reduction in toxicity. The effects of Cu and Mn together are supra-additive (synergistic); of Al and Cu are additive; and of Al + Mn, and Al + Cu + Mn, are antagonistic. Combinations of elements, from the most to the least toxic, are Cu + Mn > Al + Cu > Al + Mn > Al + Cu + Mn, with the respective 96-h LC_{50} values corresponding to 0.32, 0.70, 1.26 and 1.52 toxic units. The percentage of animals surviving after 96-hour exposures to 0.5 LC_{50} proportions were 40%, 54%, 61% and 87% respectively; and the median survival times or LT_{50} in hours were 73, 107, 139 and 147 respectively. This confirms that the toxicity of Al + Cu was higher than that of Al + Cu + Mn.

High (3354 - 14250) bioconcentration factors (BCFs) were obtained at criteria levels after 21 days with Al and Cu, while low (847 - 1500) BCFs were obtained for Mn. *Paramelita nigroculus* is an accumulator of Al, and a weak accumulator/regulator of both Cu and Mn because in almost cases the values of the bioconcentration factor changed significantly with the changes in treatment over a range of concentrations for Al, but not for Cu and especially not for Mn. High mortalities (at all concentrations) after 24-hour and 8-day exposures in both types of dilution water corresponded with periods when the rates of bioaccumulation were highest. Death in sublethal toxicity tests may therefore be attributable to bioaccumulation at low concentrations of pollutants.

Growth and reproduction occurred in all treatments but some significant differences were observed within treatments. Animals in solutions of Al + Cu + Mn showed faster growth rates and higher reproductive rates than those in mixtures of Al + Cu only. These variations cannot presently be attributed only to the effects of the metals since more information on the reproductive biology of *P. nigroculus* is still needed (e.g. the number of offspring and range of body weight in natural conditions).

Juveniles of *P. nigroculus* seem to be good test organisms for water quality control because they have not yet developed hormesis. But further information is needed on the reproductive biology and ethology of this

amphipod before deciding on its daily use for monitoring water quality. Because *P. nigroculus* is so tough, it is an ideal animal for looking, for example, at bioaccumulation and combined toxicities, but the same toughness means that it is not a good bioindicator for field conditions. However, *P. nigroculus* might be useful in chronic testing in acidic waters (e.g. mining areas) where concentrations of metals are high, because of its ability to accumulate metals and to tolerate acidic waters.

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CHAPTER 1

Introduction

1.1 The issue

Water quality is a major concern for aquatic resource managers and for those involved in the conservation of biodiversity. Indeed, as municipalities, industries, agricultural activities and so on discharge wastes, receiving water bodies become more and more polluted.

Aquatic life, and finally human life, is threatened through toxins accumulating in the food-chain. Heavy metals can be toxic and may be a real threat to humans as a result of pollution by so-called development activities (industries, urbanization, agriculture, etc.). Tissues or organs adversely affected by heavy metals include the central nervous system (CNS), the peripheral nervous system (PNS), the renal system, the liver, the blood cells, oral and nasal mucosas, hair, the respiratory tract, the skeleton, the cardiovascular system and the reproductive system. Teratogenesis and chromosomal aberrations may also result from contamination with heavy metals. The organs affected depend on the metal species involved and on the age and sex of people exposed. For instance, children are more at risk from damage to the CNS by methyl-mercury and lead, and older women are more sensitive to the effects of cadmium on bones (Walker 1975, Clarkson *et al.* 1984). The target organs for cadmium and mercury are kidney and brain, and for lead the blood system and the brain, while arsenic appears to be non-specific, though the skin is significantly affected. It is not rare to hear about poisoning by methyl-mercury or other mercury compounds of communities after continuous consumption of fish from polluted waters. Examples include human communities from Minamata Bay (1953-60s), Iraq (1956, 1960, 1971-72), Guatemala (1963-65), Niigata (1965), Ghana (1967), Pakistan (1969) and Canada (1970s), Alexander 1974, Walker 1975, Baloh *et al.* 1979, Butler 1979, Clarkson *et al.* 1984, Bruaux & Svartengren 1985, Baghurst *et al.* 1987, Patterson & Passino 1989).

Pollution of water may be understood as the introduction by humans, directly or indirectly, into aquatic environment of substances or energy that result in such deleterious effects as harm to living resources, hazards to human health, hindrance to aquatic activities such as fishing, impairment of water quality for consumption and reduction of amenities (Reish & Oshida 1986). Pollution may become the key cause of degradation in water quality, and therefore, the main constraint to the maintenance of biodiversity of ecosystems. Water quality defines all characteristics of water body for an appropriate use. Indeed water quality depends on the water use (e.g. drinking, recreational, agricultural uses). The effects of pollutants on aquatic ecosystems have caused the World Health Organization (WHO) and many governments (e.g. Dutch, South African, USA) to define standard values or criteria as the maximal concentrations of toxins permissible in natural waters for conservation of aquatic life (e.g. Lamb 1985, WHO 1987, 1990, Musibono 1992, Hespanol & Prost 1994, DWAF 1995).

The South African Water Quality guidelines for Aquatic Ecosystems (DWAF 1995) are essentially a specification of:

- the in-stream water quality required to protect aquatic ecosystems;
- numerical and narrative criteria corresponding to chronic and acute toxic effects for toxic constituents where this information is available;
- numerical and narrative criteria to protect ecosystem structure and functioning, in the case of non-toxic constituents and system variables;
- numerical and narrative criteria to protect aquatic ecosystems against changes in trophic status in the case of nutrients;
- modifications that can be made to water quality criteria on a site-specific basis, whilst still providing the same level of protection as the original criteria.

The South Africa Water Quality Guidelines for Aquatic Ecosystems are used by the Department of Water Affairs and Forestry in water quality management as the primary source of reference information and decision support required for the management and protection of aquatic ecosystems. These guidelines have been designed primarily for use by water quality managers. Educators and other interested members of the general public will also find them a valuable source of information.

The Water Quality Criteria should be understood as scientific and technical information provided for a particular water quality constituent, in the form of numerical data and narrative descriptions, of its potential effects on the health and integrity of aquatic ecosystems and the fitness of water for appropriate uses. These criteria are derived from the best available information, using species representative of the major trophic groups. The rationale for this is that if the most sensitive species within representative trophic groups are protected, then other species within the trophic group will also be protected even after long-term exposure to water of a given quality.

Three broad classes of water quality criteria can be identified for aquatic ecosystems:

- constituent-specific criteria, where a numerical value or range for each constituent of concern represents a level of ecological risk associated with the presence of that constituent in the water;

- criteria for complex mixtures, in which the whole-effluent toxicity testing approach is followed to evaluate the toxicity of complex mixtures containing several constituents, where the individual effects of each constituent cannot be resolved, and where synergistic and antagonistic effects may occur;

- biological criteria, which may be either numerical values or narrative expressions that describe the biological status of aquatic systems.

The actual criteria (DWAF 1995 as published in 1996: Document 7) deal only with constituent-specific water quality class. Four ranges of values are defined:

- the No Effect Range (NER), derived for each water quality constituent in each of four categories (i.e. toxins, system variable, non-toxic inorganics and nutrients). This is the range of concentrations or levels within which no measurable adverse effects are expected on the health and integrity of aquatic ecosystems, and which should therefore ensure their protection. They assume lifelong exposure;

- the Target Water Quality Range (TWQR) is not a water quality criterion but is rather a management objective which has been derived from numerical or narrative criteria. In keeping with the goal of protecting the health and integrity of aquatic ecosystems, the TWQR is set equal to the No Effect Range;

- the Chronic Effect Value (CEV) is defined as that concentration or level of a constituent at which there is expected to be a significant risk (5 % or less) of measurable chronic effects to the sensitive organisms in the aquatic population. If such chronic effects persist for some time and/or occur frequently, they can lead to the eventual death of individuals and disappearance of sensitive species from aquatic ecosystems. It is important to note that even if the concentration of a constituent is always below the chronic effect value there is still a significant risk of chronic effects to a small percentage of the most sensitive organisms; and if the CEV is exceeded the chronic effects will be more widespread, and the likelihood of possible acute effects will increase with increasing concentration, frequency and duration of exceedance of the chronic effect value;

- the Acute Effect Value (AEV) is defined as that concentration or level of a constituent above which there is expected to be a significant risk (5 % or less) of acute toxic effects to sensitive organism in the aquatic population. If such acute effects persist for even a short while, or occur at too high a frequency, they can quickly cause the death and disappearance of sensitive species or communities from aquatic ecosystems.

Finally, based on all the above 'ingredients', the actual South African Water Quality guidelines may be defined as a set of information provided for a specific water quality constituent. Each consists of the water quality criteria, namely the TWQR, the CEV and the AEV, together with the support information which includes the occurrence of the constituent in the aquatic environment, the norms used to assess its effects on water uses, and the conditions for case-, site- and region-specific modifications.

Table 1.1 reports national Acute Effect Values for selected metals for protection of organisms for Dutch, US, WHO, and South African cited by Enserink *et al.* 1991, Musibono 1992, Lamb 1985, Spehar & Fiandt 1986, WHO 1987 and DWAF 1996. All these criteria are based on the individual effects of single elements, usually derived from laboratory studies, or based on a comparison between actual concentrations and natural background levels (Enserink 1997: pers. com.), despite the fact that all elements in water exist in mixtures and may interact synergistically or antagonistically.

Table 1.1 *Acute Effect Values for selected dissolved metals for the protection of aquatic organisms. Concentrations are expressed in µg/L. "n.a." = not available*

Metal	***a*** ***WHO*** ***1987***	***b*** ***Dutch*** ***1989***	***c*** ***Dutch*** ***1997 MTR***	***d*** ***Dutch*** ***1997 NR***	***e*** ***South Africa*** ***1996***	***f*** ***U.S.A.*** ***1986***
Al	200	200	n.a.	n.a.	10	100
Cd	12	2.5	2.1	0.9	1.8	12
Cr(VI)	50	50	85	1.6	200	50
Cu	1000	50	3.9	1.1	1.6	100
Hg	0.5	0.5	1.5	0.06	1.7	0.5
Mn	100	50	n.a.	n.a.	1300	50
Pb	50	50	225	3.1	4	5
Zn	500	200	40	12	36	300

a WHO 1987; b Enserink et al. 1991, Musibono 1992; c Enserink 1997, maximum tolerable risk (based on single substances); d Enserink 1997, negligible risk; e DWAF 1996; f Lamb 1985, Spehar & Fiandt 1986.

1.1.1 Synergism and antagonism

Synergistic interactions may have adverse effects on aquatic organisms. For instance the combined effects of heavy metals may result in long-term toxicity (Eaton 1973, Muska & Weber 1977, Spehar *et al.* 1978, Anderson *et al.* 1979, Broderius & Smith 1979, Kiokemeister 1979, Hermens *et al.* 1984, Hutchinson & Sprague 1986). Although many works on aquatic toxicology report individual effects, a limited number deal with

the synergistic and adverse effects of pollutants on ecosystems (e.g. Hermens *et al.* 1984, Biesinger *et al.* 1986, Spehar & Fiandt 1986, SCOPE 1987, Barak & Mason 1989, Enserink *et al.* 1991, Dallinger *et al.* 1993, Roux *et al.* 1993, Arambasic *et al.* 1995, Seymore *et al.* 1995, Musibono *et al.* 1996). One of the few examples is reported by Enserink *et al.* (1991), who tested complex mixtures of eight metals (As, Cd, Cr, Cu, Hg, Ni, Pb and Zn) on *Daphnia magna* at then current Dutch criterion levels (1985-1989) and showed that the criteria for these elements were inadequate for protecting aquatic life when the elements were present in combination. Indeed, arsenic, cadmium, chromium, copper, mercury, lead, nickel and zinc were tested singly and in equitoxic mixtures based on the median survival concentration LC_{50} (individual *Daphnia magna*) or median effect concentration EC_{50} (population growth) of the individual metals. The expected toxicities of these mixtures were expressed as toxic units which are the ratios between the actual concentration of the pollutant in water (e.g. metal) and the LC_{50} or EC_{50} value. The 21-d LC_{50} and EC_{50} for the mixtures were 1.8 and 1.6 toxic units respectively, indicating an additive chronic toxicity of the metals with respect to individual survival as well as to population growth of *Daphnia magna*. Combined at the maximum levels of Dutch criteria operative 1985-89 for maintaining adequate water quality, these metals were severely toxic to *D. magna* and caused 50% mortality in rainbow trout *Salmo gairdneri* exposed for 60 days during larval development. Even a reduction of these concentrations by a factor of five produced a 10% decrease in the yield of *D. magna* populations. Similar results have been reported by Van Leeuwen *et al.* (1986, 1989), who tested binary mixtures of selected metals (i.e. As, Cd, Cr, Cu, Hg, Ni, Pb, Zn) on *D. magna* at then current Dutch criterion levels. They also reported the combined effects to be additive and to have affected reproduction. Indeed, when occurring singly, concentrations of 50 μg/L for Ni, As, Cu, Cr, and Pb; 200 μg/L for Zn; 2.5 μg/L for Cd and 0.5 μg/L for Hg did not have detrimental effects on aquatic life. In combination, though, concentrations of 31 μg/L for Ni, As, Cu, Cr and Pb; 124 μg/L for Zn; 1.55 μg/L for Cd and 0.31 μg/L for Hg provoked 50% inhibition of population growth of *Daphnia magna*. Biesinger *et al.* (1986) tested binary mixtures of Cd, Cu and Cr in chronic toxicity experiments. They concluded that concentrations of metals which caused no significant effects on the reproduction of *Daphnia magna*, if present singly, exerted a toxic action in mixtures.

Spehar and Fiandt (1986) showed that mixtures of As, Cd, Cr, Cu, Hg and Pb, at concentrations based on United States water quality criteria, induced chronic effects in two species of fish (the rainbow trout *Salmo gairdneri* and the fathead minnow *Pimephales promelas*) and one invertebrate (the daphnid *Ceriodaphnia dubia*). As, Cd, Cr, Cu, Hg and Pb combined at highest criterion concentrations caused almost 100% mortality in rainbow trout and daphnids during acute exposures. Fathead minnows *Pimephales promelas* were not adversely affected at this or twice this concentration, although a mixture of four to eight times the maximum value caused 15 to 60% mortality. Metals combined at lower criterion concentrations significantly reduced production of daphnid young after 7 days and growth of fathead minnows after 32 days. Embryo hatchability and survival of rainbow trout were reduced at four times these values. Chronic tests showed that the joint action was less than

additive for fathead minnows but close to strictly additive for daphnids, indicating that long-term interactions of metals may be different in fish than in invertebrates. Adverse effects were observed at mixture concentrations of half the maximum allowable toxicant concentration (MATC) for fathead minnows and one third the MATC for daphnids, suggesting that components of mixtures at or below 'no effect' concentrations may contribute significantly to the chronic toxicity of a mixture. Musibono (1994) and Musibono *et al.* (1996) reported mixtures of Cd, Co, Cr, Cu, Pb and Zn to be toxic to Nile lettuce, *Pistia stratiotes*, at WHO criterion levels in both short-term (96-h exposure) and long-term (21-day exposure) toxicity tests. Indeed, apical necrosis of young leaves appeared in both cases, while single-chemicals experiments did not result in necrosis. Recently, Lin *et al.* (1996), studying the individual and combined effects of copper and silver ions on the activation of the bacterium *Legionella pneumophila*, concluded that both copper and silver ions are effective in inactivating the bacterium and that the combined effect is greater than that seen with either ion alone. Indeed, at a concentration of 100 μg/L of Cu^{2+}, *L. pneumophila* was completely inactivated within 2.5 hours of exposure, while more than 24 hours was required to achieve similar reduction at the highest Ag^{+} ion concentration tested (i.e. 80 μg/L). In combination, results showed additive or synergistic effects depending on the concentration of Cu^{2+} and Ag^{+} ions. Lin *et al.* found that Cu^{2+} and Ag^{+} ions synergistically eradicated *L. pneumophila* serogroup 1 at higher combined concentrations of Cu^{2+} and Ag^{+} ions (i.e. 40 + 40 μg/L) while only an additive effect was observed at lower combined concentrations (i.e. 20 + 20 μg/L). These values were lower than those of Cu^{2+} and Ag^{+} ions when used singly (i.e. 100 μg/L for Cu^{2+} ion and 80 μg/L for Ag^{+} ion). All these results point out the need for additional studies to determine the type and degree of interaction of toxicants because single-chemical water quality criteria may not sufficiently protect some species when other toxicants are also present.

The combined effects of metals or trace elements do not always increase toxicity. They may also reduce toxicity in some cases. Thus arsenic (III), for example, is very toxic when present alone but in combination with manganese (II) it is oxidized to arsenic (V), which is not toxic. Oxido-reduction reactions seem to be responsible for either synergism or antagonism (Driehaus *et al.* 1995). Lead, which is toxic, competes with iron (an essential metal) in the intestine by inhibiting the incorporation of iron into protoporphyrin IX. Lead increases the deficiency of calcium on one hand, and on the other hand, calcium can alleviate lead toxicity (this may explain why lead workers are asked to drink milk). Lead also interferes with zinc enzymes and so added zinc can alleviate the effects of lead. Lead increases copper deficiency (Clarkson *et al.* 1984, Patterson & Passino 1989). In short, the toxicity of heavy metals in water or in organisms may increase or be reduced in mixtures.

1.1.2 Effects of heavy metals on freshwater organisms

The effects of heavy metals on freshwater organisms are reported in various works. For example, Monteiro *et al.* (1995), reporting the effects of metal stress on plankton communities of the Sado River in Portugal, concluded that dissolved metals at concentrations of Cu = 88 μg/L; Cd = 2.6 μg/L and Zn = 1800 μg/L

reduced the abundance of major taxa by 40 - 100%, of species richness by 59% and of diversity by 58 -75%, and that only a few taxa (e.g. some algae, the copepod *Acanthaclops robustus* and the protozoan *Arcella vulgaris*) tolerated high metal concentrations. The toxicity of copper to aquatic life is well documented (e.g. Viarengo *et al.* 1981, Winner & Gauss 1986, Arumugam & Ravindranah 1987, Hatekeyama 1988, Vasseur *et al.* 1988a, Roberts *et al.* 1990, Bardeggia & Alikhan 1991, Shuttleworth & Unz 1991, Maud *et al.* 1992, Nussey *et al.* 1995a, 1995b, 1995c, Pynnonen 1995, Taylor *et al.* 1995). However, the ability of this metal to bind algal exudates and other extracellular ligands may reduce the concentration of free copper ions and thus mitigate potentially toxic effects in organisms. Van Vuren *et al.* (1994) reported copper to have detrimental effects on fish blood. Indeed, according to their work on the blood of the catfish *Clarias gariepinus* from South Africa, copper causes significant changes in blood chemistry, as well as resulting in evident pathological conditions such as erythrocytopenia, leucocytosis, hyperglycemia and hyperprotonemia at concentrations of 32 and 85 μg/L, corresponding to those found in the Olifants River, Mpumalanga, South Africa during summer and winter period respectively. This shows obvious relationships between water pollution with copper and the contamination of fish with the same metal. Nussey *et al.* (1995) reported on the effects of copper on the haematology and osmoregulation of the Mozambique tilapia, *Oreochromis mossambicus*. The haematology of *O. mossambicus* investigated after short-term 96-h exposures to 0.16 mg/L and 40 mg/L, as well as long-term 4-week exposures to 40 mg/L at two temperatures, showed a physiological effect in the form of leucocytosis and morphological changes in the blood cells reflected by decreases of the concentrations of the major ions (sodium, potassium, calcium, chloride) in the plasma. Although not lethal, these changes had significant effects on respiratory and osmoregulatory functions of the gills. Indeed, morphological changes in blood cells may affect gas (oxygen and carbon dioxide) transport by red cells as well as ionic balance in the plasma. These primary changes will inevitably lead to secondary physiological responses that could affect various organ systems (e.g. liver, kidney, brain). Finally, these changes may be seen as an initial response to copper as a toxicant or as a destabilizer of homeostatic reaction. Nussey *et al.* (1995) also found copper to induce haemophilia at 29°C (summer) and at 19°C (winter) and thrombocytopenia. Thus exposure to copper causes defects in coagulation that can cause haemorrhages and may eventually cause the death of the fish. Significant increases in numbers of lymphocytes and eosinophils, combined with significant decreases in monocytes and neutrophils, are indicative of changes that set in after short-term exposures to copper.

High levels of aluminium (Al) in human brains have been implicated in Alzheimer's disease (Brady & Griffiths 1995, Mackie & Kilgour 1995). Al is reported to be toxic to zebra mussel veliger larvae, especially at pH values < 5.0 in acidic water at concentrations of 10-30 mg/L (Mackie & Kilgour 1995). Buckler *et al.* (1995), reporting the effect of Al on the survival, sublethal responses and tissue of atlantic salmon, *Salmo salar*, concluded that egg hatching and the growth of larvae were reduced significantly at pH 4.5-5.0; that larval mortality increased at pH 4.5; that larval feeding and swimming behaviour were impaired at pH 6.5 and lower,

and that at pH 5.5, reduced growth occurred in larvae exposed to concentrations of 0.071 mg/L Al or more. Fish exposed to 0.033 mg/L Al had a concentration of 0.003 mg/g Al in their whole body-tissue, whereas those exposed to 0.264 mg/L Al had concentrations of 0.096 mg/g in their tissues (Buckler *et al.* 1995). Humic acids however reduce the bioavailability of Al by binding this metal (Tipping *et al.* 1991). Biesinger & Christensen (1972) cited by Dallas and Day (1993) reported the 48-h LC_{50} for *Daphnia magna* in Al was 3.9 mg/L, while the 21-d LC_{50} for *D. magna* is 0.022 mg/L for Cu, and finally, the 21-d LC_{50} is 4-6 mg/L for Mn.

Toxicity due to manganese (Mn) is not well documented. However a deficiency of Mn in vertebrates leads to skeletal deformities but high concentrations are toxic, leading to disturbances of various metabolic pathways and also of the central nervous system by inhibition of dopamine formation (e.g. Dallas & Day 1993, Seymore *et al.* 1995, DWAF 1995).

1.2 Aquatic toxicological studies

The study of the effects of pollutants in ecosystems, or ecotoxicology, is of worldwide interest. Pollutants in water bodies may cause death, or even reduction in growth and reproduction, changes in behaviour, teratogenesis and genetic disturbances in aquatic organisms. To understand what really happens with respect to heavy metal pollution, laboratory studies are performed using selected toxicants and test organisms.

Different toxicity tests (bioassays) are run in the short term to ascertain the effects of acute toxicity and in the long term to ascertain the effects of chronic toxicity. The lengths of time commonly used are 96 hours for acute toxicity tests and 21 days for chronic toxicity tests. Test organisms are exposed to increasing concentrations of a toxicant in order to identify some change in the organisms. Death is generally used as the criterion in 96-hour acute tests. One or more controls are employed, in which some organisms are exposed to similar conditions but without toxicants in order to provide a measure of experimental acceptability. The concentration that causes death of 50% of the population is defined as the lethal concentration (LC_{50}), which illustrates aspects of acute toxicity. These experiments are conducted under controlled laboratory conditions. The data generated may be used to evaluate the effects of toxicants discharged into the aquatic environment. This practice assists in the development and application of water quality criteria for protection of the aquatic environment. This is not ideal because we have little information on the extent to which it is legitimate to apply laboratory-based results in the real world (i.e. in real ecosystems). Nevertheless these results remain an indication of what might happen in real situations. The South African guideline values, which are based on laboratory data multiplied by a safety factor, may be inadequate.

1.2.1 Types of bioassays

The term 'bioassay' signifies a test in which living tissues or organisms are used as reagents for the determination of the potency of any physiologically active substance of unknown activity (FAO 1983). In short, 'bioassay' often signifies 'toxicity test'. Many types of bioassays, from simple short-term tests to highly sophisticated long-term experiments, may be used. They are defined according to the duration of the experiment, as indicated above, and to the methods of adding toxicant to the test solution. Static tests are tests in which test solutions are not renewed (convenient for short-term tests); in renewal tests, the test solution in each experimental container is replaced periodically with fresh solution. The renewal period varies according to the species of organism and the toxicant. Finally, flow-through tests may be used in some cases; these are highly sophisticated bioassays in which the test solutions are renewed continuously. This type of test is sometimes more reflective of field conditions, but there are many inherent problems such as large space requirements, large quantities of water and toxicants used, and the need for a complex water delivery system. The choice of the type of test to be conducted depends on the selection of test organism (e.g. size, habitat, behaviour), the laboratory facilities, and the know-how of the researcher. Parameters that may be used as data include:

- the proportion of live and dead organisms
- growth as change in body size or body mass
- the number of eggs, offspring or hatchlings as a measure of reproduction
- biostimulation (a measure of the effect of a toxicant on primary productivity)
- bioaccumulation.

Bioaccumulation is defined as the increased concentration of a toxicant in an organism over time. To determine the rate of bioaccumulation, organisms which have survived a test period are analysed for the amount of the toxicant they contain. A minimum depuration period is required (i.e. 24 hours) at the end of the test if the organism has been fed during the course of experiment. The entire body of small test organisms is analysed but separate organs such as liver, kidney, muscle, brain, and digestive system can be analysed individually in larger organisms such as fish (FAO 1977, Ward & Parrish 1983, Reish & Oshida 1986, USEPA 1991, Plenet 1995).

1.2.2. Test organism used in aquatic toxicological studies

Various taxa, from bacteria and invertebrates to vertebrates such as fish, are used as test organisms in aquatic toxicology. Members of the aquatic flora (e.g. algae, macrophytes, bryophytes, diatoms, etc.) may also be used in some cases. However, most commonly used taxa seem to be Atlantic salmon *Salmo salar* and *S. trutta*, the rainbow trout *Onchorhynchus mykiss* and the brook trout *Salvelinus fontinalis* (Spehar & Fiandt 1986, Memmert 1987, Goodrich 1991, USEPA 1991, Buckler *et al.* 1995, Smith & Haines 1995, Steinberg *et al.* 1995). Many

other fishes, such as *Catastomas commensoni*, *Channa punctatus*, *Ictalurus punctatus*, *Lepomis macrocheir*, *Lepomis cyanellus*, *Micropterus salmoides*, *Micropterus dolomieui*, *Notroptis spilopterus* and *Pimephales promelas* are also used (Dallas & Day 1993). In South Africa, tilapia and catfish species have been used in aquatic toxicological studies (Van Pittius *et al.* 1992, Van Vuren *et al.* 1994, Nussey *et al.* 1995a, 1995b and 1995c). The use of invertebrates is widespread and organisms such as the waterflea *Daphnia magna*, the amphipod *Gammarus pulex*, the isopod *Asellus aquaticus* and dipterans of the genus *Chironomus* are commonly used as bioindicators of water quality (Phillips & Segar 1986, WHO 1987, Barak & Mason 1989, Gabric *et al.* 1990, Elendt 1990, Muzinger 1990, Cahon *et al.* 1991, Hatakeyama & Shiraishi 1991, Van Hattum *et al.* 1991, Whitehurst 1991, Xu & Pascoe 1993, Comber *et al.* 1993, Pascoe *et al.* 1994, Arambasic *et al.* 1995, Malby 1995, Santojanni *et al.* 1995, Plenet 1995, Mullis *et al.* 1996, Kooijman & Bedaux 1996).

Criteria need to be based on experimental results for a variety of taxa (e.g. USEPA requires at least one fish, one plant, one invertebrate). In South Africa, the use of local organisms in aquatic toxicological tests is of increasing interest because the natural conditions differ from those where most test organisms originated (i.e. Europe and North America) and the evolutionary histories of the organisms are different (different gene pools from those in Europe because of long periods of isolation and different selective pressures). Fish, snails, mayflies, amphipods, bacteria, protozoans, isopods and worms have been or are being used (Slabbert & Grabow 1986, Slabbert 1988, Dallas & Day 1993, Roux *et al.* 1993, Van Vuren *et al.* 1994, Seymore *et al.* 1995, Wepener *et al.* 1995, Reinecke *et al.* 1996a, 1996b).

In the south-western Cape, the endemic freshwater amphipod *Paramelita nigroculus* (Barnard) has been examined as a potential test organism for biomonitoring Western Cape acidic waters (Tian 1996). The life history of this local freshwater amphipod is not completely documented. However, Stewart and Davies (pers. com.) studying its life history and reproductive biology found that monthly densities of this amphipod were generally high, varying from 264 to 12 227 individuals per m^2. The mean density of 7,972 indivivduals per square meter in summer was significantly higher than the value of 1 071 calculated for winter. Sizes of animals ranged from 1 to 11 mm, with females reaching a larger size than males. As a result of continuous breeding throughout the year, size frequency distributions were similar for all months, and cohorts were difficult to discern.

P. nigroculus is abundant in the south-western Cape in mountain streams. It is robust, surviving well under laboratory conditions and in acidic humic waters (pH 4.0-6.5 and total organic carbon 75 - 135 mg/L C: Tian 1996). It is easy to feed, has no aerial phase, and lives in near-pristine "unpolluted" streams. Its one-year life cycle and its toughness make *P. nigroculus* an ideal test organism for use in chronic toxicity tests. As well as these advantages, *P. nigroculus* also has some disadvantages as a laboratory organism. It is very tolerant towards low pH and high dissolved humic materials (personal observation: Tian 1996), varying aluminium

concentrations (Tian 1996) and salinity changes (Tyson 1993), which might make it unsuitable as a test organism for short-term or acute toxicity tests.

Studies by Buchanan *et al.* (1988) on the thermal acclimation and tolerance to high temperatures in *P. nigroculus* showed that LT_{50} values (in minutes) for animal acclimated to 13.5 °C ranged from approximately 300 minutes at 27°C to 4 minutes at 31°C. LT_{50} values for individuals acclimated to 20°C were significantly longer ($p < 0.05$) than for those acclimated at 13.5°C at corresponding test temperatures. More information and data on this amphipod are still needed regarding its reproductive biology, physiology and ethology.

Tian (1996) studied the diet and feeding regime of *P. nigroculus* using data both from the natural habitat (i.e. Skeleton Gorge Window stream) and laboratory results. She concluded that *P. nigroculus* mainly feeds on dead leaves of *Rapanea melanophloeos*. She found that the consumption rates were significantly higher at 15-20°C than at 10°C ($p < 0.05$). At 20°C, all individuals died after 2 weeks only, and best results in laboratory conditions for survival, feeding and growth rates were recorded at 15°C. Tian also studied the effects of humic waters, low pH and aluminium concentrations on the survival of this amphipod. She found that no animal died during short-term (96 hours) exposure to waters even more humic than those where the animals naturally occur and that the percentage survival after 60 days ranged from 60 $\pm$ 10 to 93.33 $\pm$ 5.77 %. At pH 3.5, the median survival time (LT_{50}) for individuals (mature amphipods: size > 4mm) exposed to 1125 mg/L Al was about 25 hours. When animals were exposed to 10 mg/L Al at the same pH value (pH 3.5) LT_{50} was > 96 hours. She concluded that *P. nigroculus* was tolerant to Al at low pH. A lot of work is still required before the information on *P. nigroculus* is comparable to that on the very famous *Daphnia magna*. For example, the life history, the reproductive biology and the ethology of this amphipod are not yet well undesrtood. It is clear though that because *P. nigroculus* is tolerant of low pH and of trace elements (at least of Al), it should be a useful subject for studying aspects of synergistic and antagonistic effects. My study, on the biological responses to the combined effects of three common metals, is my contribution to the information 'bank'.

The South African Department of Water Affairs and Forestry (DWAF) is in the process of defining guidelines for water quality for sustaining aquatic life. Three types of value or criterion are defined for each constituent: the Target Water Quality Range (TWQR), the Chronic Effect Values (CEV) and the Acute Effect Values (AEV). Each of these is set for single constituents and does not take into account the potential synergistic or antagonistic reactions between constituents in real effluents. Thus these criteria might be inadequate or even more stringent than necessary for protecting aquatic life. An indication of their efficacy from toxicological studies should be obtained before these criteria can become legal requirements.

1.3 Choice of experimental material

Aluminium (Al) is one of the most toxic of the trace metals, and is probably not an essential nutrient in any organism (Dallas & Day 1993). Its solubility is strongly pH-dependent and its toxicity depends on the chemical species involved. At alkaline pH values, Al is present as soluble or insoluble and biologically unavailable hydroxide complexes. At intermediate pH values, Al is sparingly soluble and probably occurs as hydroxo- and polyhydroxo-complexes. Under acid conditions (pH < 5.0), it occurs as the soluble, available toxic hexahydrate (aquo) species (Al^{3+}). Al also precipitates at pH < 5.0 and forms toxic hydroxides $Al(OH)_3$ or $Al_4(OH)_{10}SO_4$.

Aluminium is found in soluble form in acid water draining from mines and in natural waters affected by acid rain. In waters naturally acidic because of the presence of acidic organic compounds, Al is also present but is not bioavailable because it is adsorbed onto organic molecules. Al as aluminium hydroxide is used for coagulation/flocculation processes in drinking water treatment plants (Shore & Wyatt 1983, Driscoll & Schecher 1990, Epstein 1991, Shuttleworth & Unz 1991, Tipping *et al.* 1991, Shuman 1992, Cathalifaud *et al.* 1997). However its binding capacity with alginate in the presence of calcium, and also with organics, may reduce the availability of Al, and therefore its toxicity to aquatic biotas, in natural waters.

Gregor *et al.* (1996) studied the interactions of Ca^{2+} and Al^{3+} with alginate to understand how algal extracellular organic matter (EOM) interferes with conventional aluminium-based drinking water treatment. Alginate was used as a model for algal EOM, while binding capacities and binding strengths were determined using an ion-selective electrode for calcium and equilibrium dialysis for aluminium. Calcium and aluminium ions began to bind above pH 3.5, co-incident with alginic acid deprotonation. Steady-state binding was achieved above pH 6. The variation in relative and absolute uptake of the calcium ion as a function of calcium to carboxyl ratio indicates that additional binding sites could be assessed at higher metal loadings. Alginate reached a limiting capacity for Ca ions of approximately one ion per eight carboxyl groups (or eight sugar units). Unlike Ca binding, a fixed number of aluminium binding sites was available for Al, irrespective of total concentration, corresponding to approximately one aluminium ion per seven carboxyl groups (or seven sugar units). So, on the basis of the concentration of the carboxyl groups, alginate and fulvic acids have similar capacities for aluminium ions, indicating that algal EOM can compete for Al intended for consumption by natural organic matter during coagulation. This is of course a disadvantage in the production of drinking water because of the increased amount of Al required where algal EOM has to compete with organics such as fulvic acid. However the addition of Ca ions prior to Al coagulation may minimise the influence of algal EOM on the coagulation process. Thus, the binding of Al to organics reduces the availability of this metal in aquatic environments. Do Mn and Cu affect its behaviour and bioavailability?

Manganese is an essential element at least in glycosal transferase enzymes, which are important in proteoglycan synthesis in vertebrates, and possibly in other enzymes as well. A deficiency in Mn in vertebrates leads to skeletal deformities but high concentrations are toxic, leading to disturbances of various metabolic pathways and also of the central nervous system by inhibition of dopamine formation (Dallas & Day 1993, Seymore *et al.* 1995, DWAF 1995).

Copper is an essential element and one of the world's most widely used metals. The occurrence of natural sources of copper in the aquatic environment is due to weathering or from the dissolution of copper minerals and native copper. Many copper salts are highly soluble as cupric or cuprous ions. Contamination from human source accounts for 33-60% of the total annual input of copper to the aquatic environment (Schuiling *et al.* 1994). The toxicity of copper increases with a decrease in water hardness, in dissolved oxygen and when present in combination with other metals. Copper is very toxic to algae. Fortunately, in the presence of chelating agents such as humic acids, amino acids and suspended solids, its toxicity may be reduced (Eaton 1973, Lewis 1978). Copper is also reported to be toxic to fish (Nussey *et al.* 1995).

The toxicity of heavy metals depends on their bioavailability in the absence of detoxifying agents. Copper, cadmium, zinc, lead, aluminium, manganese, nickel and iron tend to be elevated in acid, soft waters, either as a result of atmospheric input from industrial operations or because of leaching and runoff from soils. Metal chemistry is important if one wishes to make predictions about the surface activity and toxicity of a particular metal species. The ionic index and the covalent index are of particular importance in this regard. The ionic index is defined as a measure of how strong an ion pair a metal will form with a ligand, while the covalent index is a measure of the tendency of a metal to form covalent complexes. So cations of Class A, which include alkali and alkaline earth metals, have a preference for ligands where oxygen is the donor atom (e.g. in the gills of fish) whereas class B cations, which include the Period VI transition metals (from Pt to Bi), have a particularly high affinity for biological binding sites containing nitrogen or sulphur donor ligands (surface and subsurface proteins). The borderline cations, which include most of the remaining transition metals, have less well defined preferences in ligands; generally though, the class B character of these ions increases with covalent index (Nieboer & Richardson 1980, McDonald *et al.* 1989). Since class A (e.g. Al) metals have similarly low covalent indices, their interactions with surface ligands are likely to be competitive, forming increasingly stable complexes largely in proportion to their formal charges. So at equimolar concentrations, Al^{3+} would displace Ca^{2+} and Ca^{2+} would displace Na^{+}. The toxicity of the borderline metals increases approximatively with their covalent index. The key features that determine the toxicity of metal are ionic radius, redox potential, tendency to form insoluble sulphides, donor ligand preference and biological mechanisms of action of metal/H^{+} combinations. It should be clear that in many cases, trace-metal contamination of soft acid waters can be expected to have at least some impact upon the regulation of ions on gill surfaces. Toxicity may consist in

disturbances of the sodium/chloride balance (in the case of Al, Cu, H^+) and calcium balance (in the case of Cd, Zn, Mn), or neural, hepatic or renal disturbances (in the case of Cr, Pb, Hg). Thus, for metals that are surface-active (e.g. Al, Cu, Mn, Cd, Zn), the precise effect on the gills of organisms depends on the metal's concentration in the water, on its ligand preferences and binding characteristics and on water chemistry (pH, hardness and complexing capacity).

Bioaccumulation of metals by aquatic organisms may reduce their bioavailability and therefore their toxicity. Indeed, the biological uptake of soluble metals described as adsorption, complexation or binding does not explain the real effect of the metals on organisms (Driscoll *et al.* 1980, Driscoll & Schecher 1990). The vast majority of metal ions, including these of Ni, Cr, Zn, Cu, Sn and Se, are essential for the growth of organisms. Many of them function as activators in enzyme reactions (for example, Ca binds to few enzymes such as trypsin, a digestive enzyme, protecting it against denaturing agents). Some are structural components in biological molecules (for example, Fe in haemoglobin). Trace metals are in general micronutrients and may become toxic above a threshold concentration. Fortunately, most living organisms possess detoxifying mechanisms that usually involve exclusion from the cell or segregation of the metal from the cytoplasm by incorporation into granules, precipitation within the cell wall, complexation by extracellular polymers, or transformation of the metal by oxidation or reduction, so rendering it harmless to the host. These detoxifying mechanisms are used in biotechnology to reduce metal effects on the environment and are also being developed as low-cost metal-recovery agents (Brown 1990). Table 1.2 reports some important examples of the metals and their detoxifying agents.

Table 1.2 Selected metals and their detoxifying agents (Brown 1990)

Metal	***Detoxifying agent***
Al	*Aspergillus niger* (fungus)
Sb	*Staphyloccus aureus* (bacterium)
As	genus *Dunaliella* (algae)
Cd	*Zoogloea ramigera* (bacterium)
Cr	*Pseudomonas aeruginosa* (bacterium)
Co	*Chorella regualaris* (alga)
Cu	*Cladosporium resinae* (alga)
Pb	genus *Spirogyra* (algae)
Mn	genus *Chlamydomonas* (algae)
Hg	*Esherichia coli* (bacterium)
Ni	genus *Chlamydomonas* (algae)

All these examples show how complex the toxicity of trace metals is in aquatic environments.

The process of bioaccumulation is not only a means of detecting water pollution, but may also provide a process of detoxification. Indeed, if free metal ions are taken up by microorganisms as described above, they may bind to extracellular organic material (e.g. alginate by Al or Cu) and become harmless to other aquatic taxa.

Another positive fact may be hormesis, or acclimation, which is the benefit that may result from exposure to small doses of substances (e.g. heavy metals) that are toxic in larger doses. In natural waters, heavy metals such as Al, As, Cu, Pb, Mn, Zn, Cd, Cr, Co, Hg, Ni, which are found at concentrations slightly above 0.01 mg/L, are named tertiary constituents while those that normally occur at concentrations below 0.01 mg/L (e.g. Cd, Sb, Cr, Co, Hg, Ni, Sn, Ti) are constituents of the fourth class, or true trace metals according to Nalco (1984). Organisms bathe in these low concentrations without any danger. Slight increases in concentrations may provoke acclimation to exposed organisms. But hormesis may not be entirely positive. For instance, Bodar (1988, cited by Enserink 1997: pers. com.) found that *Daphnia magna* may produce more, but smaller, young when exposed to low Cd concentrations (i.e. 0.5 to 5 μg/L), which is contrary to the natural response to stress (i.e. which is the reduction of the number of offspring, eggs or juveniles: Reish & Oshida 1986).

1.4 This study

In this study, as a whole, I have attempted to ascertain the combined effects of Al, Cu and Mn in chronic exposures at low pH (< 5.8). I decided to use low pH values because the metals are available mostly in soluble forms under acidic conditions (pH < 5.0) and *P. nigroculus* occurs in natural waters at low pH.

I attempted to answer the following questions:

- Tian (1996) has shown *P. nigroculus* to be tolerant to Al at pH < 5.5 when Al was present singly. I wished to ascertain what would happen when this metal occurs together with other elements.
- What happens when *Paramelita nigroculus* is exposed to Cu in combination with Al and Mn?
- Mn, another essential element, may reduce or increase the toxicity of metals in aquatic environments; what is its effect on a combination of Cu and Al?
- Finally, synergistic or antagonistic reactions may occur in the mixtures of these three metals.

The answers should in turn provide preliminary responses to the following questions:

- Are South African interim criteria adequate for protecting aquatic life in the south-western Cape?
- Is *Paramelita nigroculus*, a good bioindicator of pollution by heavy metals?
- Is it a bioaccumulator or a regulator?
- What kinds of interactions (i.e. synergism or antagonism) occur between the three metals (Al, Cu and Mn) tested?
- What active routes are used for the uptake of these elements?

Dilution water used is 'artificial' fresh water (which is distilled water to which only appropriate inorganic salts were added) and stream water from which amphipods were collected. The use of stream water was useful in the sense that it allowed me to check if additional Al, Cu and Mn disturbed the survival, growth, reproduction and the bioaccumulation process in *P. nigroculus*. Another reason of using stream water was the fact that industries discharge their effluents in natural receiving waters and there was a need to simulate site conditions. In all cases two sets of experiments were performed, one using 'artificial' water and the other using stream water. 'Artificial' water was used because the concentrations of all elements could be controlled (within experimental limits), although the disadvantage due to the deficit in nutrients and organics. Stream water was used because organics may protect animals by binding metals and of course animals survive better in their natural habitat. But disords that levels of metals higher may provoke and speciation modelling are difficult to determine in such waters. Thus both waters used is an attempt to cope with the problems generated by using either alone.

The analysis of variance (ANOVA), the paired-test (Student Newman-Keuls test), the non-parametric Mann-Witney U-test and the multiple regression analyses are used as appropriate.

The models used in this study for growth of *P. nigroculus* (as change in body mass, and as reproduction) are not mechanistic. The aim of this thesis is not to find a mechanistic model for growth, but rather to **compare** the growth of the amphipods under different conditions (example: metal mixtures, dilution water). The multiple regression models used allow valid statistical comparisons to be made of the growth both under different chemical regimes and under different concentrations. Importantly, the method allows testing for the presence of interactions between the chemical and water types (Chapters 2 and 8), showing for instance that the effect of Mn depends upon the water type and upon its concentration. This model, simple rather than naïve, could be

regarded as a first approximation to the some unknown non-linear mechanistic models that may describe the growth, an approximation which explains 89% of the variation.

It should also be pointed out that simple extensions to this model would also allow comparisons between different environments, so the model is not as limited it may seem on first sight.

The idea of finding a mechanistic model for the growth is an intriguing one and could be a topic for further research. The experience gained from the present experiments could be used to design further experiments that would allow mechanistic models to be fitted and compared. The values of the parameters of a mechanistic model would have to be estimated from observations, and also like the empirical ones used here, are data dependent. It is only by observations that one can link a mathematical model to the 'real' world (June Juritz, Dept. of Statistics, UCT: pers. com. 1997).

The model employed for the counts (as a result of reproduction) of amphipods is somewhat mechanistic in the sense that it is based upon the assumption that the count at any time is governed by a Poisson process and that the growth rate of that mean count is exponential. Again the concept of a generalised linear model allowed to make statistically valid comparisons of the growth rate under a number of conditions, which is the aim of this investigation. Moreover this model also allows to decide if these assumptions posed were reasonable (Juritz: ditto).

1.5 Definition of concepts and explanation of terms used

Some of the terms used in this thesis may be defined as follows:

"Combined effects" refer to responses of *P. nigroculus* to mixtures of metals;

"Acidic waters" refers to freshwater bodies with pH values lower than 5.8;

"Toxicological studies" refer to the study of pollutants (e.g. Al, Cu and Mn) in living systems (i.e. in *P. nigroculus*);

"LC_{50} and LT_{50} are, respectively, concentration and time at which 50% of the exposed population dies (e.g. 96-h LC_{50} means the concentration of metals in a test solution at which 50% of the exposed population dies after 96 hours of exposure; and LT_{50} = 8 days means that 50% of the exposed population died after 8 days of exposure). LC_{50} and LT_{50} can be determined from data by using arithmetic graphic method, with % survival plotted on the ordinate against the concentration (for LC_{50}) or the exposure time (for LT_{50}) on the abscissa. Each data point is plotted and connected to form a graph. A horizontal line is drawn from the 50% survival point to intersect the plot. A vertical line from the intersection point is then dropped to the abscissa. The intersection point on

the abscissa corresponds to the LC_{50} or LT_{50} of a replicate. The average of LC_{50} or LT_{50} values is calculated by dividing the total of all replicates by the number of replicates. Logarithmic or Probit methods can also be used (Reish & Oshida 1986, Collett 1994).

"Growth" refers to changes in body weight or changes in length (size), or changes in number of individuals (essentially due to reproduction). In this study, I have used changes in body weight after 21 days of exposure to test solutions to describe the growth and changes in number of individuals after 45 days of exposure to describe reproduction.

"Juvenile" refers to an amphipod of total length less than 4 mm; unfortunately, size arbitrarily fixed for a better comparison of results since the life history of this amphipod is not well-known up-to date. Strong information on the life history is needed as a new research theme;

"Mature" refers to an amphipod of total length greater than 4mm;

"Natural water" refers to 'unpolluted' stream water from Skeleton Gorge in the Kirstenbosch National Botanical Garden (Table Mountain, Cape Peninsula);

"Artificial water" refers to distilled water to which appropriate inorganic salts have been added;

"Acute Effect" refers to severe damage caused by a toxicant leading to death in short-time exposure. In general, acute effect means lethal effect; while "chronic effect" refers to long-term effect that may not necessarily provoke the death of organism. In practice, chronic effect is named "sublethal effect". When mortality is used to assess the effect of toxicants on organisms, the median survival time may be used to compare the toxicity of these different toxicants. When LT_{50} is longer than 96 hours, the response is assumed a long-term one (Reish & Oshida 1986). The reverse is considered as a short-term effect.

"Bioaccumulation" refers to the process of taking up and retaining elements in body cells and tissues;

"Test solutions" used refer to values derived from the South African criteria (Table 1.1) levels at Target Water Quality Range (TWQR), Chronic Effect Value (CEV) and Acute Effect Value (AEV);

- 50% value above, and 50% value below the criteria levels using TWQR, CEV and AEV.
- Controls, here, refers to test solutions without metals added.

All these values derived from South African criteria are listed in the following Table 1.3, in which "A" = value at criterion levels; "B" = value at 50% above the criterion levels; "C" = value at 50% below the criterion levels; "a1" = target value for mixture Al + Cu, and "a2" = target value for mixture Al + Cu + Mn; "b1" = chronic effect value for mixture Al + Cu; "b2" = chronic effect value for mixture Al + Cu + Mn; "c1" = acute effect value for mixture Al + Cu, and "c2" = acute effect value for mixture Al + Cu + Mn; AEV2a

= new AEV (as published in 1996 by DWAF) for mixtures of Al + Cu; AEV2b = new AEV (as published in 1996 by DWAF) for mixtures of Al + Cu + Mn.

Table 1.3 Concentrations of test solutions in mg/L. as derived from the South African criteria

Test solutions	*Al*	*Cu*	*Mn*
Aa1 (TWQR)	0.062	0.001	
Aa2 (TWQR)	0.062	0.001	1.217
Ab1 (CEV)	0.185	0.004	
Ab2 (CEV)	0.185	0.004	2.434
Ac1 (AEV)	0.926	0.008	
Ac2 (AEV)	0.926	0.008	9.328
Ba1 (TWQR + 50%)	0.0925	0.0025	
Ba2 (TWQR + 50%)	0.0925	0.0025	1.825
Bb1 (CEV + 50%)	0.2775	0.005	
Bb2 (CEV + 50%)	0.2775	0.005	3.65
Bc1 (AEV + 50%)	1.388	0.0175	
Bc2 (AEV + 50%)	1.388	0.0175	13.993
Ca1 (TWQR - 50%)	0.030	0.0005	
Ca2 (TWQR - 50%)	0.030	0.0005	0.6075
Cb1 (CEV - 50%)	0.0925	0.0175	
Cb2 (CEV - 50%)	0.0925	0.0175	1.218
Cc1 (AEV - 50%)	0.4325	0.004	
Cc2 (AEV - 50%)	0.4325	0.004	4.665
AEV2a (new AEV)	0.1225	0.005	
AEV2b (new AEV)	0.1225	0.005	5.273
Control	0	0	0

Apart from Chapter 1 as an introduction, this study as a whole comprises three main sections divided into a series of Chapters. Chapter 2 discusses the effect of manganese on the mortality and growth of the freshwater amphipod *Paramelita nigroculus* exposed to a mixture of Al and Cu in acidic waters. Chapter 3 deals with reproduction in *Paramelita nigroculus* and Chapter 4 discusses the interactions between Al, Cu and Mn and toxicity modelling. Chapter 5 deals with Bioaccumulation. Chapter 6 analyses the active uptake of Al, Cu and

Mn by *P. nigroculus*. Chapter 7 presents the analysis of variance and paired-tests of comparison, while Chapter 8 reports the multiple regression analyses for survival, growth, reproduction and bioaccumulation. Finally, Chapter 9 discusses all the results and concludes the study.

CHAPTER 2

The Effect of Mn on Mortality and Growth in the Freshwater Amphipod *Paramelita nigroculus* (Barnard) Exposed to a Mixture of Al and Cu in Acidic Waters[1]

Abstract

The combined toxicity of three common trace elements - aluminium, copper and manganese - on the endemic freshwater amphipod *Paramelita nigroculus* (Barnard) was studied in acidic waters. In soft 'artificial' water (distilled water to which appropriate inorganic salts had been added), after exposure periods of 21 days and at a concentration of 1.388 mg/L Al + 0.018 mg/L Cu, survival was 55% for juveniles and 60% for adults. At a concentration of 1.388 mg/L Al + 0.018 mg/L Cu + 13.993 mg/L Mn, survival was 62% for both juveniles and adults. These concentrations are 139 times the AEV for Al(0.01mg/L), at least 10 times the AEV of Cu(0.0016mg/L) and Mn(1.300mg/L) from the official South African guidelines for each metal. Risk and multiple regression analyses show that, using juveniles in natural water, when the concentration of each element increases by 1 ppb, the risk of death increases minimally (by 1.000086) while the addition of Al + Cu mixtures increases the risk of death by 2.6 times. The addition of Al + Cu + Mn increases the risk of death by 1.5 times, suggesting that Mn reduces the risk associated with the other two elements. The risk of death for an adult is 0.8 times the risk for a juvenile, while the risk of death for a moulting individual is 5.3 times greater than that of a juvenile. The risk in artificial water (i.e. with virtually no trace metals other than those specifically added) is 0.9 times that of the risk in stream water. The relationship between log growth and log concentration is: $\log(y) = a + b * \log(x)$ and between growth and concentration is: $y = cx^b$ where y = growth; x = concentration of each element, and c = log(a) to base e. Thus growth in river water without Mn added is $y = 469\ x^{-0.194}$, while in river water with Mn added, $y = 615\ x^{-0.088}$. In 'artificial' water without Mn added, $y = 434\ x^{0.108}$ and with Mn added $y = 226\ x^{-0.070}$.

Keywords: Combined toxicity, survival, growth, amphipods, risk, heavy metals, aluminium, copper, manganese

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2.1 Introduction

Criteria for the management of water quality are usually based on the toxicity of individual trace elements. Such criteria may well be inadequate (e.g. Biesinger *et al.* 1986, Spehar & Fiandt, 1986, Enserink *et al.* 1991) because effluents seldom contain just one trace element, while synergistic and antagonistic effects are displayed by trace elements in combination.

Biesinger *et al.* (1986), for instance, tested the effects of binary mixtures of metal salts on reproduction in *Daphnia magna*. They showed that concentrations of metals having no significant effects on reproduction, when presented singly, exerted a toxic action when presented as mixtures. Enserink *et al.* (1991) tested the effects of mixtures of eight metals (As, Cd, Cr, Cu, Hg, Ni, Pb and Zn) on *Daphnia magna* and showed the (then) Dutch criterion levels to be inadequate for protecting aquatic life when the elements were present in combination. Such effects have been demonstrated for other taxa too. Spehar & Fiandt (1986), for example, used binary mixtures of metals to induce chronic toxicity in fish and *Ceriodaphnia dubia* and concluded that the combined effects were additive.

In contrast to synergistic effects, certain elements may reduce the toxicity of others. For instance, Shuttleworth & Unz (1991) examined the influences of Ca, Cu, Ni and Zn on the growth of the filamentous bacterium *Thiothrix*. As well as showing that Cu was the most toxic of the metals tested, and that mixtures of Cu + Ni and Cu + Zn appeared to act synergistically in suppressing the development of *Thiothrix*, they found that combinations of metals were less toxic at increased concentrations of Ca, and that toxicity of Ni was reduced in the presence of Zn. Wang *et al.* (1995), studying the reciprocal effects of Cu, Cd and Zn on the marine alga *Phaeodactylum*, reported antagonism if the ratio of Cd:Cu was 0.4 - 4 and synergism on either side of these ratios (i.e. <0.4 and >4). Similarly, Zn and Cu were antagonistic at ratios of Zn:Cu between 1 and 20, and synergistic at ratios >20. Total toxicity was due to Cd when Zn was low and was controlled by Zn when Zn was high.

The present paper reports on experiments examining survival and growth in a freshwater amphipod as an illustration of the acute and chronic effects of Mn on the toxicity of Cu + Al.

The amphipod, *Paramelita nigroculus*, is a local endemic of mountain streams in the south-western Cape (Stewart 1991), where natural waters are often acidic, peat-stained and rich in dissolved humic substances. We have chosen to study this species because so few toxicity data are available for organisms adapted to the conditions in which *P. nigroculus* is found. Because adults, juveniles and moulting individuals of many taxa are

known to respond differentially to the presence of toxins, we have examined the toxic effects of the metals on individuals in all three conditions.

For these experiments we have used the concentrations derived from the concentrations of each element recommended as the target water quality range (TWQR:0.005mg/L Al; 0.002mg/L Cu and 0.180mg/L Mn), the acute effect value (AEV:0.010mg/L Al; 0.0016 mg/L Cu and 1.3 mg/L Mn) and the chronic effect value (CEV:0.001mg/L; 0.00053 mg/L Cu and 0.370mg/L Mn) in the interim South African water quality guidelines for aquatic ecosystems (DWAF 1995). We have chosen to use Al not only because it is a very abundant element but also because it is at its most toxic at the acidic conditions prevalent in the streams inhabited by *P. nigroculus*. Cu is one of the most widely used metals in the world and is known to be very toxic to aquatic organisms. Finally, as well as being an abundant metal in nature, Mn is known to react synergistically or antagonistically with a variety of other heavy metals.

The streams in which *P. nigroculus* occurs are rich in dissolved humic substances, which may adsorb metal ions (and thus act as antagonists to the metals) (Tian 1996). Indeed, the concentrations of Al in particular are relatively high for natural stream ecosystems. For these reasons two sets of experiments were performed: one using 'artificial' water containing no organics or heavy metals (within experimental limits) and stream water, with measurable quantities of both.

2.2 Materials and methods

2.2.1 Materials

Test solutions were prepared using Al(III) in $Al_2(SO_4)_3.18H_2O$ (Merck extra pure), Cu(II) in $CuSO_4.5H_2O$ (Analar 99.5%) and Mn(II) in $MnSO_4.4H_2O$ (BDH 97%). Soft 'artificial' water was prepared using 85mg L^{-1} NaCl (Univar 99.5%), 10mg L^{-1} $CaCl_2.2H_2O$ (Merck 99.5%) and 5mg l^{-1} $MgSO_4$ (Merck 70%) in distilled water (USEPA 1991, Tian 1996) and a pH value of 5.0 $\pm$ 0.8.

Stream water (pH 5.0 $\pm$ 0.8; Total Hardness or T.H. < 40 mg/L as $CaCO_3$), from Skeleton Gorge on Table Mountain in the extreme south-west of South Africa, was used both to acclimatise amphipods to laboratory conditions prior to their exposure to pollutants and also to prepare a suite of test solutions.

Containers were cleaned in a phosphate-free surfactant solution (Contrad, Merck) and rinsed in distilled water (reducing thus contamination by surfactants) before being used in the experiments. Concentrated nitric acid was used to adjust the pH to 5.0$\pm$0.8 and also to preserve samples when chemical analyses could not be performed on the same day.

2.2.2 Methods

(a) Experimental set up

A hundred and twenty-six cylindrical white plastic containers approximately 80 mm in depth and 60 mm in diameter (volume of 500 mL) were washed in Contrad, rinsed with distilled water and filled with 200 mL of test solution. Three replicates were run for each treatment and each experiment was repeated five times.

Tests were run using Al + Cu and Al + Cu + Mn at concentrations corresponding to the South African interim TWQR, CEV and AEV (DWAF 1995) and also at concentrations 50% above and 50% below each of these criterion values. Additional tests were performed at concentrations corresponding to the interim acute effect values modified in the later (1996) version of the interim guidelines (DWAF 1995 as published in 1996).

The diluent for all solutions in one set of 63 containers (21 treatments) was the 'artificial' water described above and for all those in the other set (another 21 treatments) was unpolluted stream water from the natural habitat of *Paramelita*. Stock solutions were filtered through 0.45μm GF/C Watman filter paper using a plastic funnel. Total hardness (TH) was determined using a Hach model 16900-01 digital titrator and a 0.800mol l^{-1} EDTA cartridge 14364-01 (Hach 1992). A Crison pH.mV meter, model 506, was used to measure pH. Conductivity and temperature were measured with a Hach model 44600 Conductivity/TDS meter (at 0.1μS/cm).

(b) Test solutions

The concentrations of experimental metals used in the stock test solutions are indicated in Table 1 in this chapter and some values for the stream water are indicated in Table 2. The code for the combinations listed in Table 1 is as follows: A = criterion values, B = criterion values + 50%; C = criterion values - 50%; a = TWQR; b = AEV; c = CEV; 1 = without Mn; b = with Mn; AEV2 = later AEV + 50%

(c) Test animals

In the laboratory, amphipods were acclimated for at least a week in water from Window Stream (also named Skeleton Gorge), a small tributary of the Liesbeek River, which drains the eastern flank of Table Mountain. The temperature was kept constant at 14$\pm$1°C and water was replaced every day. The animals, which are shredders, were fed only on leaves of a riparian tree, *Rapanea melanophloeos*. Light intensity was at laboratory levels (10-20 μE m^{-2} s^{-1}) and the photoperiod was set at 12h dark:12h light. The water was not aerated (Stewart 1991). Adult animals (body length >4mm: Tian 1996) of either sex were selected using a 125-950μm sieve, a magnifying glass and graph paper (Stewart 1991, Winger *et al.* 1993, Tian 1996). Mature breeding females

Table 1: *Concentrations of Al, Cu and Mn (mgL^{-1}) used in the experimental setups. TWQR = target water quality range; CEV = chronic effect value; AEV = acute effect value*

Experimental setups	*Al*	*Cu*	*Mn*
Aa1 (TWQR, no Mn)	0.062	0.001	
Aa2 (TWQR, with Mn)	0.062	0.001	1.217
Ab1 (CEV, no Mn)	0.185	0.004	
Ab2 (CEV, with Mn)	0.185	0.004	2.434
Ac1 (AEV, no Mn)	0.926	0.008	
Ac2 (AEV, with Mn)	0.926	0.008	9.328
Ba1 (TWQR + 50%, no Mn)	0.0925	0.0025	
Ba2 (TWQR + 50%, with Mn)	0.0925	0.0025	1.825
Bb1 (CEV + 50%, no Mn)	0.2775	0.005	
Bb2 (CEV + 50%, with Mn)	0.2775	0.005	3.65
Bc1 (AEV + 50%, no Mn)	1.388	0.0175	
Bc2 (AEV + 50%, with Mn)	1.388	0.0175	13.993
Ca1 (TWQR - 50%, no Mn)	0.030	0.0025	
Ca2 (TWQR - 50%, with Mn)	0.030	0.0025	0.6075
Cb1 (CEV - 50%, no Mn)	0.0925	0.0175	
Cb2 (CEV - 50%, with Mn)	0.0925	0.0175	1.218
Cc1 (AEV - 50%, no Mn)	0.4325	0.004	
Cc2 (AEV - 50%, with Mn)	0.4325	0.004	4.665
AEV2a (new AEV, no Mn)	0.1225	0.005	
AEV2b (new AEV, with Mn)	0.1225	0.005	5.273
Control	0	0	0

were excluded because of their sensitivity to pollution, and because of environmental ethics and also because it would be difficult to distinguish offspring generated in the natural environment from those produced in the laboratory at the end of the experiment. Individuals <4 mm were considered to be juveniles; moulting individuals were identified by colour and transparency (Stewart 1991, Tian 1996).

Each container of 500 mL capacity received 200 mL of test solution and, depending on the test, 10 mature amphipods, 10 moulting individuals or 10 juveniles as test animals. All organisms were randomly selected by numbering each container consecutively and selecting test organisms for a particular concentration and replicate by following the 'random numbers from the hat' method (Clarke 1980, Zar 1995). Each experiment was repeated five times, giving 15 replicates per concentration and thus 150 individuals (per concentration).

The mean values (X ± SD) for each concentration were used as results.

Table 2: Concentrations (µg/L ± SD) of dissolved Al, Cu and Mn in dilution waters, R. melanophloeos leaves and P. nigroculus prior to experiments. n/a = not analysed; number of samples n = 5, corresponding to five collections of amphipods from Skeleton Gorge stream

	Stream water	*'Artificial' water*	*Rapanea leaves*	*P. nigroculus*
Al	221 ± 15	0	0	157
Cu	9 ± 6	0	<1	14 ± 7
Mn	323 ± 34	0	<1	52 ± 23
Suspended organics (%)	1.1 ± 0.5	0	n/a	n/a
pH	4.5 - 5.5	4.5 - 5.8	n/a	n/a
Total hardness (as mg/L $CaCO_3$)	30 ± 6	30 ± 4	n/a	n/a
Conductivity (S/m)	12 - 35	12 - 35	n/a	n/a

In line with standard practice (USEPA 1991), during the first 96 hours of exposure, amphipods were not fed. From the fifth day, 0.5g dry mass of leaves of *Rapanea melanophloeos*, conditioned previously by being soaked in distilled water for 24 hours, were introduced into each container. Test solutions and food were renewed twice a week (as static-renewal type simulating natural 'vleis' in which amphipods live during summer time). Dead amphipods were counted and removed from test containers every day.

Each experiment lasted 21 days. During the experiments, containers were not washed. After 21 days, live amphipods were removed from the test solutions and kept for 30 hours in clean 'artificial' water to allow depuration. No food was given during this period. Fifteen animals were then washed in distilled water and dried at 75°C to constant mass. The dry weight was recorded. Finally, the dried amphipods were ignited at 650°C in a muffle furnace for 10 hours. The ash obtained was weighed, 0.5 g of each ash sample digested in 5 mL of concentrated nitric acid for at least 8 hours and filtered into a 50 mL Erlenmeyer flask. The volume was made up with distilled water and analyzed by Inductively-Coupled Plasma spectrometry.

At the end of each experiment, 50 mL of each test solution were filtered through 0.45 µm Watman GF/C filter paper. Concentrated nitric acid (1mL per container) was used to preserve samples, which were stored at 4°C prior to analysis for Al, Cu and Mn. Stream water, 'artificial' water, *Rapanea melanophleos* leaves and control amphipods were also chemically analyzed for these elements.

After filtering 1L of stream water through 0.45 μm Watman GF/C filter paper, the residue was dried at 75°C to constant weight, and then ignited at 650°C. The difference between dry weight and ash weight (expressed as a percentage) was considered to represent the organic fraction of the suspended material in stream water.

Acute toxicity was expressed as 96-h LC_{50} and chronic toxicity as LT_{50} after 8 and 21 days.

Growth was assessed as change in dry mass. Three replicates of twenty juveniles of known length were dried at the beginning of the experiments. The average dry weight of these animals was used as the reference value when assessing growth (e.g. Reish & Oshida 1986, USEPA 1991, Sidoumou *et al.* 1992, Winger & Lasier 1993, Kooijman 1993).

Results were statistically analyzed using ANOVA, Student's *t*, the Neuman-Keul t test were performed to compare differences in mortality an growth within different test concentrations (Zar 1995). Multiple regression analysis (Collett 1994) provided simple models explaining possible interactions between elements (Al, Cu, Mn) and water types (stream or 'artificial' water). Cox's proportional hazards model (1987) describes the risk of death of a given organism exposed to various concentrations of pollutants; this risk is proportional to the change in concentrations and the exposure time.

2.3 Results

Aspects of the chemical composition of the dilution waters, *Rapanea* leaves and amphipods before the tests are reported in Table 2 of this paper.

Survival and mortality of *P. nigroculus* after 24-h, 96-h, 8 days and 21 days of exposure to test solutions are reported in Tables 3 and 4 for mature and for juvenile individuals,respectively (in this paper). Due to high mortality in controls (90% in 'artificial' water, and 73% in stream water after 21 days of exposure) test results for moulting individuals were disregarded and no further experiments have been attempted on moulting amphipods.

Each experiment started with 3 x 10 amphipods and each result given in the Tables is the mean of five experiments (i.e. 150 individuals per concentration after 21 days of exposure).

Chronic effects: growth as measured by change in the body weight.

Table 3: *Number of adults [mean(±SD)] surviving exposure to mixtures of Al + Cu, or Al + Cu + Mn, in river water (R) or 'artificial' water (A). Concentrations of metals in mg/L. Actual concentrations in river water at the end of the experiments in parentheses.*

Mixture	*Al*	*Cu*	*Mn*	*24-h*		*96-h*		*8 days*		*21 days*	
				R	*A*	*R*	*A*	*R*	*A*	*R*	*A*
Aa1	0.062(0.02)	0.001(0.02)		26	26	24	25	18	17	16(0.6)	15(0.0)
Aa2	0.062(0.02)	0.001(0.02)	1.217(0.34)	28	29	28	27	23	22	20(1.2)	20(0.6)
Ab1	0.185(0.02)	0.000(0.01)		27	28	26	26	18	17	15(0.0)	15(0.0)
Ab2	0.185(0.01)	0.004(0.01)	2.434(0.45)	29	29	28	28	23	23	20(0.6)	19(0.6)
Ac1	0.926(0.04)	0.008(0.01)		28	29	26	28	15	14	13(0.6)	13(0.6)
Ac2	0.926(0.03)	0.008(0.01)	9.328(0.08)	29	29	28	28	22	21	19(0.6)	19(0.6)
Ba1	0.092(0.06)	0.002(0.05)		25	25	23	20	18	16	16(0.6)	14(0.6)
Ba2	0.092(0.04)	0.002(0.02)	1.825(0.07)	28	28	27	27	21	21	19(1.2)	18(0.0)
Bb1	0.277(0.3)	0.005(0.07)		27	25	26	24	18	18	15(0.0)	14(0.6)
Bb2	0.277(0.1)	0.005(0.04)	3.65(1.2)	29	29	27	26	23	19	20(0.6)	17(0.6)
Bc1	1.388(0.12)	0.017(0.02)		27	26	25	25	14	14	12(0.0)	11(0.0)
Bc2	1.388(0.09)	0.017(0.01)	13.99(4.33)	29	28	28	28	22	22	18(1.0)	19(0.6)
Ca1	0.030(0.02)	0.002(0.01)		29	29	29	28	25	26	25(0.6)	24(0.0)
Ca2	0.030(0.01)	0.002(0.01)	0.607(0.45)	29	29	28	28	26	26	25(0.6)	24(1.0)
Cb1	0.092(0.05)	0.017(0.01)		29	30	27	29	19	21	16(0.6)	16(0.5)
Cb2	0.092(0.03)	0.017(0.01)	1.218(0.75)	29	29	28	27	25	26	24(0.0)	25(0.6)
Cc1	0.432(0.24)	0.004(0.04)		28	28	27	28	20	20	16(0.6)	24(1.0)
Cc2	0.432(0.10)	0.004(0.02)	4.665(0.36)	29	29	28	29	25	26	24(0.0)	25(0.6)
AEV2a	0.122(0.09)	0.005(0.01)		29	28	27	27	21	21	17(1.0)	16(0.5)
AEV2b	0.122(0.07)	0.005(0.01)	5.273(1.20)	29	29	28	28	25	24	24(0.6)	23(0.6)
Control	0	0	0	29	29	29	29	26	26	26(0.6)	25(0.6)

The results of the experiments examining the chronic effects of mixtures of the metals, as shown by effects on growth rates, are reported in Table 3 (in this paper).

Table 4: *Number of juveniles surviving exposure to mixtures of Al + Cu, or Al + Cu + Mn, in river water (R) or 'artificial' water (A). Concentrations of metals in mg/L.*

Mixture	*Al*	*Cu*	*Mn*	*24-h*		*96-h*		*8 days*		*21 days*	
				R	*A*	*R*	*A*	*R*	*A*	*R*	*A*
Aa1	0.062	0.001		27	21	23	20	17	16	13 (0.6)	14 (0.6)
Aa2	0.062	0.001	1.217	27	24	27	24	21	20	18 (0.0)	16 (0.0)
Ab1	0.185	0.004		25	24	24	22	16	16	12 (0.0)	14 (0.6)
Ab2	0.185	0.004	2.434	28	26	26	25	20	19	14 (0.6)	18 (1.2)
Ac1	0.926	0.008		23	25	20	21	15	14	11 (0.0)	12 (0.6)
Ac2	0.926	0.008	9.328	23	27	20	26	15	19	11 (0.6)	17 (0.6)
Ba1	0.092	0.002		20	21	19	20	17	16	13 (0.6)	14 (0.6)
Ba2	0.092	0.002	1.825	24	24	21	24	18	20	17 (0.6)	18 (0.0)
Bb1	0.277	0.005		22	23	19	19	14	16	10 (0.6)	14 (0.0)
Bb2	0.277	0.005	3.65	25	26	24	23	19	20	17 (1.2)	17 (0.6)
Bc1	1.388	0.017		23	22	19	20	14	12	10 (0.6)	9 (0.6)
Bc2	1.388	0.017	13.99	25	25	23	20	20	19	17 (0.6)	16 (0.6)
Ca1	0.030	0.002		28	27	27	27	24	24	21 (0.6)	22 (0.6)
Cb1	0.092	0.017		28	27	27	26	23	22	22 (0.6)	18 (0.0)
Cb2	0.092	0.017	1.218	29	29	28	26	25	24	23 (0.0)	22 (0.6)
Cc1	0.432	0.004		27	29	26	24	15	14	14 (0.6)	12 (0.0)
Cc2	0.432	0.004	4.665	29	29	27	25	21	21	20 (0.6)	19 (0.6)
AEV2a	0.122	0.005		28	27	27	25	23	21	20 (0.6)	17 (0.6)
AEV2b	0.122	0.005	5.273	28	29	26	26	23	21	21 (0.0)	20 (0.6)
Control	0	0	0	29	29	28	28	24	25	23 (0.0)	24 (0.0)

Table 5: *Growth as measured by change in dry mass (mg) of juvenile P. nigroculus after 21 days of exposure to mixtures of solutions of Al, Cu and Mn in river water and 'artificial' water (n = 150). Mean dry mass per individual before the experiment was 1.30 mg. Result = mean (± SD)*

Experimental setups	*Al*	*Cu*	*Mn*	*River water*	*'Artificial' water*
Aa1 (TWQR, no Mn)	0.062	0.001		3.25(0.06)	1.90(0.01)
Aa2 (TWQR, with Mn)	0.062	0.001	1.217	3.64(0.03)	2.46(0.01)
Ab1 (CEV, no Mn)	0.185	0.004		2.81(0.14)	1.79(0.01)
Ab2 (CEV, with Mn)	0.185	0.004	2.434	3.78(0.15)	2.03(0.02)
Ac1 (AEV, no Mn)	0.926	0.008		2.18(0.17)	1.72(0.01)
Ac2 (AEV, with Mn)	0.926	0.008	9.328	3.32(0.14)	1.91(0.00)
Ba1 (TWQR + 50%, no Mn)	0.0925	0.0025		2.80(0.21)	1.83(0.01)
Ba2 (TWQR + 50%, with Mn)	0.0925	0.0025	1.825	3.16(0.04)	2.06(0.00)
Bb1 (CEV + 50%, no Mn)	0.2775	0.005		2.47(0.13)	1.74(0.02)
Bb2 (CEV + 50%, with Mn)	0.2775	0.005	3.65	3.78(0.09)	2.05(0.01)
Bc1 (AEV + 50%, no Mn)	1.388	0.0175		1.83(0.08)	1.69(0.02)
Bc2 (AEV + 50%, with Mn)	1.388	0.0175	13.993	2.75(0.06)	1.90(0.01)
Ca1 (TWQR - 50%, no Mn)	0.030	0.0025		3.83(0.09)	2.50(0.01)
Ca2 (TWQR - 50%, with Mn)	0.030	0.0025	0.6075	4.07(0.06)	2.90(0.01)
Cb1 (CEV - 50%, no Mn)	0.0925	0.0175		3.49(0.20)	1.84(0.01)
Cb2 (CEV - 50%, with Mn)	0.0925	0.0175	1.218	3.72(0.09)	2.05(0.01)
Cc1 (AEV - 50%, no Mn)	0.4325	0.004		2.06(0.02)	1.79(0.01)
Cc2 (AEV - 50%, with Mn)	0.4325	0.004	4.665	3.68(0.09)	2.04(0.01)
AEV2a (new AEV, no Mn)	0.1225	0.005		3.43(0.09)	1.85(0.01)
AEV2b (new AEV, with Mn)	0.1225	0.005	5.273	2.92(0.06)	1.93(0.00)
Control	0	0	0	3.90(0.00)	2.61(0.01)

2.4 Statistical analyses

ANOVA for the survival of mature amphipods after 21 days of exposure to the Al + Cu mixtures in artificial water provided an F value of $10.5 > 2.3$, $p = 0.05$ (DF = 10, s.e.= 0.26). The null hypothesis was therefore rejected and observed differences in survivorship were not due to chance. The application of Student's t test and Newman-Keuls paired-test of comparison showed that survival was significantly different from the control ($p<0.05$) for Ac1, Bc1, Ba1, Cc1, Aa1, Cb1, Ab1, Bb1, and AEV2a. Survival in Ca1 was significantly different from the above treatments except for AEV2a and survival in all other treatments was not significantly different. The F value for mature amphipods in 'artificial' water of Al + Cu + Mn was $2 < 2.30$ and H_0 was therefore accepted, namely that the variations observed in survivorship were due to chance.

ANOVA for the survival of mature amphipods in stream water solutions of Al + Cu mixtures provided an F value of $7.7 > 2.3$, $p<0.05$ (D.F. = 10, s.e. = 0.32). The paired-test of comparison showed that the survival in the control was significantly different from that in Bc1, Ac1, Ab1, Bb1, Aa1, Ba1, Cb1, AEV2a and Cc1. Survival in Ca1 was statistically different from Bc1, Ac1, Ab1, Bb1, Aa1, Ba1, Cb1, AEV2a and Cc1; survival in Aa1, Ba1, Cb1, AEV2a.

Other F values were:

- for juveniles in river water with mixtures of Al + Cu: $0.94 < 2.30$ (not significant) and therefore null hypothesis accepted and differences were random;

- for mature amphipods in river water with mixtures of Al + Cu + Mn: 3.2 (H_0 rejected). So the survival of mature amphipods in the mixtures of aluminium, copper and manganese in river water showed for the control significant difference from Bc2, Ac2 and Ca2 was significantly different from Bc2. All the other treatments showed no significant difference.

- for juveniles in 'artificial' water with mixtures of Al + Cu: 11 (H_0 rejected). So the control was significantly different from Bc1, Cc1, Ac1, Aa1, Ba1, Ab1, Bb1, AEV2a and Cb1.

- for juveniles in 'artificial' water with mixtures of Al + Cu + Mn: 2 (not significant); so null hypothesis accepted and differences were therefore random.

- for juveniles in river water with mixtures of Al + Cu: 7.5 (H_0 rejected); so the survival in the control was significantly different from Bb1, Bc1, Ac1, Ab1, Aa1, Ba1 and Cc1.

- for juveniles in river water with mixtures of Al + Cu + Mn: 3.3 (H_o rejected). So the survival in the control was significantly different from Ab2, Ac2, Bc2, Bb2, Ba2.

Cox's proportional hazards model (1987) was used to estimate the risk of death for various combinations of metals, using juveniles in mixtures of metals in river water as a baseline. Concentrations used are the highest values tested (i.e. 1.388 mg/L Al; 0.018 mg/L Cu, and 13.993 mg/L Mn). The addition of Al + Cu increases the risk of death 2.6 times, whereas the addition of Al + Cu + Mn increases the risk of death by only 1.5 times, so Mn reduces the risk of death somewhat. The risk of death for an adult is 0.8 of the risk for a juvenile, while the risk of death for a moult is 5.3 times that of a juvenile, giving an overall increase in risk in the presence of Al + Cu + Mn of 9.8 times. When Al and Cu are present, the risk for a moulting individual increases a further 1.8%. The risk of death in the 'artificial' water was only 90% of the risk in natural water.

If the concentration increases by one μg/L, the risk increases minimally (by a factor of 1.000086).

Multiple regression analyses of growth *vs* various combinations of metals, using data from Table 3, gave the following results. All the variables (lconc, Mn, water, lMn, lwat, Mnwat) except the interaction between log concentration of manganese (lMn) and water type (river or artificial water) were significantly different from zero at 5% level. This means that the growth of *P. nigroculus* is affected by water-type and by both the presence and the concentration of Mn. A multiple regression model using all the variables above (i.e. lconc, Mn, water, lMn, lwat and Mnwat) explained 89.5% of the variation in log growth. Because of the interactions between variables and because the residuals tended to be larger than the observed values for the high concentrations and smaller than the observed values for low concentrations, we decided to fit separate models for each water type and each combination of chemicals. The equation linking log growth to log concentration for individual growth curves for each combination of manganese and water type has the form

$$\log (y) = a + b * \log (x)$$

and in terms of growth and concentration, the equation has the form

$$y = cx^b$$

where y = growth in grams; x = concentration in mg/L and a,b are the estimates for constant and log-concentration respectively; c = log(a) to base e.

The terms in the individual equations are given in Table 6.

Table 6: *Regression equation for growth as change in body weight (variations statistically significant at $p < 0.05$)*

Water-type	*log y = a + b log x*		*$y = cx^b$*	*% of variation in growth rate explained by the model*
	a	*b*	*c*	*%*
River water, no Mn	6.151	-0.194	468.7	83.3
River water with Mn	6.421	-0.089	614.6	47
'Artificial' water, no Mn	6.072	0.109	433.6	64
'Artificial' water with Mn	5.418	-0.070	225.5	56

2.5 Discussion

Mortality of mature amphipods after 24 hours was lower than that of juveniles (16% as opposed to 29% in the tests using river water, and 16 *vs* 32% in the tests using 'artificial' water). On the other hand, the 8-day mortality of mature amphipods was higher than that of juveniles in both types of solution (53% as opposed to 36% in the tests using river water, and 52% as opposed to 35% in the tests using 'artificial' water). This seems to be due to pre-exposure and hormesis. Indeed, since amphipods from Skeleton Gorge stream are exposed to mixtures of metals in natural conditions, they may have developed adaptative responses and this may be why they showed low mortalities after 24-hour exposures. But as the concentrations of metals increase in their bodies, their resistance becomes reduced and death may result. It may be that mortality of juveniles is higher because they have not had the opportunity to develop a protective response. It is also possible that juveniles are more at risk because their metabolic rates are higher than are those of adults.

The presence of Al, Cu and Mn in the water of the stream from which the amphipods were collected (Table 2) may play an important role in their ability to withstand the toxic effects of these metals. Indeed, pre-exposure in chronic toxicity experiments may develop hormesis, as reported by Munzinger (1990), Klerks & Levinton (1993) for invertebrates. This is why it is often better to use juveniles in toxicity experiments. Furthermore, pre-exposure of generations of organisms to low concentrations of pollutants in their natural environment may have resulted in natural selection of somewhat resistant forms.

It appears that in the case of *P. nigroculus*, 24-hour mortality data are useful for predicting the acute effects of pollutants and 8-day chronic tests are useful for predicting chronic effects of pollutants in the routine control of water quality. These times are shorter than the 96-hour and 21-day exposures that are normally applied.

From short-term exposure data on mortality in acute toxicity tests on *Daphnia magna*, Santojanni *et al.* (1995) used regression models in an attempt to predict mortality following longer exposure times. Mortality, defined as probability of death, was expressed as $q_{x-x+n} = (l_x-l_{x+n})/l_x$ where l_x is the proportion of animals surviving to day x and l_{x+n} is the proportion of animals surviving to day x+n so that q_{x-x+n} is the ratio of the number of dead individuals in the n day interval to the number of live individuals at time x. The results described by Santojanni *et al.* (1995) suggested that both acute and chronic tests should be shortened to 24-48 hours for acute tests, and 8 days for chronic tests. Our present results using *P. nigroculus* confirm that this modelling approach may be useful under some circumstances. Shortening of the time needed to evaluate the chronic effects of pollutants would be useful in environmental management but would need further careful evaluation.

Combined effects of Al + Cu were more toxic than that of Al + Cu + Mn both on survival and growth rates. These are additive for Al + Cu and antagonistic for Al + Cu + Mn (Musibono & Day: in preparation).

2.6 Conclusions

The combinations of metals tested at concentrations derived from South African criteria levels were not acutely toxic to adult amphipods since LT_{50} values were longer than 21 days. Moulting individuals, on the other hand, showed high mortalities even in the controls and should not be used as test organisms at this stage. They are extremely sensitive and the presence of metals may not have been the sole cause of death.

Mixtures of Al + Cu were more toxic than mixtures of Al + Cu + Mn. This allows us to conclude that Mn reduces the toxicity of mixtures of Al + Cu. The fact that stream water contains Mn, Al, and Cu may have allowed adults to develop tolerances to certain levels of metals. From this point of view, juveniles are the best test organisms to use.

Growth occurred in all treatments but was fastest in river water without Mn added (but with Al + Cu). This may be due to the presence of interactions with other elements in river water.

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CHAPTER 3

Reproduction in *Paramelita nigroculus*

3.1 Introduction

Reproduction is the ultimate biological function that can be used to assess the effects of toxicants on aquatic organisms. The numbers of eggs laid or of offspring produced are convenient measures of the effects of toxicants on an organism. Unfortunately, experiments on reproduction require long periods of time and are often not feasible because of economic demands. Long life cycles of test organisms may also constrain the use of reproductive effects in toxicology studies. I decided to examine the effects of mixtures of Al, Cu and Mn on reproduction in *Paramelita nigroculus* because *P. nigroculus* presents some advantages, such as having no aerial and no larval phases (Buchanan *et al.* 1988, Stewart 1991, Tian 1996) and surviving and breeding well in the laboratory. My study may provide preliminary information on possible combined effects of Al, Cu and Mn on reproductive rates of *Paramelita nigroculus*. Indeed, the aim of this Chapter is to examine the extent to which reproduction in *P. nigroculus* is toxicity-dependent in laboratory tests.

I have assumed that the number of eggs laid, or of offspring produced, by an organism in polluted water is a function of the concentration of pollutants in water. So, high concentrations of pollutants should result in low numbers of eggs or offspring produced, and vice-versa. Because of the possibility of a reduction in the size and not the number of eggs and offspring (Bodar 1988 cited by Enserink 1997, Musibono 1992) when exposed to low concentrations of pollutants, the reproductive biology of test organisms must be known before using reproduction as a test parameter. I do not know a lot about reproduction in *P. nigroculus* but using it as a test organism may provide preliminary information on the toxicity response. Reproductive biology of *P. nigroculus* may be a new topic of study before using this response in routine toxicological tests. Nevertheless, because of the presence of controls in these tests, the relative numbers of offspring produced may be a good indication of toxic effects.

3.2 Methods

The same test solutions as for the Chapters 1 and 2 (Table 1.3 in Chapter 1, and Table 1 in Chapter 2 as a paper) were used in the experiments on reproduction. Each concentration of test solution in each of three replicates received 10 mature individuals (excluding breeding females and moulting individuals) of *P. nigroculus* (sex ratio: 1:1) and each set of experiments was run three times, giving 9 replicates per concentration. Dilution of toxins was done in both river and 'artificial' water. All other experimental conditions were the same as for the experiments on mortality and growth (Chapter 2): semi-static type, pH $< 5.0 \pm 0.8$; TH < 40 mg/L as $CaCO_3$; temperature 14 ± 1°C, etc. Dilution water was replaced twice a week and *Rapanea melanophloeos*

leaves as feed were provided twice a week. Nutrient media (such as Elendt M4 used for *D. magna*) are not available for *P. nigroculus* but it is known that this species can survive and breed adequately for months or years in stream water with *R. melanophloeos* leaves (Stewart 1991). This is one of the reasons for duplicating the experiments with natural stream water and with 'artificial' water.

The growth rate of the population, estimated as changes in the number of individuals in the exposed populations, was determined by the model adapted from Hyne *et al.* (1993) at 95% confidence limits, as follows:

$$kt = [\ln(N_t) - \ln(No)]/T,$$

where k = the growth rate per day for each concentration; ln = log to base e; N_t = number of individuals after exposure time T; and N_0 = number of individuals at the beginning of experiments (time t = O); T = exposure time in days (45 days). Since migration into the test populations was zero, the increase in numbers was due only to production of juveniles during the tests. The count regarded all live individuals after 45 days of exposure.

3.3 Results

The results for the reproduction of *P. nigroculus* are reported in the Tables 3.1 and 3.2 with reference to natural and 'artificial' waters respectively. Juveniles were produced in all treatments.

Results showed that more juveniles were produced in solutions with Al + Cu + Mn than in solutions containing Al + Cu only, generally more in stream than 'artificial' water; most produced was 267 in Ca1 in stream water (Al + Cu) and fewest produced was 61 in Bc1 in 'artificial' water (Al + Cu).

Differences in growth rates will be analysed in detail in Chapter 7, which deals with all statistical calculations. The Mann-Whitney U-test of significance was performed, however, to determine if the mixtures of Al + Cu vs the mixtures of Al + Cu + Mn showed significant differences in the numbers of juveniles produced and also to determine the effect of dilution water (river water or 'artificial' water) of reproductive rates. The dependent variable was the number of juvenile amphipods produced after 45 days.

Calculated R (R defined as total of ranks for all scores) values (R = 64 in river water and 63 in 'artificial' water) were lower than R value from the statistical Table (R = 65) at p = 0.002 for n1 = n2 = 10, the null hypothesis was rejected. So the metal mixtures (Al + Cu and Al + Cu + Mn) in stream and 'artifical' water significantly differed in their effect on the reproduction of the amphipods (at α = 0.002) in the sense that in both dilution waters, reproductive rates were higher in the solutions of Al + Cu with Mn than without Mn.

Table 3.1: Reproduction of P. nigroculus after 45-day exposures to Al, Cu and Mn mixtures in natural water (concentrations in mg/L). N_o = 30; T = 45 days; $N_t \pm SD$ = number of amphipods after 45 days of exposure; k_t = reproductive rate.

Mixture	*Al*	*Cu*	*Mn*	*Nt (±SD)*	k_t
Aa1 (TWQR)	0.062	0.001		189 (12)	0.041
Aa2 (TWQR)	0.062	0.001	1.217	237 (15)	0.046
Ab1 (CEV)	0.185	0.004		131 (12)	0.033
Ab2 (CEV)	0.185	0.004	2.434	269 (8)	0.049
Ac1 (AEV)	0.926	0.008		127 (9)	0.032
Ac2 (AEV)	0.926	0.008	9.328	250 (12)	0.047
Ba1 (TWQR + 50%)	0.0925	0.0025		149 (7)	0.036
Ba2 (TWQR + 50%)	0.0925	0.0025	1.825	247 (5)	0.047
Bb1 (CEV + 50%)	0.2775	0.005		120 (3)	0.031
Bb2 (CEV + 50%)	0.2775	0.005	3.65	239 (9)	0.046
Bc1 (AEV + 50%)	1.388	0.0175		83 (5)	0.023
Bc2 (AEV + 50%)	1.388	0.0175	13.993	234 (10)	0.046
Ca1 (TWQR - 50%)	0.030	0.0005		267 (19)	0.049
Ca2 (TWQR - 50%)	0.030	0.0005	0.6075	260 (15)	0.048
Cb1 (CEV - 50%)	0.0925	0.0175		152 (5)	0.036
Cb2 (CEV - 50%)	0.0925	0.0175	1.218	250 (11)	0.047
CC1 (AEV - 50%)	0.4325	0.004		122 (5)	0.031
Cc2 (AEV - 50%)	0.4325	0.004	4.665	240 (9)	0.046
AEV2a (new AEV)	0.1225	0.005		145 (7)	0.035
AEV2b (new AEV)	0.1225	0.005	5.273	241 (12)	0.046
Control	0	0	0	257 (14)	0.048

Table 3.2: Reproduction of P. nigroculus after 45-day exposures to Al, Cu and Mn mixtures in 'artificial' water (concentrations of metals in mg/L). N_o = 30; T = 45 days; N_t (± SD) = Number of amphipods after 45 days of exposure = mean values of 3 experiments; k_i = reproductive rate

Mixture	*Al*	*Cu*	*Mn*	N_t (±SD)	k_i
Aa1 (TWQR)	0.062	0.001		167 (8)	0.038
Aa2 (TWQR)	0.062	0.001	1.217	243 (12)	0.046
Ab1 (CEV)	0.185	0.004		119 (6)	0.031
Ab2 (CEV)	0.185	0.004	2.434	259 (18)	0.048
Ac1 (AEV)	0.926	0.008		113 (5)	0.029
Ac2 (AEV)	0.926	0.008	9.328	233 (9)	0.046
Ba1 (TWQR + 50%)	0.0925	0.0025		150 (7)	0.036
Ba2 (TWQR + 50%)	0.0925	0.0025	1.825	234 (17)	0.046
Bb1 (CEV + 50%)	0.2775	0.005		116 (5)	0.030
Bb2 (CEV + 50%)	0.2775	0.005	3.65	244 (18)	0.047
Bc1 (AEV + 50%)	1.388	0.0175		61 (4)	0.016
Bc2 (AEV + 50%)	1.388	0.0175	13.993	224 (8)	0.045
Ca1 (TWQR - 50%)	0.030	0.0005		248 (14)	0.047
Ca2 (TWQR - 50%)	0.030	0.0005	0.6075	251 (14)	0.047
Cb1 (CEV - 50%)	0.0925	0.0175		145 (3)	0.035
Cb2 (CEV - 50%)	0.0925	0.0175	1.218	247 (18)	0.047
Cc1 (AEV - 50%)	0.4325	0.004		118 (7)	0.030
Cc2 (AEV - 50%)	0.4325	0.004	4.665	231 (11)	0.045
AEV2a (new AEV)	0.1225	0.005		143 (6)	0.035
AEV2b (new AEV)	0.1225	0.005	5.273	237 (15)	0.046
Control	0	0	0	193 (8)	0.041

Examining the effects of the same mixtures of metals in stream water and in 'artificial' water (i.e. Al + Cu in both waters or Al + Cu + Mn in both waters), production of juveniles was not significantly affected by the type of water used. Thus the rate of population growth was not significantly different in river and in 'artificial'

water, calculated R (R = 93 for mixture of Al + Cu in both dilution water and R = 87 for mixture of Al + Cu + Mn in both dilution water) being higher than the value from the table at 0.05 (R = 82 at α = 0.05). Observed differences were therefore attributed to chance.

These calculations were done separately for each U-test, as follows:

I looked at all Al + Cu (stream water) vs Al + Cu + Mn (stream water) and Al + Cu ('artificial') vs Al + Cu + Mn ('artificial') for each water-type first. I concluded that in both waters, more juveniles were produced if Mn was present than if it was absent. Then I looked at all Al + Cu (stream) vs Al + Cu ('artificial') and Al + Cu + Mn (stream) vs Al + Cu + Mn ('artificial') for both water-types, and I concluded that water type did not affect significantly the production of juveniles.

3.4 Conclusion

The growth rate for the populations of *P. nigroculus* exposed to a mixture of Al + Cu or Al + Cu + Mn was shown to depend on the presence of Mn and not on the type of the dilution water used.

Mortality, growth and reproduction showed that the mixtures of Al + Cu in either dilution water (river water or 'artificial' water) were more toxic than that with Al + Cu + Mn. Mn appeared to reduce the toxicity of mixtures of Al + Cu.

Some explanations for these differences are explored in the following Chapters.

CHAPTER 4

Chemical speciation and toxicity of Al, Cu and Mn

4.1 Introduction

The results from Chapters 2 and 3 show the combination Al + Cu to be more toxic than the combination of Al + Cu + Mn in acidic waters in both acute and chronic tests. I performed individual toxicity tests of each element on *P. nigroculus* to determine the median survival time for each metal. The arithmetic graphic method was used to calculate the LC_{50} values after 96-hour exposures (values based on nominal concentrations which are the concentrations derived from the dissolution of a given amount of chemical in a given volume of dilution water, these concentrations being not necessarily bioavailable): 360 mg/L Al, 20 mg/L Cu and 375 mg/L Mn at pH < 5.0. From these values, test concentrations were derived. Tian (1996) reported *P. nigroculus* to be tolerant to high nominal concentrations of Al. She found that at pH 5.0-5.4, at nominal concentration of 211 mg/L, only 45% of *P. nigroculus* died after 96 hours of exposure. Al is a non-essential element. From her data, the 96-h LC_{50} for Al is 363 mg/L. No information was available regarding LC_{50} values for Cu and Mn in *P. nigroculus*. This is why I started this Chapter by determining the 96-h LC_{50} for Cu and Mn.

As stated in Chapters 1 and 2, Cu and Mn are two essential metals, are abundant in aquatic environments. Cu is reported to be very toxic to aquatic life for various taxa such as algae (Brown 1977, Viarengo *et al.* 1981, Roesijadi *et al.* 1987, Botley 1989a) and fish (McDonald *et al.* 1989). Not much is known about Mn toxicity in aquatic environments but Mn toxicity has been described for some fish such as *Barbus marequensis* from the Olifants River in South Africa (Nath & Kumar 1987, Seymore *et al.* 1995), and *Salmo gairdneri* (McDonald 1989) in acidic water. Mn^{2+} predominates in dissolved form at low pH (< 5.5) and Mn^{2+} is readily oxidised to the insoluble manganic (Mn^{4+}) form when the pH increases. Mn bound to dissolved organic carbon (DOC) presents a minor ecological risk as colloidal manganese oxyhydroxides and permanganate ions (Mn^{7+}) do not persist in the environment because they rapidly oxidise organic materials and are therefore reduced (Lazerte & Burling 1990). The most important effect of Mn is its action in mixtures because dissolved Mn (Mn^{2+}) may increase or reduce the toxicity of other metals (Richard & Bourg 1991, Driehaus *et al.* 1995).

Based on ionic and covalent indices, the toxicity of the three elements are ranged, from the most to the least toxic, as follows: Cu > Al > Mn. As mentioned in Chapter 1, class A cations (which include Al, alkali and the alkali earth metals), have a preference for ligands where oxygen is the donor atom (e.g. ligands that are found on the gill surfaces) whereas class B cations (mostly the Period VI transition metals, Pt to Bi and including Cu), have a particularly high affinity for biological binding sites containing N and S donor ligands (e.g. surface and subsurface proteins). The borderline cations (most of the remaining transition metals, including Mn) have less well defined preferences for ligands (Nieboer & Richardson 1980). This is confirmed by the 96-h

LC_{50} values: 20 mg/L for Cu, 360 mg/L for Al (Tian 1996) and 375 mg/L for Mn for *P. nigroculus* that I determined.

Chemical speciation and toxicity modelling for Al, Cu and Mn were also performed in this study using the MINTEQA2/PRODEFA2 chemical equilibrium package for all calculations (Botley 1989a, Allison *et al.* 1990, Shuman 1992, Filella *et al.* 1995, Serkiz *et al.* 1996). The aim was to determine metal species present in solutions and the interactions betweem the metals and at last the induced toxicity. Gaddum diagrams were used to determine different interactions between Al, Cu and Mn (Ward & Parrish 1983). According to the Gaddum method, the LC_{50} proportion or toxic unit (T.U.) is the ratio between the actual concentration of a pollutant in water and the 96-h LC_{50} value for this pollutant. This method suggests the simple way of determining the interactions between two metals, A and B. Test organisms are exposed to solutions with concentrations corresponding to half-values of the 96-h LC_{50} (i.e. T.U.= 0.5 LC_{50}) for each metal. When the biological response (e.g. mortality) is the same as for the median survival concentration (LC_{50}) of each metal alone, the interaction is additive. When the response is above that obtained for the 96-h LC_{50}, the interaction is supra-additive (i.e. synergistic) and when the response in terms of LC_{50}, is below that obtained for 96-h LC_{50}, the interaction may be less than additive (i.e. antagonistic: see Figure 4.1). So from the three 96-h LC_{50} values (360 mg/L for Al; 20 mg/L for Cu and 375 mg/L for Mn), I prepared test concentrations corresponding to different LC_{50} proportions (Table 4.1).

A Chi-squared test was used to check if the difference in the proportions of populations surviving was significant or not.

4.2 Material and methods

Single acute toxicity tests were run to determine the 96-h LC_{50} values for each element. Stock solutions were prepared by dissolving a given amount of salt in 1 litre of distilled water. So for Al, I dissolved 24.701 g of $Al_2(SO_4)_3.18H_20$ (Merck extrapure); for Cu, 3.918 g of $CuSO_4.18H_2O$ (Analar 99.5%) and for Mn, 3.970 g of $MnSO_4.4H_2O$ (BDH 97%). These stock solutions contained 1 gram of metal each. From these stock solutions, test concentrations were derived by dilution method, 1 mL of each stock solution representing 1 mg of the metal species. Only 'artificial' water was used for dilution. Mature *P. nigroculus* (excluding breeding females and moulting individuals) were used as test organisms. Ten individuals per replicate in 3 replicates, giving 30 individuals per concentration.

I used the same test features as for Chapters 2 and 3. Tests lasted only 96 hours and no food was offered. Solutions were not aerated. Each replicate was checked twice a day and dead amphipods were removed. After 96-h exposures, the median survival concentration (LC_{50}) were found by arithmetic graphic method. These

values were then used in Gaddum method. These results are reported in Table 4.1 as preliminary information necessary in the application of Gaddum diagrams.

4.2.1 Introductory results for 96-h LC_{50} (as part of the Gaddum method)

Preliminary results as 96-h LC_{50} for Al, Cu and Mn are reported in Table 4.1.

These values were later used to prepare test solutions for the application of Gaddum diagrams.

Table 4.1: Preliminary results for 96-h LC_{50} (values are in mg/L)

Metal	*Replicate No.*			*Mean (±SD)*
	(1)	*(2)*	*(3)*	
Al	361	359	359	*360 (1)
Cu	0.019	22	19	20 (2)
Mn	376	376	373	375 (2)

* Tian (1996) found the 96-h LC_{50} for *P. nigroculus* to be 363 mg/L for Al.

Test concentrations were then made from LC_{50} proportions, which are toxic units, according to Gaddum method (Ward & Parrish 1983) as mentioned in the introduction. These concentrations (Table 4.2) were used in determining the interactions, while TWQR (Chapters 1-2: Table 1.3) values were used for the modelling of chemical speciation.

Table 4.2: Test concentrations (in mg/L) based on 96-h LC_{50} proportions of Al, Cu and Mn in 'artificial' water

96-h LC_{50} Proportions	*Al*	*Cu*	*Mn*
0.25	90	5	93.8
0.50	180	10	187.5
0.75	270	15	281.3
1.0	360	20	375
1.25	450	25	469
1.50	540	30	563
1.75	630	35	656

After one week of acclimation, three replicates of 10 mature but non-breeding and non-moulting amphipods were exposed to each concentration. Experiments were run three times, so in all 90 individuals were used per concentration. Each experiment lasted at least 96 hours and the only parameter used to assess toxic effects was the median survival time (LT_{50}), assuming that the time after which 50% of an exposed population dies is an inverse function of toxicity.

After comments by Enserink S.L. (1997: pers. com.) on the use of LC_{50}, I have also calculated the 96-h LC_{50} values using the arithmetic graphic method described in Chapter 1.

At the beginning and at the end of the experiments, pH, TH and electric conductivity were measured (see Chapter 2: Table 2 in the paper). The MINTEQA2/PRODEFA2 chemical equilibrium package (Allison *et al.* 1990, Serkiz *et al.* 1996) was used for modelling toxicity and chemical speciation. Reactions and equilibrium constants used in the calculations are listed in Table 4.10, as are all the minerals considered in the calculations (Table 4.11).

The following metal combinations were used both in Gaddum diagrams and in MINTEQA2/PRODEFA2 program : (a) Al + Cu; (b) Al + Mn; (c) Cu + Mn and (d) Al + Cu + Mn. Concentration data used for input in the calculations for MINTEQA2 are given Table 1.3 at TWQR (i.e. 0.062 mg/L Al; 0.001 mg/L Cu and 1.217 mg/L Mn). The pH value was fixed at 5.0. Total dissolved solids (TDS) was calculated by MINTEQA2 and equilibrium constants were corrected at the same time (Allison *et al.* 1990, Serkiz *et al.* 1996). Thus errors related to expressing the reaction in terms of MINTEQA2 were corrected by writing all reactions in MINTEQA2 as formation reactions from the MINTEQA2 components. For solid species, the log K and standard enthalpy of reaction needed in MINTEQA2 were of opposite sign. All solutions were assumed to be in equilibrium with atmospheric CO_2; supersaturated solids were allowed to precipitate when present. Finally, the modelling results and their interpretation were subject to the following assumptions:

(a) the data collected in the MINTEQA2/PRODEFA2 database are complete and the best available;

(b) the experimental systems are in equilibrium with atmospheric CO_2;

(c) redox processes are disregarded;

(d) effects of natural organic matter are disregarded (no organics are present in 'artificial' water solutions); and

(e) free metal species (Cu^{2+}, Mn^{2+} and Al^{3+}) are biovailable (due to acidic conditions: pH < 5.0), and the influence of concentration on the metal species was also disregarded.

A summary of concentrations used as input is given in Table 4.3.

Table 4.3: Components and concentrations used in calculations

Components	*Concentration (mol/L)*
H^+	**
Na^+	1.453×10^{-3}
Ca^{2+}	6.798×10^{-5}
Mg^{2+}	4.153×10^{-5}
SO_4^{2-}	4.711×10^{-5}
Cl^-	1.589×10^{-3}
CO_3^{2-}	***
Cu^{2+}	4.000×10^{-9}
Mn^{2+}	5.500×10^{-6}
Al^{3+}	1.860×10^{-7}

Note: ** = pH fixed at 5.0 (i.e. $[H^+] = 10^{-5}$ mol l^{-1}); *** = solution in equilibrium with CO_2.

The No Observed Effect Concentration (NOEC) at 95% level of confidence ($p<0.05$) for each metal species was calculated using the equation of Sloof *et al.* (1986) as modified by Forbes and Forbes (1994) as follows:

$$NOEC = 0.52 \times (Acute)^{0.95}$$

where 'acute' means 96-h LC_{50}.

Median survival times (LT_{50}) were calculated by interpolating between the time periods which contained the 45th and 46th deaths and taking the averages of these two times. For example for Al + Cu, the median survival time must lie in the interval between 96 and 120 hours (Table 4.4), a time interval of 24 hours. At 96 hours, there were 49 individuals alive and at 120 hours, there were 41 alive. So in 24 hours there were 8 deaths. I assume the deaths to have occurred uniformly over 24 hours, so there was a death every 3 hours. The 46th death occurred 9 hours after 96 hours, which is 105 hours, and the 45th death occurred 12 hours after 96, that is at 108 hours. The median survival time is thus (105 hours + 108 hours)/2 = 106.5 hours, which is rounded to 107 hours.

The 95% confidence intervals for the percentage of *P. nigroculus* surviving 96 hours or more were obtained from Chi-squared Table (for Chi-squared = 43.03, df = 3).

The 96-h LC_{50} was calculated by arithmetic method as stated above.

4.3 Results and discussion

4.3.1 Survival / Mortality

Results of acute tests for individual 96-h LC_{50} experiments are given in Table 4.1.

All other results are reported in Tables 4.4 - 4.7. Table 4.4 gives the number of survivors of *P. nigroculus* in mixtures of Al + Cu, Table 4.5 for mixtures of Cu + Mn, Table 4.6 for Al + Mn, and Table 4.7 for Al + Cu + Mn after 24, 48, 72, 96, 120 and 150 hours of exposure using LC_{50} proportions for each metal.

Table 4.4: Total number of survivors of P. nigroculus in the mixtures of Al + Cu after 0-, 24-, 48-, 72-, 96-, 120- and 150-hour exposures using LC_{50} proportions for each metal species

Al + Cu:	*Al*	*Cu*	*Exposure time*						
Properties of LC_{50}	*mg/L*	*mg/L*	*0-h*	*24-h*	*48-h*	*72-h*	*96-h*	*120-h*	*150-h*
0.25	90	5	90	81	70	62	53	31	12
0.50	180	10	90	75	61	56	49	41	6
0.75	270	15	90	70	59	48	44	23	0
1.00	360	20	90	11	0	0	0	0	0
1.25	450	25	90	6	0	0	0	0	0
1.50	540	30	90	0	0	0	0	0	0
1.75	630	35	90	0	0	0	0	0	0
2.00	720	50	90	0	0	0	0	0	0
Control	0	0	90	90	88	87	87	83	79

The 96-h LC_{50} for mixture of Al + Cu is 0.70 toxic units (T.U.).

Survival after 96-hour exposure at 0.50 LC_{50} proportions for Al and Cu (Table 4.4) is 54.4%. So $LT_{50} > 96$ hours. The toxicity of this mixture is mitigated since the median survival time (LT_{50}) is equal to 107 hours.

Interaction is infra-additive and antagonistic reactions may be occuring. Indeed, as LT_{50} is longer than 96 hours, there is a possibility of antagonism between the actions of Al and Cu.

Table 4.5: Total number of survivors of P. nigroculus in the mixtures of Cu + Mn after 0-, 24-, 48-, 72-, 96-, 120- and 150-hour exposures using LC_{50} proportions for each metal species

Cu + Mn:	*Cu*	*Mn*	*Exposure time*						
Properties of LC_{50}	*mg/L*	*mg/L*	*0-h*	*24-h*	*48-h*	*72-h*	*96-h*	*120-h*	*150-h*
0.25	5	93.8	90	79	70	51	49	14	0
0.50	10	187.5	90	71	52	45	36	9	2
0.75	15	281.3	90	68	49	35	24	2	0
1.00	20	375	90	15	3	0	0	0	0
1.25	25	469	90	6	0	0	0	0	0
1.50	30	563	90	1	0	0	0	0	0
1.75	35	656	90	0	0	0	0	0	0
2.00	40	750	90	0	0	0	0	0	0
Control	0	0	90	90	88	87	87	83	79

The 96-h LC_{50} for mixture of Cu + Mn is 0.32 T.U.

Survival after 96-hour exposure at concentration corresponding to 0.50 LC_{50} proportions for Cu and Mn (Table 4.5) is 40%, so LT_{50} < 96 hours. This combination is therefore toxic since the median survival time (LT_{50}) is equal to 73 hours. The interaction between the elements is supra-additive since the toxic effect resulting in the death of the amphipod is magnified in the mixture of Cu + Mn at half-lethal concentrations (0.50 LC_{50}).

Survival after 96-hour exposures at concentration corresponding to 0.50 LC_{50} proportions for Al, Cu and Mn (Table 4.6) is 86.7%, so LT_{50} > 96 hours. The interactions are antagonistic and the mixture of Al + Cu + Mn is not acutely toxic since the median survival time (LT_{50}) for Al + Cu + Mn is 147 hours.

Table 4.6: Total number of survivors of P. nigroculus in the mixtures of Al + Cu + Mn after 0-, 24-, 48-, 72-, 96-, 120- and 150-hour exposures using LC_{50} proportions for each metal species

Al + Cu + Mn:	*Al*	*Cu*	*Mn*	*Exposure time*						
Properties of LC_{50}	*mg/L*	*mg/L*	*mg/L*	*0-h*	*24-h*	*48-h*	*72-h*	*96-h*	*120-h*	*150-h*
0.25	90	5	93.8	90	88	87	83	80	63	46
0.50	180	10	187.5	90	87	87	81	78	60	44
0.75	270	15	281.3	90	84	80	71	62	51	39
1.00	360	20	375	90	81	76	70	58	49	39
1.25	450	25	469	90	78	69	61	53	44	23
1.50	540	30	563	90	71	66	58	46	35	11
1.75	630	35	656	90	60	41	29	16	8	0
2.00	720	40	750	90	20	6	0	0	0	0
Control	0	0	0	90	90	88	87	87	83	79

The 96-h LC_{50} for mixture of Al + Cu + Mn is 1.52 T.U.

Table 4.7: Total number of survivors of P. nigroculus in the mixtures of Al + Mn after 0-, 24-, 48-, 72-, 96-, 120- and 150-hour exposures using LC_{50} proportions for each metal species

Al + Mn:	*Al*	*Mn*	*Exposure time*						
Properties of LC_{50}	*mg/L*	*mg/L*	*0-h*	*24-h*	*48-h*	*72-h*	*96-h*	*120-h*	*150-h*
0.25	90	93.8	90	80	73	68	61	50	43
0.50	180	187.5	90	77	71	62	55	49	38
0.75	270	281.3	90	72	66	61	50	45	21
1.00	360	375	90	67	60	53	49	44	12
1.25	450	469	90	68	63	54	46	31	14
1.50	540	563	90	62	50	42	23	13	0
1.75	630	656	90	53	31	10	0	0	0
2.00	720	750	90	38	13	0	0	0	0
control	0	0	90	90	88	87	87	83	79

The 96-h LC_{50} for mixture of Al + Mn is 1.26 T.U.

Survival after 96-hour exposures at concentration 0.50 LC_{50} of Al and Mn (Table 4.7) is 61.1%, so LT_{50} > 96 hours. The interactions are therefore antagonistic since LT_{50} > 96 hours. This combination is sparingly toxic. The median survival time (LT_{50}) for Al + Mn is 139 hours.

As mentioned in the section of methods, the NOEC values for the three metals were derived from the equation of Sloof *et al.* (1986) as modified by Forbes & Forbes (1994), which is

$$NOEC = 0.52 \times (acute)^{0.95}.$$

The values are 139.479 mg/L for Al, 8.953 mg/L for Cu and 144.988 mg/L for Mn. These values being greater than the actual South African interim guidelines (i.e. at TWQR: 0.5 μg/L Al, 0.2 μg/L Cu and 180 μg/L Mn; at CEV: 1 μg/L Al, 0.53 μg/L Cu and 370 μg/L Mn; at AEV: 10 μg/L Al, 1.6 μg/L Cu and 1300 μg/L Mn) remain valueless in my study because based on nominal concentrations and mortality only. Another reason is that the use of NOEC in the standard clones models is misleading because of several problems inherent in NOEC (e.g. extreme standardization of cultured (cloned) organisms (Kooijman 1996) eliminates the physiological differences between individuals; the distribution that describes the variation in threshold values (log-logistic, log-probit) represents an arbitrary choice from a large set of possible distributions; sublethal effects show no big variations in the physiological conditions of standardized test organisms (e.g. *Daphnia magna*). So when a toxicant affects reproduction, for instance, it does in all individuals to about the same extent; there are no records of individuals that cease reproduction, while others continue at the control rate). Another reason is that LC_{50}/EC_{50} values depend on exposure time. By standardizing toxicity tests to a fixed exposure period (e.g. 48 hours with *Daphnia magna*) independently of the type of compound tested, there is a mistake. For example, surfactants react quickly and if no effect shows up after a few hours of exposure, it unlikely that any effect will show up at that concentration, while things are totally different for metals such as Cd. So the LC_{50}-time behaviour depends also on what you can observe and on properties of the chemical as well as those of the organism (especially body size). For these reasons, some alternatives such as 'Small' Effect Concentration and 'No' Effect Concentration (NEC) are under discussion and might replace the NOEC (Laskowski 1995, Kooijman 1996). I calculated the NOEC just to have an idea on the extent to which these values are above the S.A. criteria.

I used the median survival concentrations (LC_{50}) of each metal species (i.e. 96-h LC_{50} = 360 mg/L for Al, 20 mg/L for Cu and 375 mg/L for Mn) in the calculation of the NOEC. These NOEC values are based on mortality only since I do not know the role of each metal species in the physiology of this amphipod. This should be studied before standardizing *P. nigroculus* as test organism for aquatic ecosystem.

The toxicity tests using physiological endpoints (e.g. sublethal effects) instead of mortality may lower these NOEC values (Kooijman 1996, Hallam 1996).

According to the results for the experiment on survival, the toxicity of different mixtures is, from the most to the least toxic, as follows : Cu + Mn (LT_{50} = 73 hours) > Al + Cu (LT_{50} = 107 hours) > Al + Mn (LT_{50} = 139 hours) > Al + Cu + Mn (LT_{50} = 147 hours), assuming that the median survival time (LT_{50}) is reverse of toxicity for a given pollutant or mixture of pollutants. Using Gaddum diagrams (as explained above), the interactions between:

- Cu and Mn are supra-additive (strong synergism, LT_{50} < 96 hours, Figure 4.3 , and 96-h LC_{50} = 0.32 toxic units);
- Al and Cu are infra-additive (with mitigated synergism and LT_{50} slightly above 96 hours, Figure 4.2, and 96-h LC_{50} = 0.70 toxic units);
- Al and Mn are antagonistic (with antagonism and LT_{50} > 96 hours, Figure 4.4 , and 96-h LC_{50} = 1.26 toxic units); and finally
- Al, Cu and Mn are also antagonistic (with LT_{50} above 96 hours: 139 hours for Al + Mn and 147 hours, Figure 4.4, and 96-h LC_{50} = 1.52 toxic units). However, the percentage of survivors after 96 hours of exposure in the Al + Cu mixture (54.4%) and Al + Mn (61.1%) mixture is not statistically different using Chi-squared test (Chi-squared = 43.03, df = 3, $p<0.001$), suggesting that the toxicity of these mixtures may be mitigated. The median survival times (= LT_{50}) are 73h for Cu + Mn, 107h for Al + Cu, 139h for Al + Mn and 147h for Al + Cu + Mn. This confirms the fact that the combination of Cu + Mn is the most toxic, followed by Al + Cu and Al + Mn. The combination of Al + Cu + Mn is the least toxic.

E.L. Enserink (1997: pers. com.) using her 'unspecified' method found the following 96-h LC_{50} (Table 4.8) and my values are in (...) and I used arithmetic method)

Table 4.8: 96-h LC_{50} calculated from data in Tables 4.4 - 4.7 and interaction types

Data from Tables 4.4 - 4.7	*96-h LC_{50} (...) in T.U.*	*Interaction*
4.4	0.75 (0.70)	infra-additive
4.5	0.30 (0.32)	supra-additive
4.6	1.50 (1.52)	antagonistic
4.7	1.25 (1.26)	antagonistic

4.3.2 Chemical speciation and toxicity of Al, Cu and Mn

The results of modelling using MINTEQA2/PRODEFA2 and test concentrations of 0.062 mg/L Al, 0.001 mg/L Cu and 1.217 mg/L Mn (TWQR) showed for the following combinations that

- Cu + Mn were present predominantly as Cu^{2+} and Mn^{2+} and no supersaturated solids were identified; speciation did not differ from that of the metals in isolation.
- Al + Cu: Cu was present mainly as Cu^{2+}; the solution was supersaturated with respect to diaspore, an aluminium trihydroxide solid or $Al(OH)_3$ (the remainder being Al^{3+}); approximately 85% of Al^{3+} was removed from the solution as diaspore;
- Al + Mn: again, the solution was supersaturated with respect to diaspore; Mn was present predominantly as Mn^{2+};
- Al + Cu + Mn: Mn and Cu predominated as Mn^{2+} and Cu^{2+}; when precipitation was allowed, all Al^{3+} was removed from the solution as diaspore and Cu^{2+} and Mn^{2+} were adsorbed onto the surface of the diaspore surface, reducing the bioavailable concentration of the metal species and therefore the toxicity. Mn (II) allows the precipitation of Al (III). This may explain why the combination of the three elements is the least toxic.

The above results suggest that in acidic waters the combination of Cu + Mn should be very toxic (In fact, it is for *P. nigroculus*: only 40% survival after 96 hours of exposure). The combination of Al + Cu is less toxic and 54 % of *P. nigroculus* survived after 96 hours of exposure, while the combination of Al + Mn not acutely toxic (61% of *P. nigroculus* survived after 96 hours of exposure) and nor is that of Al + Cu + Mn (87% of *P. nigroculus* survived after 96 hours of exposure).

Indeed, as the model did not show any changes in the aqueous speciation of the metals from that in isolation when different combinations of metals were used, the observed changes in toxicity of the mixtures cannot be

explained on the basis of aqueous speciation at TWQR concentrations (0.062 mg/L Al, 0.001 mg/L Cu and 1.217 mg/L Mn). However, the fact that mixtures including Al are supersaturated with respect to $Al(OH)_3$ solids may affect metal speciation and therefore toxicity. Firstly, the precipitation of Al removes some of it from solution, thereby decreasing its bioavailability; secondly, Mn^{2+} increases the precipitation of Al; thirdly, $Al(OH)_3$ solids are regarded as being unavailable for uptake by aquatic organisms (Degremont 1979, Hem 1986, Driscoll *et al.* 1980). Thus the behaviour of the Al in mixtures may be the key factor regulating the combined toxicity of the three metals.

Results of the chemical equilibrium calculations from MINTEQA2/PRODEFA2 simulations support those as predicted, using Gaddum diagrams, that the mixture Cu + Mn would be the most toxic, followed by Al + Cu or Al + Mn and that Al + Cu + Mn would be the least toxic. The results from the experiments on mortality and growth reported in Chapter 2 and experiments on reproduction reported in Chapter 3, are well explained by these analyses, in that combinations Al + Cu affect biological functions more than combinations Al + Cu + Mn do. This may also explain the tolerance of *P. nigroculus* to Al reported by Tian (1996). Nominal concentrations do not mean bioavailable. Indeed, the quantities of metals used to prepare test solutions will not be necessarily ready to be taken up by *P. nigroculus*. A lot of Al, for example, may precipitate and becomes therefore unavailable.

Results of the Chi-squared test used to check for significant differences in the proportions of amphipods surviving for at least 96 hours when exposed to the pollutants at a concentration of 0.50 LC_{50} proportions (as reference concentration) for Al, Cu and Mn starting with 90 amphipods for each combination (Table 4.9).

The hypothesis that there was no significant difference in the proportions of amphipods surviving was rejected (Chi-squared = 43.03, df = 3, $p<0.001$). Examination of the residuals from the fitted values showed that the proportion of amphipods surviving in the mixture of Cu + Mn was significantly lower than with the proportions surviving in mixtures of Al + Cu or Al + Mn. There was no significant difference in survival Al + Cu and Al + Mn and the proportion surviving in Al + Cu + Mn was significantly greater than in the other three. Table 4.9 reports the results of Chi-squared tests for the proportion of amphipods surviving at least 96 hours.

Table 4.9: Chi-squared test results for the proportion of amphipods surviving at least 96 hours (n = 90, pfit = 0.6056, $p<0.05$)

Metal mixtures	*No. of amphipods surviving at least 96h*	*Proportion surviving*	*Residual*	*Lower %*	*Upper %*	*Median survival time or LT_{50} in hours*
Cu + Mn	36	0.4000	-4.538	0.2981	0.5078	73
Al + Cu	49	0.5444	-1.359	0.3396	0.5530	107
Al + Mn	55	0.6111	0.125	0.5026	0.7121	139
Al + Cu + Mn	78	0.8667	6.313	0.7787	0.9292	147

4.4 Conclusion

According to the analysis of the interactions of metal mixtures using

a) the Gaddum method based on LC_{50} proportions or toxic units;

b) the chemical speciation using MINTEQA2/PRODEFA2 computer programme and finally

c) the Chi-squared analysis of the median survival times

the toxicity of different mixtures ranges (from the most to the least toxic) as follows:

$$Cu + Mn > Al + Cu > Al + Mn > Al + Cu + Mn,$$

with 96-h LC_{50} values of 0.70, 0.30, 1.26 and 1.52 T.U., respectively; and with LT_{50} values of 73, 107, 139 and 147 hours respectively. Differences in toxicity between the mixtures of Al + Cu and of Al + Mn were not significant.

Does the bioaccumulation process play an important role in the toxicity of these metals in chronic exposures? What might be the routes of active uptake? These questions are addressed in Chapters 5 and 6.

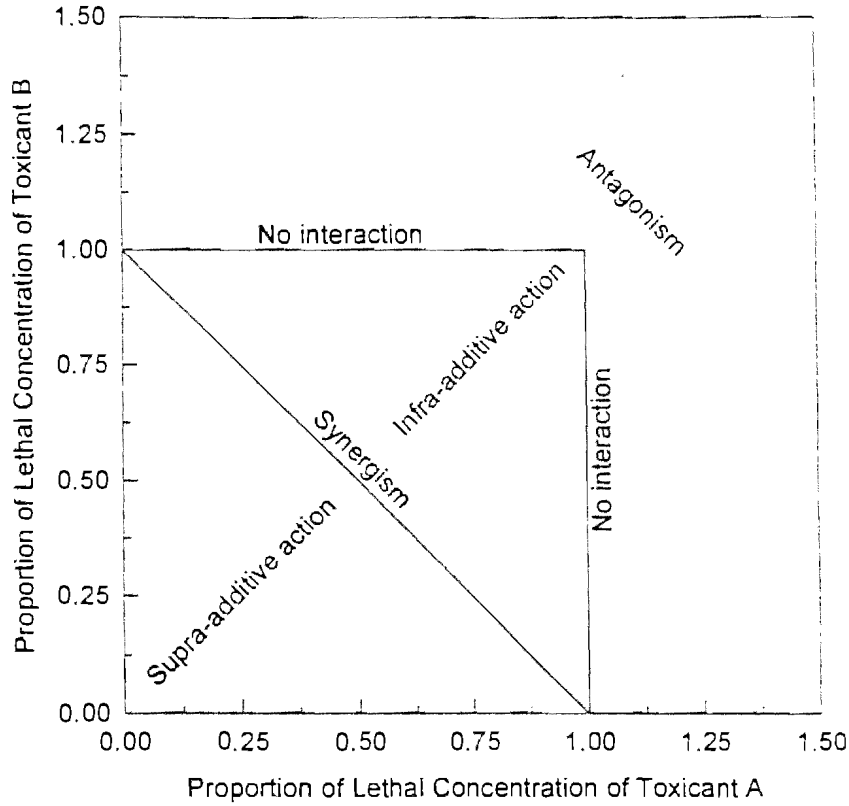


Figure 4.1 Gaddum diagram showing possible interactions that might occur in metal mixtures (after Ward and Parrish 1993). Concentrations are given as mass per unit volume.

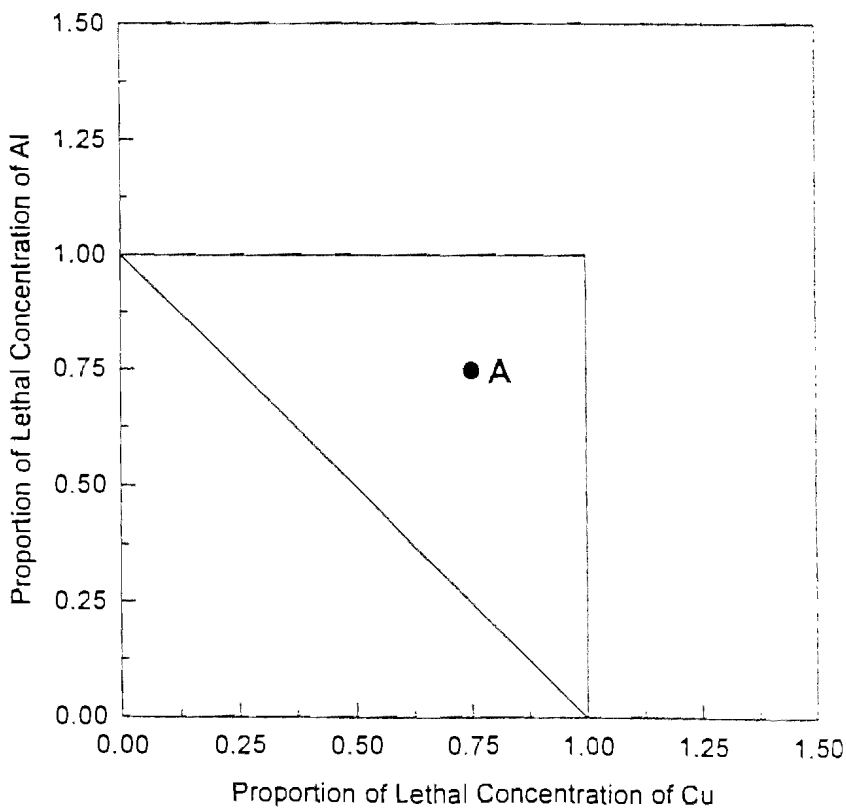


Figure 4.1.1 Gaddum diagram showing the interaction between Cu and Al, where A =Infra-additive action at 0.75 T.U. of both metal species.

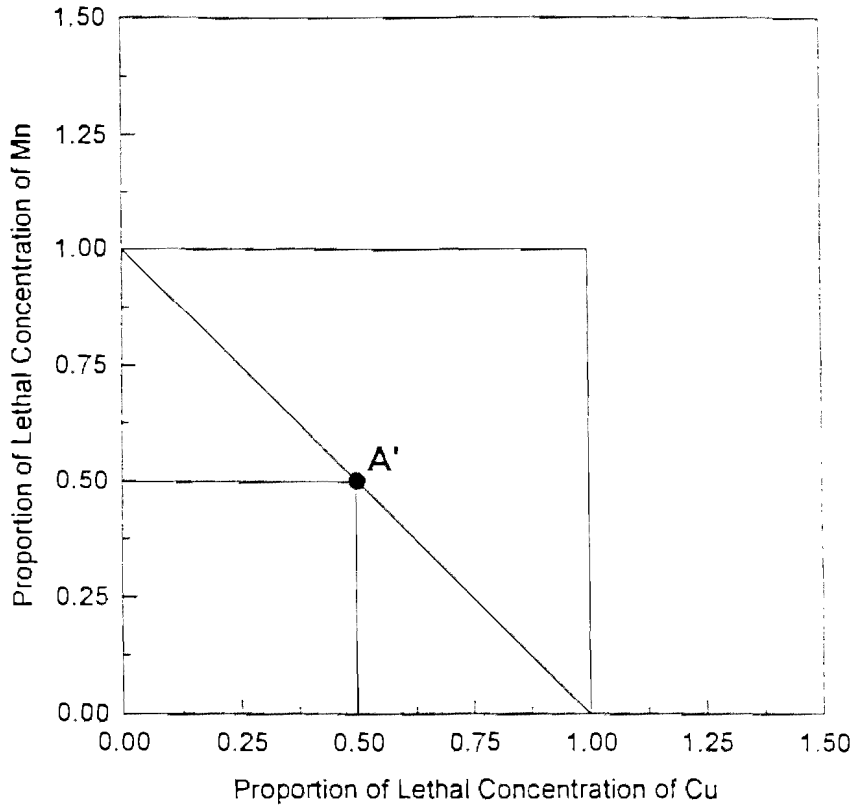


Figure 4.1.2 Gaddum diagram showing the interaction between Cu and Mn, where A' =additive action at 0.50 T.U. of both metal species.

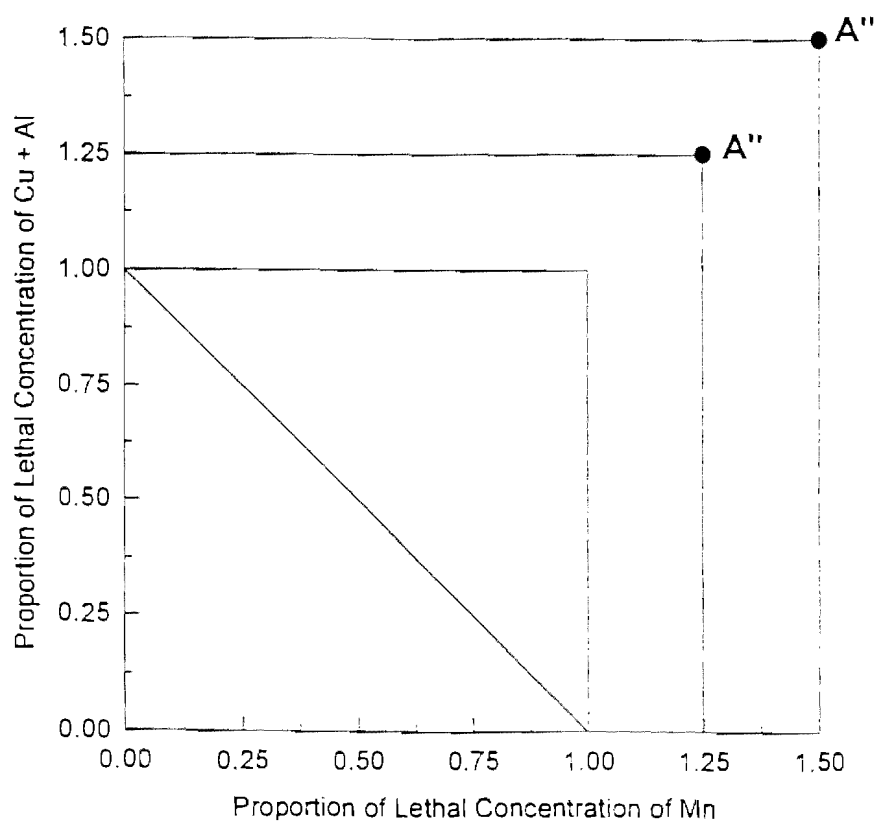


Figure 4.1.3 Gaddum diagram showing the interaction between Cu + Al and Mn, where A'' and A''' = antagonistic action at 1.25 and 1.50 T.U. of all metal species respectively.

Table 4.10: List of species and equilibrium constants considered in chemical speciation (Chapter 4)

Reaction identity & species name	Log K (I = 0.00; T = 298 K)
3301401 H_2CO_3 AQ	16.681
3307320 HSO_4^-	1.987
3300020 OH^-	-13.998
4603300 $MgOH^+$	-11.197
4603304 $Mg(OH)_4^{-2}$	-39.710
4601801 $MgCl^+$	0.600
4601400 $MgCO_3$ AQ	2.920
4601401 $MgHCO_3^+$	11.340
4601403 $Mg_2CO_3^{+2}$	3.680
4607320 $MgSO_4$ AQ	2.250
1503300 $CaOH^+$	-12.697
1501400 $CaHCO_3^+$	11.600
1501401 $CaCO_3$ AQ	3.200
1507320 $CaSO_4$ AQ	2.300
1501403 $CaMgCO_3^{+2}$	4.180
5001400 $NaCO_3^-$	1.268
5001401 $NaHCO_3$ AQ	10.080
5007320 $NaSO_4^-$	0.700
5003300 NaOH	-13.897
303300 $Al(OH)^{+2}$	-4.990
303301 $Al(OH)_2^+$	-10.100
303302 $Al(OH)_4^-$	-23.000
307320 $AlSO_4^+$	3.020
307321 $Al(SO_4)_2^-$	4.920
303303 $Al(OH)_3$ AQ	16.000
4701800 $MnCl^+$	0.607
4701801 $MnCl_2$ AQ	0.041
4701802 $MnCl_3^-$	-0.305
4703300 $MnOH^+$	-10.590
4703302 $Mn(OH)_2$	-22.200
4703301 $Mn(OH)_3^-$	-34.800
4703303 $Mn(OH)_4^{-2}$	-48.300
4703304 Mn_2OH^{+3}	-7.200
4703305 $Mn_2(OH)_3^+$	-23.900
4707320 $MnSO_4$ AQ	2.260
4701401 $MnCO_3$	4.320
4701400 $MnHCO_3^+$	11.600
2311402 $CuHCO_3^+$	12.130
2311400 $CuCO_3$ AQ	6.730
2311401 $Cu(CO_3)_2^{-2}$	10.200
2311403 $CuOHCO_3^-$	-2.410
2311800 $CuCl^+$	0.200
2311801 $CuCl_2$ AQ	0.160
2311802 $CuCl_3^-$	-2.290
2311803 $CuCl_4^{-2}$	-4.590
2313300 $CoOH^+$	-7.500
2313301 $Cu(OH)_2$ AQ	-16.200
2313302 $Cu(OH)^{3-}$	-26.899
2313303 $Cu(OH)_4^{-2}$	-39.600
2313304 $Cu_2(OH)_2^{+2}$	-10.359
2317320 $CuSO_4$ AQ	2.310
2317321 $Cu(SO_4)_2^{-2}$	1.100
2317322 $Cu(SO_4)_3^{-4}$	-3.900
3301400 HCO_3^-	10.330
3301403 CO_2 (g)	21.600

Table 4.11 All minerals considered in chemical speciation using MINTEQA2/PRODEFA2 method

Identity Name	Stoichiometry in [brackets]
200300 $AlOH_3$ (A)	[1.000]Al+3[3.000] H_2O[-3000]H^+
6003000 $AlOHSO_4$	[-1.000]H^+ [1.000]Al^{+3} [1.000]SO_4^{-2} [1.000]H_2O
6003001 $Al_4(OH)_{10}SO_4$	[-10.000]H^+[4.000]Al^{+3}[1.000]SO_4^{-2} [10.000]H_2O
6015000 Anhydrite	[1.000]Ca^{+2} [1.000]SO_4^{-2}
5015000 Aragonite	[1.000]Ca^{+2} [1.000]CO_3^{-2}
5046000 Artinite	[-2.000]H^+ [2.000]Mg^{+2} [1.000]CO_3^{-2} [5.000]H_2O
2003001 Boehmite	[-3.000]H^+ [1.000] Al^{+3} [2.000]H_2O
2046000 Brucite	[1.000]Mg^{+2} [2.000]H_2O [-2.000]H^+
5015001 Calcite	[1.000]Ca^{+2} [1.000]CO_3^{-2}
2003002 Diaspore	[-3.000]H^+ [1.000]Al^{+3} [2.000]H_2O
5015002 Dolomite	[1.000]Ca^{+2} [1.000]Mg^{+2} [2.000]CO_3-2
6046000 Epsomite	[1.000]Mg^{+2} [1.000]SO_4^{-2} [7.000]H_2O
2003003 Gibbsite (C)	[-3.000]H^+ [1.000]Al^{+3} [3.000]H_2O
3003000 Al_2O_3	[2.000]Al^{+3} [3.000]H_2O [-6.000]H^+
6015001 Gypsum	[1.000]Ca^{+2} [1.000]SO_4^{-2} [2.000]H_2O
4150000 Halite	[1.000]Na^+ [1.000]Cl^-
5015003 Huntite	[3.000]Mg^{+2} [1.000]Ca^{+2} [4.000]CO_3^{-2}
5046001 Hydromagnesite	[5.000]Mg^{+2} [4.000]CO_3^{-2} [-2.000]H^+ [6.000]H_2O
5046002 Magnesite	[1.000]Mg^{+2} [1.000]CO_3^{-2}
6050001 Mirabilite	[2.000]Na^+ [1.000]SO_4^{-2} [10.000]H_2O
3050000 Natron	[2.000]Na^+ [1.000]CO_3^{-2} [10.000]H_2O
5046003 Nesquehonite	[1.000]Mg^{+2} [1.000]CO_3^{-2} [3.000]H_2O
6050002 Thenardite	[2.000]Na^+ [1.000]SO_4^{-2}
5050001 Thermonatron	[2.000]Na^+ [1.000]CO_3^{-2} [1.000]H_2O
2047003 Pyrocroite	[-2.000]H^+ [1.000]Mn^{+2} [2.000]H_2O
5047000 Rhodochrosit	[1.000]Mn^{+2} [1.000]CO_3^{-2}
4147000 $MnCl_2.4H_2O$	[1.000]Mn^{+2} [2.000]Cl^- [4.000]H_2O
6047000 $MnSO_4$	[1.000]Mn^{+2} [1.000]SO_4^{-2}
4123100 Melanothalli	[1.000]Cu^{+2} [2.000]Cl^-
5023100 $CuCO_3$	[1.000]Cu^{+2} [1.000]CO_3^{-2}
2023100 $Cu(OH)_2$	[-2.000]H^+ [1.000]Cu^{+2} [2.000]H_2O
4123101 Atacamite	[-3.000]H^+ [2.000]Cu^{+2} [3.000]H_2O [1.000]Cl^-
6023100 Antlerite	[-4.000]H^+ [3.000]Cu^{+2} [4.000]H_2O [1.000]SO_4^{-2}
6023101 Brochanite	[-6.000]H^+ [4.000]Cu^{+2} [6.000]]H_2O [1.000]SO_4^{-2}
6023102 Langite	[-6.000]H^+ [4.000] Cu^{+2} [7.000]H_2O [1.000]SO_4^{-2}
2023101 Tenorite	[-2.000]H^+ [1.000]Cu^{+2} [11.000]H_2O
6023103 $CuOCuSO_4$	[-2.000]H^+ [2.000] Cu^{+2} [1.000]H_2O [1.000]SO_4^{-2}
6023104 $CuSO_4$	[1.000]Cu^{+2} [1.000]SO_4^{-2}
6023105 Chalcanthite	[1.000]Cu^{+2} [1.000]SO_4^{-2} [5.000]H_2O
5023101 Malachite	[2.000]Cu^{+2} [2.000]H_2O [1.000]CO_3^{-2} [-2.000]H^+
5023102 Azurite	[3.000]Cu^{+2} [2.000]H_2O [2.000]CO_3^{-2} [-2.000]H^+
2015000 Lime	[-2.000]H^+ [1.000]Ca^{+2} [1.000]H_2O
2015001 Portlandite	[-2.000]H^+ [1.000]Ca^{+2} [2.000]H_2O
2046001 Periclase	[-2.000]H^+ [1.000]Mg^{+2} [1.000]H_2O
3046000 Spinel	[-8.000]H^+ [1.000]Mg^{+2} [2.000]Al^{+3} [4.000]H_2O

CHAPTER 5

Bioaccumulation: experimental results

5.1 Introduction

Aquatic invertebrates bathe in a medium (marine or fresh water) containing dissolved trace metals at various concentrations, generally lower than those found in their body cells or tissues (Rainbow & Dallinger 1993).

The flux of these elements into and out of the body is strongly concentration-dependent and the toxicity of heavy metals to aquatic invertebrates is usually due to the effects of toxins accumulated over a period of time. Bioaccumulation is the process through which organisms concentrate any substance in their cells and tissues. The process comprises the following phases: uptake; transport, distribution and sequestration within the body; and, sometimes, excretion. The degree of accumulation in an organism depends on the interrelationship of these phases, and varies both within and between species, and also between metals. This leads to a distinction between "strong net accumulation", "weak net accumulation" and "regulation". Rainbow and Dallinger (1993) define net accumulation as "the result of the balance of absolute uptake and excretion of metals". Strong accumulators, therefore, show no (significant) excretion of metal (e.g. the barnacle *Elminius modestus* is a strong net accumulator of Zn: Rainbow & White 1989). A regulator, referring to a whole animal, may be defined as "an organism that shows no significant changes in body metal content over time on exposure to raised bioavailability of a metal" (Doherty *et al.* 1987).

To reduce metal uptake, some species have developed various short-term adaptations such as closing their shell valves for several hours, thus avoiding contact with the ambient water and so temporarily preventing uptake of dissolved metals (e.g. the freshwater bivalve *Corbicula fluminea*: Doherty *et al.* 1987). These short-term strategies cannot, however, explain the entire regulation process. Indeed, invertebrates cannot close their shells for long periods, thereby excluding at the same time food and essential metals. They may also have regulatory systems that allow regulation of a metal only when a critical concentration is reached.

Kraak *et al.* (1993) studied regulation in two species of freshwater bivalves exposed to a wide range of Cd, Cu and Zn concentrations and found that metals do not accumulate evenly in animal body. For example, *Unio pictorum* (a freshwater mussel of the family Unionidae) exposed to Cu accumulated this metal in the gills, mantle, and digestive gland. For the kidneys and the gonads, no significant difference compared to the controls was recorded even at the highest concentration tested (121 μg/L). The "no observed effect" concentration (NOEC) for accumulation is defined as the highest metal concentration in the water that does not result in a measurable increase in the metal concentration in the organism (Rainbow & Dallinger 1993). The NOECs for the different organs of *U. pictorum* were: gills (34 μg/L) < digestive gland (74 μg/L) = mantle (74 μg/L) <

kidney = gonads (> 121 μg /L), indicating that *U. pictorum* was able to regulate the amount of Cu in the tissues and that this regulation capacity was organ-specific. This view brings me to think that in the body, some organs may play a key role during the uptake phase, whereas others store or excrete the metal, depending on the concentrations of metals and the role of the metal in the animal's physiology. Thus, obviously, essential metal concentrations will depend on the physiological demands of the organisms via their organs; non-essential metals will be taken up proportional to the metal bioavailability in water up to the upper tolerance limits, after which death or excretion may occur. This presupposes the absence of detoxifying mechanisms (e.g. excretory mechanism).

The toxic effects of metals fall into three categories, as follows:

- blocking the essential biologically functional groups of biomolecules (e.g. enzymes and other proteins);
- displacing the essential metal ion in biomolecules; and
- modifying the active conformation of biomolecules (Viarengo *et al.* 1981, Barak & Mason 1989, Krantzberg & Stokes 1989, McDonald *et al.* 1989, Rainbow & White 1989, Phillips & Rainbow 1989, Alikhan *et al.* 1990, Metayer *et al.* 1990, Rainbow & Dallinger 1993, Kraak *et al.* 1993, Timmermans 1993, Shutes *et al.* 1993, Mullis *et al.* 1996, Jak *et al.* 1996, Hallam 1996). Although differences occur in accumulation and regulation of metals or other pollutants in different organs in the same invertebrate, I worked at the whole-body level because of the small size (total length < 10 mm) of the test organisms used.

Furthermore I did not use the NOEC for accumulation (NOEC-accumulation) despite the values calculated in Chapter 4 with respect to the equation of Sloof *et al.* (1986) because of the lack of data on the behavioural, developmental and morphological responses of *P. nigroculus* to chronic exposure to pollution, and also because the very low criteria values tested are assumed to be harmless to aquatic life (Gerhardt & Bisthoven 1995, Vuori & Kukkonen 1996). The use of NOEC values is also being abandoned as the new approach of No Effect Concentration (NEC) or "small-effect concentration" is receiving increasing interest (Laskowski 1995, Kooijman 1996). This new concept, as described by Kooijman (1996), is based on an examination of physiological responses at a cellular level. Indeed, standard tests of the lethality of toxicants usually give the number of surviving animals as a function of the concentration of toxicant, which has been constant during a standardized exposure. The probability of survival of control animals is typically larger than 90% and a sigmoid curve is fitted to the number of survivors as a function of the concentration of toxicant in water. This model is based on the idea that death is certain as soon as toxicant in the organism exceeds a certain individual-specific threshold value. But individuals vary in physiological condition, and therefore in threshold values. The effect

is described deterministically at the level of the individual and stochastically at the level of the cohort of tested organisms. Many problems inherent in standardized method suggest that it should be abandoned (for example the extreme standardization of culture conditions eliminates the physiological differences between individuals, but experimental practice shows that the gradient parameter (e.g. concentration of toxicants in an exposed organism cannot be increased above a given level: there seems to be an upper limit for the maximum slope of the concentration response curve. This means that there is a substantial variation of threshold values between individuals and the effect is stochastic at the level of the individual and not deterministic, as is considered to be the case in standardized tests).

The bioaccumulation process in aquatic environments is very important in toxicological studies and may explain the long-term effects of non-biodegradable compounds, such as heavy metals, on aquatic life. For example, human illnesses and deaths in Minamata Bay in the 1950-60s, Iraq (1956, 1960, 1971-1972), Guatemala, Niigata (1965), Ghana (1967), Pakistan (1969) and Canada (1970) were due to continuous consumption of Hg-contaminated products (fish, grain) and bioaccumulation of the metal in the cells and tissues of the consumers.

Finally, the bioaccumulation of a given element depends on its bioavailability (some chemical species are easily taken up by the organism) and also on the source and routes of uptake.

This chapter discusses the bioaccumulation of Al, Cu and Mn as a function of exposure time, and as a function of the metal concentration in water. As I worked on a 2-compartment model (amphipod + water, but no sediment and food source negligible: metal concentrations < 1 μg/kg food), bioaccumulation was evaluated in terms of bioconcentration factor (BCF), which is the ratio between the concentration of metal in the amphipod and the residual concentration of metal in water at the end of the exposure time (BCF = Concentration in the amphipod (or C_a) / concentration in water (or C_w) after 21 days of exposure for example). The depuration process also reduces the impact of the food source on bioaccumulation (Reish & Oshida 1986, Kooij *et al.* 1991, Sidoumou *et al.* 1992, Tsuda *et al.* 1995, Jak *et al.* 1996, Chaisuksant *et al.* 1997, Tsuda *et al.* 1997). Thus bioconcentration becomes the simple ratio of metal concentration in the animal to the concentration in water at steady-state equilibrium:

$$BCF = C_p / C_w$$

where C_p = concentration of metal in the animal at the end of the exposure time (i.e. 24h, 96h, 8 or 21 days) expressed in μg/g of dry body weight; and Cw = actual concentration of metal species in the residual solution after the same exposure time (Enserink *et al.* 1991, Xu & Pascoe 1993, Kishino *et al.* 1995, Musibono *et al.* 1996, Thomann 1996, Tsuda *et al.* 1997).

I also wanted to determine if *P. nigroculus* was an accumulator or a regulator of Al, Cu and Mn.

5.2 Materials and methods

For this topic, experiments were performed to check the variation of BCF as a function of exposure time, and also as a function of concentration of test elements.

The design was the same as that in Chapter 2 (except for sampling period), namely:

- test type: static-renewal simulating natural 'vleis' (small ponds) in which amphipods live during summer, and test solutions were renewed twice a week;
- dilution water: 'artificial' (pH 5.0 $\pm$ 0.8; TH < 40 mg/L as $CaCO_3$; temperature = 14 $\pm$ 1°C; electric conductivity range: 120 - 350 μS/cm);
- the test animal was the same endemic freshwater amphipod *Paramelita nigroculus* as described in Chapters 1 and 2;
- number of amphipods per concentration = 30 (3 replicate and 10 animals per replicate; only mature and juvenile individuals were used together);
- feeding regime: simple, only *Rapanea melanophloeos* leaves were used as described in Chapters 1 and 2.

5.2.1 BCF as a function of exposure time

Five test solutions, as mixtures of Al + Cu + Mn, in 3 replicates with 10 amphipods each were used to study BCF as a function of exposure time. These were AEV2b, Cc2, Cb2, Bb2 and Bc2 (Table 1.3). Animals to be analysed were sampled after 1, 4, 8 and 21-day exposures. Food and test solutions were replaced twice a week and, animals were fed from the first day, in contrast to the situation regarding experiments on survival, growth and reproduction reported in earlier Chapters. Only 'artificial' water was used.

After exposures of 24h, 96h, 8 days or 21 days to different test solutions of Al, Cu and Mn mixtures described above, survivors (both juveniles and mature amphipods) from test solutions were collected and kept in unpolluted 'artificial' water for 30 hours to allow depuration, and then rinsed with distilled water. They were then put into foil dishes, dried at 75°C to constant weight (about 72 hours), weighed to an accuracy of 0.1 mg and the values recorded as dry biomass. Dry amphipods were then ashed at 650°C for 8 hours. From the grey ash obtained, 0.5 g (about 15 animals) was digested with 5 mL of concentrated nitric acid for 5 hours, then diluted with 50 mL of distilled water. This solution was filtered through 0.45 μm filter paper (Watman GF/F)

using a filtering turret under pressure and the volume was made up to 50 mL with distilled water. Samples were finally analysed using Inductively Coupled-Plasma Spectrometry methods (ICP-S) at wavelengths of 257.610, 279.553, 308.215, 324.754, 393.754, 589.592 and 766.490 nm for Mn, Mg, Al, Cu, Ca, Na and K respectively. Water samples (residual solutions at the end of each exposure time), *Rapanea* leaves and control amphipods were also analysed in a similar way.

When chemical analyses were not done on the same day, samples were kept at 4°C, but also 5 mL per litre of HNO_3 were added to samples before storage for a maximum period of 4 weeks (Winge *et al.* 1984, Keliher 1987, Degremont 1989).

5.2.2 BCF as a function of the bioavailable concentrations of metals

Various concentrations of mixtures of Al, Cu and Mn were tested to see how the concentration of metals might affect the bioaccumulation process. The effects of thirteen new test solutions were thus examined at high concentrations (namely 360 mg/L Al, 20 mg/L Cu and 375 mg/L Mn, corresponding to 96-h LC_{50}, or 1 toxic unit (T.U.) for each metal as discussed in Chapter 4, but in mixtures); eleven at moderate concentrations (36 mg/L Al, 2 mg/L Cu and 37.5 mg/L Mn, corresponding to 0.1 T.U. for each metal type); and six at low concentrations (3.6 mg/L Al, 0.2 mg/L Cu and 3.75 mg/L Mn, corresponding to 0.01 T.U. for each metal species).

Using concentrations derived from South African criteria for protection of aquatic ecosystems, values at criteria levels were assumed to be moderate; values at 50% above the criteria levels were assumed to be high, and values at 50% below the criteria levels were assumed to be low. This classification was arbitrarily made to allow comparison of results. All other experimental conditions remained the same as above (section 5.2.1).

5.3 Statistical analysis

Differences between BCFs from different test solutions were analysed statistically using the Student's t-test of significance (Clarke 1980, Liorzou 1980, Zar 1984, Bonhivers & De Ketele 1986, Kooijman & Bedaux 1996). Regression analysis results using the Genstat 5 Release 3.1 package (DEC ALPHA / OpenVMS, Copyright 1994, Lawes Agricultural Trust at Rothamsted Experimental Station) are reported in Chapter 8.

Only the results of the Student's t-test are presented in this chapter to allow earlier discussion on bioaccumulation and regulation.

5.4 Results

5.4.1 Bioaccumulation as a function of exposure time

All results are reported in Tables 5.1 to 5.10.

Results as averages of three replicates are reported in Table 5.1. Details of the actual concentrations of metals in the amphipods and in the residual water at the end of the experiments are found in Appendix 1, for illustration.

Table 5.1: BCFs for individuals P. nigroculus as a function of exposure time to test solutions. Concentrations of metals in this table refer to test solutions used and not to residual solutions

BCFs for	*Al*	*Cu*	*Mn*	*BCF*			
	(mg/L)	*(mg/L)*	*(mg/L)*	*1-d*	*4-d*	*8-d*	*21-d*
Mn							
1	1.388	0.0175	13.993	300	429	7130	7120
2	0.0925	0.0175	1.218	428	438	7080	7170
3	0.2775	0.005	3.65	400	420	7120	7180
4	0.4325	0.004	4.665	420	429	7120	7180
5	0.1225	0.005	5.273	420	437	7003	7091
Al							
1	1.388	0.0175	13.993	563	1799	14238	18246
2	0.0925	0.0175	1.218	442	917	10090	14100
3	0.2775	0.005	3.65	510	1559	13810	16750
4	0.4325	0.004	4.665	497	1004	11230	15200
5	0.1225	0.005	5.273	493	1001	11230	15010
Cu							
1	1.388	0.0175	13.993	500	1200	10300	13600
2	0.0925	0.0175	1.218	435	803	8599	11400
3	0.2775	0.005	3.65	495	1098	9899	12300
4	0.4325	0.004	4.665	457	995	8987	11980
5	0.1225	0.005	5.273	473	999	9000	11960

Table 5.1 shows that BCF values increased with the duration of exposure to test solutions (e.g. BCF = 300 after 1 day and 7120 after 21 days for Mn in case 1; BCF = 563 after 1 day and 18246 after 21 days for Al in case 1; BCF = 500 after 1 day and 13600 after 21 days for Cu in case 1). This increase is largest when reported per day (Table 5.2) at 8-day exposures.

Table 5.2: Bioconcentration factors per day derived from Table 5.1, for exposure periods in the intervals of 0-1 day; 2-4 days; 5-8 days and 9-21 days

Metal	*0-1day*	*2-4days*	*5-8 days*	*9-21 days*
Mn				
1	300	43	1675	-1
2	428	3	1653	30
3	400	7	1675	20
4	420	3	1673	5
5	420	6	1642	7
Mean (±SD)	394(±53)	11(±17)	1665(±15)	< 1(±18)
Al				
1	563	412	3110	308
2	442	158	2293	308
3	510	350	3063	226
4	497	169	2612	288
5	493	169	2557	291
Mean (±SD)	501(±43)	556(±361)	2727(±350)	284(±34)
Cu				
1	500	233	2275	254
2	435	123	1949	215
3	495	201	2200	185
4	457	179	1998	230
5	473	175	2000	228
Mean (±SD)	472(±27)	182(±81)	2084(±144)	222(±25)

Table 5.2 shows noticeable variations in BCF values per day between 4- and 8-day exposures, corresponding with the high mortalities found during this time interval (Tables 3 - 4 in Chapter 2 presented as a paper).

It seems obvious that the mortality is essentially due to bioaccumulation during exposures longer than 4 days. This assumption is tested statistically in Chapter 8.

5.4.2 Bioaccumulation as a function of the bioavailable concentrations of metals

All results are reported in Tables 5.3 to 5.9.

Table 5.3: BCFs for P. nigroculus at high concentrations of Al (360 mg/L), Cu (20 mg/L) and Mn (375 mg/L) after 21-day exposure to metal mixtures. Each set of values (1 - 13) represents values calculated for an initial replicate of 10 animals

	BCF for Al = a	*BCF for Cu = b*	*BCF for Mn = c*	*Ranking*
1	5400	8800	1000	b>a>c
2	32500	7100	1000	a>b>c
3	12600	8800	4000	a>b>c
4	15800	4300	2300	a>b>c
5	22900	15300	40	a>b>c
6	54700	90700	100	b>a>c
7	7400	3300	300	a>b>c
8	4400	3000	3800	a>c>b
9	7900	7500	5500	a>b>c
10	3100	5500	100	b>a>c
11	2100	12800	1000	b>a>c
12	200	100	100	a>b>c
13	4600	9000	2300	b>a>c
Mean (±SD)	13354 (15448)	13553 (23536)	1480 (1805)	b>a>c

Table 5.3 shows that the BCF for Mn is only about a tenth of that for Al and Cu.

Table 5.4: BCFs of Al, Cu and Mn in P. nigroculus at moderate concentration after 21-day exposure to metal mixtures (36 mg/L Al + 2 mg/L Cu + 37.5 mg/L Mn); NR = not recorded.

Mixtures label codes	*BCF for Al (n = 11)*	*BCF for Cu (n = 11)*	*BCF for Mn (n = 9)*
1	27000	9000	2800
2	18800	4900	1300
3	28400	26700	1000
4	3700	6900	200
5	1100	1400	NR
6	1900	1000	1000
7	5700	1300	1000
8	1600	1300	1000
9	1000	4800	NR
10	15800	4300	3300
11	18500	4000	1700
Mean (±SD)	11227 (10719)	5964 (7334)	1478 (981)

Table 5.4 shows how noticeable BCF is for Al (almost twice as great as that of Cu and 8 times greater than that of Mn).

Table 5.5: BCFs of Al, Cu and Mn in P. nigroculus after 21-day exposures at low concentrations of metal mixtures (3.6 mg/L Al + 0.2 mg/L Cu + 3.75 mg/L Mn)

Mixtures	*BCF for Al (n = 6)*	*BCF for Cu (n = 6)*	*BCF for Mn (n = 6)*
1	2500	900	1000
2	10300	4000	1000
3	4600	3600	1000
4	4000	300	2500
5	2200	6700	1000
6	4500	5300	2500
Mean (±SD)	4683 (2932)	3467 (2479)	1500 (775)

Table 5.5 shows that BCF for Mn remains the lowest. Results in Tables 5.3 - 5.5 showed that BCFs did not vary with concentration for Mn, while it did for Al and Cu.

Tables 5.6 - 5.8 showed BCFs for Al, Cu and Mn at concentrations derived from South African criteria for protecting the aquatic life. I want to check if the result they showed is different from that in Tables 5.3 - 5.5.

Table 5.6: BCF of Al, Cu and Mn in P. nigroculus at 50 % values above criteria levels (that I have assumed to be high concentrations) after 21-day exposures.Bc2a...Bc2i are the same concentration containing 1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn; Bb2a...Bb2j are the same concentration containing 0.2775 mg/L Al, 0.005 mg/L Cu and 3.65 mg/L Mn; Ba2a = Ba2b and contains 0.0925 mg/L Al, 0.0025 mg/L Cu and 1.825 mg/L Mn; ACM (1) contains 0.360 mg/L Al, 0.020 mg/L Cu and 3.75 mg/L Mn; NR = value not recorded

Mixtures label codes	*BCF for Al (n = 17)*	*BCF for Cu (n = 18)*	*BCF for Mn (n = 18)*
Bc2a	370	900	385
Bb2a	2475	1700	3789
Bc2b	749	1500	6429
ACM(1.0)	1093	1198	729
Bb2b	772	142	779
Bc2c	563	458	300
Bb2c	3818	1580	100
Bb2d	3344	4000	344
Bc2e	1760	333	109
Bc2f	3690	1330	878
Bc2g	NR	110	300
Bc2h	292	7000	100
Bb2e	1050	7565	230
Bb2i	8750	875	591
Bb2j	5400	1300	58
Bc2i	1186	40	60
Ba2a	284	90	15
Ba2b	271	71	50
Mean(±SD)	2100(2294)	1677(2222)	847(1637)

Table 5.6 shows that BCF for Mn is the lowest (almost 2.5 times lower than that for Al, and almost 2 times lower than that for Cu).

Table 5.7: BCFs of Al, Cu and Mn in P. nigroculus after 21-day exposures at criteria level concentrations (that I have assumed to be moderate), test solutions Aa2a...Aa2c are the same solution containing 0.062 mg/L Al, 0.001 mg/L Cu and 1.217 mg/L Mn; AEV2b1...AEV2b2 contain 0.1225 mg/L Al, 0.005 mg/L Cu and 5.273 mg/L Mn; Ab2a...Ab2b contain 0.185 mg/L Al, 0.001 mg/L Cu, 2.434 mg/L Mn; Ac2a...Ac2e contain 0.926 mg/L Al, 0.008 mg/L Cu and 9.328 mg/L Mn

Mixtures label codes	*BCF for Al (n = 12)*	*BCF for Cu (n = 12)*	*BCF for Mn (n = 12)*
Aa2a	1210	1156	359
AEV2a1	1654	6800	300
AEV2a2	5833	375	190
Aa2b	462	667	80
Aa2c	292	6000	500
Ab2a	1490	2250	250
Ab2b	24000	2233	1350
Ac2a	11000	1600	3513
Ac2b	74000	1857	2857
Ac2c	39600	14727	670
Ac2d	4462	1250	5958
Ac2e	7000	1333	2867
Mean (±SD)	14250 (22185)	3354 (4106)	1575 (184)

Table 5.7 shows the high BCF values for Al (which is almost 10 times as great as that for Mn and 4 times greater than that for Cu).

Table 5.8: BCF of Al, Cu and Mn in P. nigroculus after 21-day exposures at concentrations of 50 % values below criteria levels (that I have assumed to be low concentrations); Cc2a...Cc2g contain 0.4325 mg/L Al, 0.004 mg/L Cu and 4.665 mg/L Mn; Cb2a...C3b2l contain 0.0925 mg/L Al, 0.0175 mg/L Cu and 1.218 mg/L Mn; Ca2b...Ca2h contain 0.030 mg/L Al, 0.0025 mg/L Cu and 0.6075 mg/L Mn

Mixtures	*BCF for Al (n = 27)*	*BCF for Cu (n = 27)*	*BCF for Mn (n = 27)*
Cc2a	11200	5778	439
Cb2a	1757	917	189
Cb2b	662	455	21
Cb2c	900	1030	40
Cc2b	1006	1148	168
Ca2b	3891	2244	100
Ca2c	4000	2000	2333
Ca2d	1100	200	2300
Ca2e	100	1100	132
Ca2f	16818	8300	1926
Cb2c	800	1667	378
Cb2d	975	250	108
Cb2e	9229	1857	15196
Cb2f	8550	2000	2183
b2g	462	167	2000
Ca2g	5186	1833	2175
Ca2h	84400	3500	2111
Cb2h	25000	15000	1795
Cb2i	40667	13600	1321
Cb2j	42000	69600	4100
Cc2c	778	143	655
Cc2d	18548	629	1900
Cc2e	9333	2000	346
Cb2k	27500	4380	4556
Cb2l	6111	22000	2560
Cc2f	17235	43667	7750
Cc2g	10000	13000	16000
Mean (±SD)	12897 (20429)	8091 (15457)	2696 (4099)

Table 5.8 shows that BCF for Al is higher than for Cu or Mn and that the BCF for Mn is the lowest.

Results in Tables 5.6 - 5.8 showed the same tendency for BCF values (higher for Al and lower for Mn). Table 5.9 provides mean BCF values at all concentrations given in Tables 5.3 to 5.8.

Table 5.9: Mean of BCF values at all concentrations given in Tables 5.3 - 5.8

Relative concentration	***BCFs for Al***	***BCFs for Cu***	***BCFs for Mn***
High	13354 (Table 5.4)	13553 (Table 5.4)	1480 (Table 5.4)
	2110 (Table 5.8)	1677 (Table 5.8)	847 (Table 5.8)
Mean at high concentrations	7732	7615	1164
Moderate	11227 (Table 5.6)	3354 (Table 5.6)	1478 (Table 5.6)
	14250 (Table 5.9)	5964 (Table 5.9)	1575 (Table 5.9)
Mean at moderate concentrations	12739	4659	1526
Low	4683 (Table 5.7)	3467 (Table 5.7)	1500 (Table 5.7)
	12897 (Table 5.10)	8091 (Table 5.10)	2696 (Table 5.10)
Mean at low concentrations	8790	5779	2098

The information in Table 5.9 suggests that:

- at high concentrations, bioaccumulation of Al and Cu was not different but the BCF for Mn was about 1/7 of these values.
- at moderate concentrations, bioaccumulation was very high for Al (almost 3 times greater than for Cu and 8 times greater than for Mn);
- at low concentrations, bioaccumulation was higher for Al than for Cu and Mn (1.5 times greater than that for Cu and 4 times greater than that for Mn).

This suggests that *P. nigroculus* may be a good accumulator of Al, a weak accumulator of Cu and a poor accumulator of Mn. The extent to which each of the metals affects bioaccumulation of the others is discussed in Chapter 8 on the multiple regression analysis.

Discussion

Dallinger and Rainbow (1993) defined 'strong' accumulators as organisms with BCFs increasing with increased concentrations of metal in solution, while 'weak' accumulator refers to organisms with excretion process under certain circumstances. Regulators have almost constant BCFs despite increased concentrations of metal in solutions. They have a stromg excretion.

According to the above definition given by Dallinger and Rainbow (1993), *P. nigroculus* is a good or strong accumulator of Al (BCF = 7732 at high concentrations of the metal, 12739 at moderate concentrations and 8790 at low concentration), an accumulator of Cu (BCF = 7615 at high concentrations of the metal, 4659 at moderate concentrations and 5779 at low concentrations) and a poor accumulator of Mn (BCF 1164 at high concentrations, 1526 at moderate concentrations and 2098 at low concentrations) as shown in Tables 5.3 - 5.9.

Bioaccumulation of Cu, the most toxic of the 3 metals under discussion, is high at high concentrations (mean = 7615), whereas bioaccumulation of Al is high at moderate concentrations (mean = 12739), while bioaccumulation of Mn is roughly the same at high (mean = 1164) and moderate (mean = 1562) concentrations despite the mean value of 2098 recorded at low concentrations. This indicates that *P. nigroculus* may to some extent regulate the quantity of Mn in the body. Results of the statistical analysis (Table 5.10 in section 5.3) discuss differences in BCFs at various concentrations as mentioned above.

The link between mortality and bioaccumulation of toxicants in chronic tests seems to be obvious, if one compares the results shown in Table 5.1 and the results on mortality reported in Chapter 2. Indeed, highest mortalities occurred after 8-day exposures and the greatest increases in BCF values were also recorded after 8 days (see Tables 2.3 - 2.6 in relation to the effect of Mn on the mortality and growth of *P. nigroculus*, and Table 5.1 relating BCF to exposure time). The fact that the accumulation process, and therefore mortality, are important at 24-h and at 8-d exposures, may be explained as follows.

When an organism is exposed chronically to a low concentration of pollutant, the organism will inevitably take up some of the pollutant through ordinary metabolic processes such as feeding, filtering through the gills or digestion. But if the pollutant is not biodegradable, its concentration in the body will increase up to the first signal of danger. At this point the organism may develop a protective response (such as closing the shell or gills), as reported by Barnes (1982), Rainbow and Dallinger (1993) and Barnes et al. (1995). Because it cannot

maintain this state for long (it needs to feed and respire), it will open the organs and pollutant will again enter the body. The concentration of pollutant in animal body will increase and will either lead to death (for the unfortunate) or to survival (for the fortunate) if they have long-term protective mechanism like excretion or storage in on-toxic form. Pre-exposure and the physiology of the animal may play a key role in survival (Munzinger 1990, Roux *et al.*1993, Hallam 1996), possibly due to hormesis. The link between 24-h, and 8-d mortalities and bioaccumulation suggests that useful information regarding the acute test with *P. nigroculus* may be obtained after 24 hours of exposure, while useful information for chronic toxicity tests might be obtained after 8 days of exposure using mortality as test response.

Previous studies on *Daphnia magna* by Enserink *et al.* (1991) reported the following BCF values for some heavy metals: As = 75; Cd = 79000; Cr = 550; Cu = 4900; Hg = 55000; Ni = 3100; Pb = 14000; and Zn = 16000. Previously Memmert (1987) had reported BCF values for Zn in *D. magna* from 100 000 to 800 000 and in the fish *Brachydanio rerio* from 200 000 to 450 000 respectively. Reinert (1972) and Moriarty (1984) reported a BCF value of 49307 for Cd in *Poecilia reticulata*, the guppy. Xu and Pascoe (1993) reported a BCF value = 380000 for Zn in the dry amphipod *Gammarus pulex* after 12-day exposure in solution of Zn at the concentration of 1.78 mg/L. So *P. nigroculus* may provide key information regarding a water body history via the study of bioaccumualtion. From this point of view, this amphipod is a good bioindicator of water quality.

Results given in Tables 5.6 - 5.8 suggest BCFs to be low at high concentrations of pollutants (i.e. 2109.8 for Al; 1677.3 for Cu and 847.4 for Mn at concentrations of 50% above criteria levels) and high at moderate and low concentrations (i.e. 14250.3 and 12896.6 for Al; 3354 and 8091.3 for Cu and 1574.5 and 2695.6 for Mn at criteria levels and at 50% below criteria levels respectively). This suggests the importance of the bioaccumulation process when defining guidelines for aquatic environments and chronic toxicity essentially results from bioaccumulation. However more information is needed for confirming the importance of bioaccumulation in chronic toxicity studies.

Finally, the fact that different BCF values were obtained for the same treatments shows the variability in the ability of each individual to accumulate the pollutant, some individuals being faster accumulators than others, depending possibly on the genetic difference and body size.

5.5 Statistical Analysis - Tests of significance

To determine if *Paramelita nigroculus* is an accumulator or a regulator of each of the three metals studied, I analysed statistically the results for bioaccumulation as a function of the concentrations of the heavy metals (see Tables 5.3 - 5.8).

It is assumed that if BCFs vary significantly with changes in the concentration of a toxicant, then the test organism is an accumulator for this substance. Conversely, when BCFs do not vary significantly with changes in concentrations of toxicants, the subject is assumed to be a regulator. Different observations reported from the Tables 5.3 to 5.8 are assumed to be from a random sample of a normal distribution, and therefore having the same unknown variance, but with different known means. Given these assumptions, I used Student's t-test for differences between two means with variance unknown but assumed to be the same (Clarke 1980, Liorzou 1980, Zar 1984, Bonhivers & De Ketele 1986, Reish & Oshida 1986, USEPA 1991, Kooijman & Bedaux 1996). Nine cases are reported in Table 5.10.

*Table 5.10: Summary of Student's t-test for BCFs calculated for Al, Cu and Mn (see Tables 5.3 - 5.6); * = significantly different from each other; H_o = null hypothesis accepted and variations attributed to chance*

Relative sample or population	*Mean*	*df*	*t*	*t(table)*	*p*
Al: N = 13 (Table 5.3)	13354	17	2.239	2.110	0.025*
M = 6 (Table 5.5)	4683	17	2.239	2.110	0.025*
N = 13 (Table 5.3)	13354	22	0.56	1.717	0.05 H_o
M' = 11 (Table 5.4)	11227	22	0.56	1.717	0.05 H_o
M' = 11 (Table 5.4)	11227	15	1.444	1.753	0.05 H_o
M = 6 (Table 5.5)	4683	15	1.444	1.753	0.05 H_o
Cu: N = 13 (Table 5.3)	13553	17	1.020	1.740	0.05 H_o
M = 6 (Table 5.5)	3467	17	1.020	1.740	0.05 H_o
N = 13 (Table 5.3)	13553	22	1.030	1.717	0.05 H_o
M' = 11 (Table 5.4)	5964	22	1.030	1.717	0.05 H_o
M = 6 (Table 5.5)	3467	15	0.774	1.753	0.05 H_o
M' = 11 (Table 5.4)	5964	15	0.774	1.753	0.05 H_o
Mn: M = 6 (Table 5.5)	1500	17	-	-	H_o
N = 13 (Table 5.3)	1480	17	-	-	H_o
N = 13 (Table 5.3)	1480	20	-	-	H_o
M' = 9 (Table 5.4)	1478	20	-	-	H_o
M = 6 (Table 5.5)	1500	13	-	-	H_o
M' = 9 (Table 5.4)	1478	13	-	-	H_o

Table 5.10 showed no significant difference in BCFs related to variation in concentrations of test solutions (data from Tables 5.3 - 5.5) for Cu and Mn, but did for Al at high (Table 5.3) and low (Table 5.5) concentrations ($p<0.025$ for df = 17 and t = 2.239).

Statistical analyses of a second series of BCF values, based on concentrations related to criteria levels, 50 % above and 50% below the criteria levels (data from Tables 5.6 - 5.8), are shown in Table 5.11.

*Table 5.11: Summary of Student's t-test for BCFs calculated for Al, Cu and Mn for concentrations at criteria levels, 50% above and 50% below criteria levels; * = significantly different; H_o = null hypothesis accepted and variations attributed to chance*

Relative sample or Population	*Mean*	*df*	*t*	*t (table)*	*p*
Al: N = 17 (Table 5.6)	2110	42	2.400	1.297	0.05*
M = 27 (Table 5.8)	12897	42	2.400	1.297	0.05*
N = 17 (Table 5.6)	2110	27	1.906	1.699	0.05*
M' = 12 (Table 5.7)	14250	27	1.906	1.699	0.05*
M = 27 (Table 5.8)	12897	37	0.190	1.464	0.05 H_o
M'= 12 (Table 5.7)	14250	37	0.190	1.464	0.05 H_o
Cu: N = 18 (Table 5.6)	1677	43	1.684	1.575	0.025*
M = 27 (Table 5.8)	8091	43	1.684	1.575	0.025*
N = 18 (Table 5.6)	1677	28	1.416	1.699	0.05 H_o
M' = 12 (Table 5.7)	3354	28	1.416	1.699	0.05 H_o
M = 27 (Table 5.8)	8091	37	1.022	1.464	0.05 H_o
M' = 12 (Table 5.7)	3354	37	1.022	1.464	0.05 H_o
Mn: N = 18 (Table 5.6)	847	43	1.727	1.264	0.025*
M = 27 (Table 5.8)	2696	43	1.727	1.264	0.025*
N = 18 (Table 5.6)	847	28	1.333	1.699	0.05 H_o
M' = 12 (Table 5.7)	1575	28	1.333	1.699	0.05 H_o
M = 27 (Table 5.8)	2696	37	0.917	1.464	0.05 H_o
M' = 12 (Table 5.7)	1575	37	0.917	1.464	0.05 H_o

Table 5.11 shows:

- that BCFs for Al vary significantly with the concentrations of test solutions, particularly for BCF values at concentrations 50% above South African criteria for protecting the aquatic ecosystems (Table 5.6) and BCFs at concentrations 50% below criteria levels (Table 5.8)(for $p<0.05$; df = 42 and t = 2.400);

- significant difference in BCFs for Al at concentrations 50% above criteria levels (Table 5.6) and concentrations at criteria levels (Table 5.7) ($p<0.05$; df = 27 and t = 1.906);

- that BCFs for Cu and Mn vary significantly with variation in concentrations only for BCFs at 50% above (Table 5.6) and 50% below (Table 5.8) criteria levels ($p<0.025$; df = 43 and t = 1.684 for Cu, and t = 1.727 for Mn).

Discussion

Results from Student's t-test for mean (for Mn) of 1480 (Table 5.3), 1477.8 (Table 5.4), 1574.5 (Table 5.7) and 1500 (Table 5.8) are not significantly different at $p<0.05$, despite differences in concentration (high, moderate and low) to which animals were subjected. This indicates that *P. nigroculus* may to some extent regulate the quantity of Mn in its body and that *it* is an good accumulator for Al but a poor accumulator for Cu and Mn.

Differences in BCF, from Tables 5.1 - 5.8, revealed the complexity of the bioaccumulation process, which seems to be a function of exposure time (Table 5.1), concentration (Tables 5.3 - 5.8), and element (all Tables). Test organisms also vary at an individual level. For example, in the single species *P. nigroculus*, for the same element and at the same concentrations, big differences in BCF values for individuals animals are shown in Tables 5.4 and 5.5. Naylor *et al.* (1990), working on the isopod *Asellus aquaticus* and on the amphipod *Gammarus pulex*, came to the same conclusion, as did Emson and Crame (1994), Metayer *et al.* (1990) and Mackie and Kilgour (1995) working on bivalves.

5.6 Conclusions

The results of the statistical analyses suggest that the bioaccumulation process depends on the concentrations of the pollutants in the case of Al, Cu and Mn.

P. nigroculus seems to be an accumulator of Al, a weak accumulator or a regulator of Cu and a regulator of Mn. Cu and Mn are two essential metals. Indeed, the fact that differences between BCF values for Cu and Mn from the Tables 5.3, 5.4 and 5.5 are not statistically significant at $p < 0.05$ suggests that *P. nigroculus* regulates the concentrations of both of these two metals in the body, while the statistically significant differences between the BCFs for Cu and Mn in some cases suggest that accumulation occurs. This suggests that the regulation of an essential metal may start after uptake and accumulation have reached a maximum level tolerated by the organism. This depends on the concentration of the metal in water.

Indeed it may be presupposed that some sort of mechanism such as copious urine, low permeability of eperdemis allows excretion of specific elements. Xu and Pascoe (1993), studying the bioconcentration of Zn by the amphipod *Gammarus pulex*, showed that the elimination of Zn by the amphipod started when a certain concentration of metals was reached in its body. This level of metal in the body changed with the concentration of test solutions. For example, when the amphipod was exposed at concentration of 0.41 mg/L Zn, elimination started when the metal content in the amphipod reached 0.23 mg/g dry animal weight. Exposed at concentrations of 0.85 mg/L, elimination started when the concentration in the amphipod reached 0.28 mg/g dry animal weight. When the amphipod was exposed at concentrations of 2.02 mg/L, elimination started after the concentration of Zn in the amphipod has reached 0.4 mg/g dry animal weight. This aspect may be studied by further works.

What active routes of uptake for Al, Cu and Mn may be used by *P. nigroculus*? Chapter 6 provides an answer.

CHAPTER 6

Active routes for uptake of Al, Cu and Mn by *Paramelita nigroculus*

6.1 Introduction

In estuarine environments, invertebrates are immersed in diluted seawater. Their body fluids either follow the osmotic conditions of the environment faithfully (i.e. they are osmoconformers) or they resist the dilution of their body fluids (i.e. they are osmoregulators). Animals living in fresh water are similar to osmoregulators from brackish water, but have to regulate their body fluids throughout their lives. Most freshwater animals regulate by having an epidermis of low permeability and by producing copius amount of urine (e.g. the decapod crustacean *Astacus* and the peracarid *Gammarus* (an amphipod) produce 40% of body weight per day as urine, the cladoceran *Daphnia* more than 200% of body weight, and the freshwater bivalve *Adononta* more than 400% (Barnes *et al.* 1995). The urine of freshwater invertebrates is hypo-osmotic to body fluids and useful ions are selectively removed from the urine in appropriate parts of the excretory organs and retained in the body. However, despite urine production and the impermeabilty of the epidermis, some ions are lost and need to be replaced. This occurs through food and so is not fully under the control of the animal's physiology. Ions obtained in this way enter the tissues via the passive route of facilitated diffusion (Rainbow & Dallinger 1993, Barnes *et al.* 1995).

Many freshwater invertebrates are also capable of the direct active uptake of ions from the surrounding medium (e.g. freshwater crustaceans such as the crayfish *Astacus*, the isopod *Asellus*, and amphipods like *Gammarus*: Robertson 1968, Barnes 1982, Barnes *et al.* 1995). The whole body surface may play an important role in this process, although the gills are obvious organs of active ion transport in crustaceans. On the other hand, the larvae of the mosquito *Aedes aegypti* and of dragonflies use anal papillae in the active uptake of ions (Barnes *et al.* 1995).

6.2 Active routes of uptake via active pumps of major cations

As stated by Barnes (1982), Rainbow and Dallinger (1993), Timmermans (1993) and Barnes *et al.* (1995), the active uptake of metal ions by freshwater crustaceans is brought about by active pumps of the major cations Na, Ca, K. Mg, another major cation, may play an important role during the active uptake of some ions by arthropods. Indeed, Mg levels are generally low in the body fluids of arthropods (Barnes *et al.* 1995) due to the fact that high concentrations of Mg will reduce the animal's activity, acting as an anaesthetic. Ions following the Mg route of uptake will therefore be present in low concentration (Silby & Calow 1986, Barnes *et al.* 1995) in the body fluids of arthropods. In this chapter, attention is paid only to the active uptake of the metals Al, Cu and Mn, the passive route being neglected. The interest of this topic may be from management purposes.

Indeed, when an active route of uptake for a given metal is known, water managers may play on the concentration of the major cation (active pump) to reduce or to increase the uptake of the metal and thus the toxicity.

The absorption of ions by cells depends on membrane potential; indeed, an ion in aqueous solution is acted on by at least two physical forces, one arising from chemical potential gradients and another from electrical potential differences. Chemical potential is related to the concentration of the ion and electrical potential results from the net positive or negative charge carried by the ion. When a salt is added to water, it diffuses through the solution from regions of higher concentration to those of lower concentration until a uniform concentration is achieved. The cell membrane is usually the main barrier to the diffusion of molecules into and out of cells. In general, one of any two kinds of ion will have a higher mobility than the other and will cause a slight separation of charges, setting up an electrical potential gradient and leading to a diffusion potential, which is termed the membrane diffusion potential. The relationship between the electrical potential difference across a membrane and the accompanying distribution of an ion across it at equilibrium is given the well-known Nernst equation of ion species as follows:

$$E_j = (RT/z_jF)\ln (Co/Ci),$$

where E_j is the electrical potential between inside and outside for ion species j; R is the gas constant; T is the absolute temperature; z_j is the valency of the ion j; F is the Faraday; ln is the natural log and Co and Ci are external and internal concentrations of ion species j respectively (e.g. Sutcliffe and Baker 1978). The simplified form of the above equation at 18°C is Ej = 58 log Co/Ci for cations, and Ej = 58 log Ci/Co for anions. No work is done during this uptake and it is termed passive uptake.

In active uptake, work is done and metabolic energy is supplied. A useful hypothesis to account for the active transport of solutes is that a membrane constituent or carrier selectively binds certain ions or molecules and then ferries them across the membrane. According to this hypothesis, a penetrating ion (M) combines with the carrier (R) at the outer surface of the membrane and the complex (MR) is formed. This might involve adsorption, exchange-adsorption or some kind of chemical reaction. The complex (MR) cannot leave the membrane but is mobile within it and may move more to the inner side of the membrane, where it is broken down, releasing the ion and forming a carrier precursor (R'). This precursor moves back across the membrane and is reconverted to R, which can now accept another ion at the surface (e.g. Robertson 1968, Sutcliffe & Baker 1978, Schmidt-Nielsen 1982). Another possible mechanism of active uptake is micro-pinocytosis: ions are selectively bound at specific sites on the membrane and are transported inwards by an invagination which results in the formation of a micro-vesicle within the cytoplasm. The subsequent breakdown of the vesicular membrane could lead to

the release of the bound ions within the cytoplasm. This process requires energy, which is obtained from respiration (Robertson 1968, Sutcliffe & Baker 1978, Kishino & Koboyashi 1995).

Wright (1980) presented evidence that Cd accumulation by the freshwater amphipod crustacean, *Gammarus pulex*, may be at least partially accounted for by a process of 'accidental' active Cd uptake by the Ca pump.

Bjerregaard and Depledge (1990) have evidence that the uptake of Cd by the intertidal winkle *Littorina littorea* is significantly related to the Ca concentration of the medium, independently of a salinity effect. In the Australian freshwater mussel *Velesunio angasi* there is a strong correlation between accumulated labelled Cd and labelled Ca concentrations (Jeffree 1991), explicable by the uptake of both metals through the Ca pump.

This chapter examines the possibility of active uptake of Al, Cu and Mn by *P. nigroculus* via the major cation (Ca^{2+}, Na^{+}, K^{+} and Mg^{2+}) pumps. I wished to see if the three metals of interest (i.e. Al, Cu and Mn) are indeed incorporated into *P. nigroculus* in this way. I reasoned that, if a cation pump is actively used in the transport of another metal, then a reduction or an increase in the concentration of that cation in the body should result in a reduction or an increase in bioaccumulation of the second metal. My work remains exploratory and further investigations regarding the active transport of metal ions in aquatic environments need to be done.

For the purposes of these analyses, I have made three assumptions. These are:

- That if the concentration of a trace metal in an animal is proportional to the concentration of a particular major cation, then the active pump of that major cation is responsible for bringing the trace metal into the body (Bjerregaard and Depledge 1990, Jeffree 1991). Thus high or low concentrations of Al, Cu or Mn in *P. nigroculus* should correspond with high or low concentrations of at least one of the major cations Na, K, Ca or Mg. For instance, the rate at which the pump works, and the initial concentration of each major ion in the body, will affect the results. The link however, between the amount of trace metal and of a major ion in the body should be a useful indication of the active pump used. Similarities of charge between Al, Cu and Mn, and Ca, Na and Mg, as well as similarities of valency, have allowed me to predict the active uptake of the three metals under study via at least one of the major ions. The size of the ionic radii may differ and may thus affect my predictions.

- That facilitated diffusion is negligible, as is uptake via food route; and that excretion,if present, does not affect the active uptake.

- That for single trace elements, an active pump plays a role during the uptake process; and also, that each single trace element may be transported by more than one active pump.

Some data on Al, Cu and Mn concentrations in the amphipod, as described in Chapter 5, are used in this chapter. Additional data on the concentrations of Ca, Na and Mg in the amphipods are also provided. K was not examined as I was working with 'artificial' water with K included.

6.3 Methods

Experiments were run as described in Chapter 2. After 21 days of exposure to test solutions of 'artificial' water, 20 survivors of *P. nigroculus* per concentration were dried and ashed as described in Chapter 5. Concentrations of Al, Cu, Mn, Ca, Na and Mg in the body of *P. nigroculus* were measured by ICP-S (as described in Chapter 5) at wavelengths of 257.610 nm for Mn, 279.553 nm for Mg, 308 nm for Al, 324.754 nm for Cu, 393.367 nm for Ca and 589.592 nm for Na.

I compared the concentrations of the major cations (i.e. Ca, Na and Mg) with those of the trace metals under study (i.e. Al, Cu and Mn). When the changes in trace metal concentration in the amphipod over time were proportional to those of a given major cation, I concluded that the active uptake may have used the active route of this major element. Regression analysis, using the Microsoft Excel program (Book 1, Copyright UCT, 1997), was done to identify any statistically significant correlations between pairs of metals: Ca/Cu, Ca/Al, Ca/Mn, Na/Cu, Na/Al, Na/Mn, Mg/Cu, Mg/Al, and Mg/Mn. Student's t-test was used to test differences in bioaccumulation of Mg and Mn by *P. nigroculus* according to the experiment recorded in section 6.6.

6.4 Results

Tables 6.1 to 6.4 show the concentrations of Al, Cu, Mn, Ca, Mg, Na and K in *P. nigroculus* after 21 days of exposure to test solutions at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn. I chose these concentrations because they were the highest I derived from the South African guidelines for aquatic ecosystems. Only 'artificial' water was used because its composition was known as stated in Chapter 2. To allow comparison of results, ranges of concentrations for major cations and metals under study were arbitrarily defined according to the levels of metals in the amphipods.

Table 6.1: The amounts (in μg/g) of Al, Cu, Ca and Na in P. nigroculus body ash after 21-d exposures to the test solution. Amounts in amphipods of Ca ≤ 25000 μg/g, Na ≤ 4000 μg/g, Cu ≤ 100 μg/g and Al ≤ 500 μg/g (dry weight)

Replicate numbers	*Ca*	*Na*	*Al*	*Cu*
1	20	504	504	165
2	20	5890	999	1070
3	30	5080	505	43
4	3200	3670	357	69
5	1620	8190	70	30
6	4200	862	880	80
7	2320	591	137	147
8	4190	1010	135	16
9	2060	1440	202	971
10	4700	740	211	236
11	1110	843	476	120
12	12000	874	236	29
13	7930	767	412	597
14	2450	18200	54	14
15	4980	601	190	238
16	3820	564	107	128
17	960	460	37	10
18	1910	548	115	115
19	5030	639	177	19
20	3190	571	57	50
21	3020	553	63	67
22	15100	1690	246	199
23	1960	499	49	50
24	19700	1470	214	84
25	8510	838	143	38

Table 6.1 shows that when Ca concentrations in animals were ≤ 25000 μg/g, Al concentrations were ≤ 500 μg/g in 21/25 cases (84%) and Cu concentrations were ≤ 100 μg/g in 17/25 cases (68%). In 23/25 cases (92%) when Na concentrations were ≤ 4000 μg/g and Al ≤ 500 μg/g and Cu concentrations were ≤ 100 μg/g in only 15/25 (60%). This suggests that Al and Cu may be actively taken up via Ca or Na pumps.

Table 6.2: Concentrations of Al, Cu, Ca and Na in amphipods after 21 days of exposure to the test solutions. Concentrations in amphipods of Ca > 25000 μg/g, Cu >100 μg/g and Al < 500 μg/g (dry weight)

Replicate numbers	*Ca*	*Na*	*Al*	*Cu*
1	54300	3770	667	118
2	38500	2530	259	140
3	54900	4980	747	78
4	47400	4200	754	127
5	60000	6880	848	230
6	60000	5540	518	81
7	60000	5350	766	1360

Table 6.2 indicates that when Ca concentrations were higher than 25000 μg/g, Al concentrations were ≥ 500 μg/g in 6/7 (86%) and Cu concentrations were ≥ 100 μg/g in 5/7 (71%) of cases.

When Na concentrations were above 4000 μg/g, Al concentrations were ≥ 500 μg/g in all cases (100%) and Cu was above 100 μg/g in 5/8 cases (63%). Tables 6.1 and 6.2 suggest a proportional link between the amount of Ca, Na, Al and Cu. Is this a significant correlation? Results of the regression analysis (Table 6.5) give the answer.

Table 6.3: Concentrations of Ca, Mg, Na and Mn in amphipods after 21 days of exposure to the test solutions. Concentrations in amphipods of Ca ≤ 25000 μg/g, Na and Mg ≤ 4000 μg/g each, and Mn ≤ 100 μg/g (dry weight)

Replicate numbers	*Ca*	*Na*	*Mg*	*Mn*
1	20	5040	995	40
2	20	5890	1320	21
3	30	5080	1020	33
4	3200	3570	1700	159
5	1620	8190	991	39
6	4200	862	116	10
7	2320	591	37	30
8	4190	1010	163	10
9	3200	3670	1750	159
10	20600	1440	324	90
11	4700	740	840	38
12	11100	843	173	10
13	12000	874	218	10
14	7930	767	103	88
15	2450	18200	1840	539
16	4980	601	122	293
17	3820	564	52	10
18	960	460	18	10
19	1910	548	29	10
20	5030	639	115	10
21	3190	571	50	10
22	3020	553	48	10
23	15100	1690	255	49
24	1960	499	32	10
25	19700	1470	298	30
26	8510	838	1540	80

Table 6.3 indicates that when the Ca concentrations in the amphipod were ≤ 25000 μg/g, Mn was ≤ 100 μg/g in 20/26 cases (77%) and when Na concentration was ≤ 4000 μg/g, Mn was ≤ 100 μg/g in 20/23 (87%) of cases. This suggests that Mn may use the active pumps of Ca and Na to actively enter into the amphipod.

Table 6.4: Concentrations of Al, Na, Mg and Mn in amphipods after 21 days of exposure to the test solutions. Ca concentrations in amphipods of Ca were above 25000 μg/g, Na and Mg above 4000 μg/g and Mn above 100 μg/g (dry weight)

Replicate numbers	*Ca*	*Na*	*Mg*	*Mn*
1	54300	3670	1700	20
2	38500	2530	505	20
3	54900	4980	1030	14
4	47400	4200	902	13
5	60000	6880	1220	18
6	60000	5540	1100	17
7	60000	5350	1040	41

Table 6.4 indicates that when Ca concentrations in amphipods were above 25000 μg/g, Mn was never above 100 μg/g; when Na was above 4000 μg/g, Mn was above 100 μg/g in only 1/7 (14%) of cases; when Mg was ≥ 4000 μg/g, Mn was never above 100 μg/g; when Mg ≤ 4000 μg/g, Mn was ≤ 100 μg/g in 29/33 cases (88%). This suggests that at high concentrations of Ca and Na, the active uptake of Mn does not depend on Ca and Na, but rather on Mg uptake.

Tables 6.1 - 6.4 show a link between the concentrations of Al, Cu and Mn and that of Ca, Na and Mg. Does the link between low concentrations of Mg and low concentrations of Mn in the amphipod suggests an important control of Mn uptake by Mg? The answer is reported in section 6.6.

Table 6.5 lists the results of the regression analysis performed on the data from Tables 6.1 - 6.4.

*Table 6.5: Correlations between Al, Cu, Mn, Ca, Na and Mg concentrations in dry body weight of P. nigroculus (n = 32; df=30 and * = significant, $p<0.05$; H_o = not significant.*

Metals	*Regression equation*	*r*	*Significant at 95% level*
Ca vs Al	Y = 0.0076x + 232.08	0.5626	*
Ca vs Na	Y = 0.0404x + 2347.1	0.2396	H_o
Ca vs Mg	Y = 0.011x + 454.34	0.3927	*
Ca vs Cu	Y = 0.0034x + 130.52	0.2470	H_o
Ca vs Mn	Y = -0.0009x + 69.277	0.1811	H_o
Na vs Al	Y = 0.0183x + 293.59	0.2296	H_o
Na vs Cu	Y = 0.0094x + 154.92	0.1145	H_o
Na vs Mg	Y = 0.1189x + 269.11	0.7194	*
Na vs Mn	Y = 0.0182x + 1.7424	0.6253	*
Al vs Mn	Y = -0.0797x + 83.421	0.2189	H_o
Al vs Mg	Y = 0.8825x + 314.67	0.4264	*
Al vs Cu	Y = 0.5256x - 0.2309	0.5129	*
Cu vs Mg	Y = 0.3454x + 558.65	0.1709	H_o
Cu vs Mn	Y = -0.0147x + 58.381	0.0412	H_o
Mg vs Mn	Y = 0.0647x + 15.463	0.3676	*

6.5 Discussion

Table 6.5 indicates significant correlations between the amounts of metals in amphipods for Ca vs Al, and Mg; Na vs Mg, and Mn; Al vs Mg, and Cu; and Mg vs Mn.

Tables 6.1 - 6.4 show the variability in the extent of metal uptake by *P. nigroculus* in the same treatments and suggests that individuals may have intrinsic differences in the rate or extent of accumulation, a phenomenon previously noted by e.g. Metayer *et al.* (1990), Naylor *et al.* (1990) and Maud *et al.* (1992).

6.6 Effects of Mg on uptake of Mn

6.6.1 Material and methods

Stock solutions containing 1g/L of Mg were prepared by dissolving 4.95 g of $MgSO_4$ in 1L of distilled water; and stock solutions containing 1g/L Mn were prepared by dissolving 2.75 g of $MnSO_4$ in 1L of distilled water. Thus 1 mL of stock solution contained 1 mg of the corresponding metal species. Drops of concentrated nitric acid were used to adjust the pH values to between 4.5 and 5.8. Test concentrations were derived from the stock solutions through dilutions and concentrations of 1000 mg/L, 100 mg/L, 10 mg/L, 1 mg/L and 0.1 mg/L of each metal species were tested since these two metals occur in most natural fresh waters at concentrations much lower than 5 mg/L (e.g. NALCO 1984). Three series of experiments were performed: test solutions with Mg only; test solutions with Mn only; and test solutions containing both metals at a mass ratio of 1:1.

Each test concentration was replicated three times and each replicate received at least 10 individual amphipods of various sizes but excluding breeding females and moulting individuals. Results are reported as the number of the survivors after 24, 72, 96, 144 and 192 hours of exposure to test solutions, and also as the median survival time (LT_{50}). After 192 hours of exposure, survivors of *P. nigroculus* were collected from different solutions and controls and kept separately for 30 hours in soft artificial water without Mg and Mn for depuration. This might be a problem if excretion occurs rapidly after the animal is removed from the solution. I neglected this possible route of excretion, however. Amphipods were rinsed in distilled water, dried and ignited at 650°C for 8 hours. The ash was weighed and 0.5 g of ash from each sample was digested with 5 mL of concentrated nitric acid. The volume was made up to 50 mL with distilled water as described in Chapter 5. The solutions were filtered through Watman 0.45 μm GF/C filter paper and analysed by ICP-S. The wavelengths used were 257.610 nm for Mn and 279.553 nm for Mg.

6.6.2 Results

(a) Survival and LT_{50} records

All results based on survival and on LT_{50} are reported in Tables 6.6 - 6.9. Table 6.6 indicates the survival of *P. nigroculus* exposed to test solutions containing different concentrations of Mg. Table 6.7 indicates the survival of *P. nigroculus* exposed to test solutions containing different concentrations of Mn. Table 6.8 indicates the survival of *P. nigroculus* exposed to different mixtures of Mg + Mn at a mass ratio 1:1. Table 6.9 indicates the concentrations of Mn and Mg in amphipod ash (in μg/g) after 8 days of exposure to different test solutions.

Table 6.6: Survival of P. nigroculus exposed to test solutions containing different concentrations of Mg

Mg (mg/L)	n	24 hrs	72 hrs	96 hrs	6 days	8 days	LT_{50} (hrs)
1000	15,15,10	0,1,0 (3%)	0,0,0 (0%)				12
100	20,20,20	9,9,10 (47%)	0,0,1 (2%)	0,0,0 (0%)			22
10	15,15,15	10,9,9 (62%)	6,7,7 (44%)	5,4,5 (31%)	5,4,4 (29%)	4,4,3 (24%)	54
1	20,20,18	18,20,17 (95%)	18,18,17 (91%)	14,15,15 (76%)	11,13,12 (62%)	11,12,10 (57%)	>192
0.1	18,18,20	16,18,17 (91%)	16,17,17 (89%)	15,15,16 (82%)	13,14,13 (71%)	13,13,12 (68%)	>192
Control	18,20,20	18,19,20 (98.3%)	17,19,19 (94.8%)	17,18,19 (93%)	15,17,17 (84.5%)	15,16,16 (81%)	>192

Table 6.7: Survival of P. nigroculus exposed to test solutions containing different concentrations of Mn

Mn (mg/L)	n	24 hrs	72 hrs	96 hrs	6 days	8 days	LT_{50} (hrs)
1000	16,16,20	10,11,15 (69%)	9,9,13 (60%)	9,8,12 (56%)	4,4,5 (25%)	4,3,3 (19%)	103
100	20,20,18	18,19,18 (95%)	17,18,17 (90%)	15,14,14 (74%)	12,11,12 (57%)	11,11,10 (55%)	>192
10	15,15,15	15,14,14 (96%)	14,14,14 (93%)	12,13,14 (87%)	9,12,12 (73%)	9,11,11 (69%)	>192
1	15,15,16	14,13,14 (89%)	14,13,14 (89%)	13,13,13 (85%)	13,13,12 (83%)	13,13,12 (83%)	>192
0.1	20,15,15	17,13,14 (88%)	16,13,13 (84%)	16,12,13 (82%)	16,12,13 (82%)	15,12,13 (80%)	>192

Table 6.8: Survival of P. nigroculus exposed to different mixtures of Mg + Mn at a mass ratio of 1:1

Mg + Mn (mg/L)	*n*	*24 hrs*	*72 hrs*	*96 hrs*	*6 days*	*8 days*	*LT_{50} (hrs)*
1000 + 1000	20,18,20	13,11,12 (62%)	13,10,11 (59%)	9,8,11 (48%)	9,7,10 (45%)	5,3,5 (22%)	90
100 + 100	15,15,15	11,12,11 (76%)	10,10,9 (64%)	9,10,9 (62%)	8,8,7 (51%)	6,7,7 (44%)	144
10 + 10	15,15,15	11,13,12 (80%)	11,12,12 (78%)	9,10,10 (64%)	9,10,9 (62%)	8,9,9 (58%)	>192
1 + 1	15,15,15	14,15,14 (96%)	14,14,13 (91%)	14,13,13 (89%)	12,13,11 (80%)	10,12,11 (73%)	>192
0.1 + 0.1	20,20,18	18,17,15 (86%)	17,17,15 (84%)	16,16,15 (81%)	16,15,13 (76%)	15,14,13 (72%)	>192

Table 6.6 indicates that at a concentration of 1 mg/L Mg, LT_{50} for *P. nigroculus* is longer than 8 days. Table 6.7 indicates that LT_{50} was longer than 8 days and that % of survival after 8 days of exposure was above 50% in all treatments except one (i.e. 1000 mg/L and 19% survival). Table 6.8 indicates the reduction of Mg toxicity in *P. nigroculus* when Mn is added (e.g. at concentration of 1000 mg/L Mg, % of survival for Mg after 8 days of exposure = 0% and LT_{50} = 12 hours, while in mixture with Mn the same concentration allows 22% of survival and LT_{50} = 90 hours).

(b) Chemical analyses

Results of the chemical analyses are summarised in Table 6.9, which indicates the amount of Mg and Mn (in μg/g dry body weight) in *P. nigroculus* after 8 days of exposure to each metal solution alone and to metal mixtures. Ash was made with 20 survivors from each test concentration and control. Values in the Table 6.9 are mean values for each set of 3 replicates.

Student's t-test result showed that observed differences in bioaccumulation of Mn, when alone (c) and when combined with Mg (d), were not statistically different at $p<0.05$ ($t = 1.403$; $df=8$). Differences in bioaccumulation of Mg, when alone (a) and when combined with Mn (b), were statistically significant at $p<0.002$ ($df=6$). These results indicate that Mn lowers the Mg uptake by *P. nigroculus*.

*Table 6.9: Concentrations of Mn and Mg in amphipod ash (µg/g) after 8 days of exposure to different test concentrations; * = no live amphipods after 8-day exposure*

Conc. (mg/L)	Mg in ash (µg/g)		Mn in ash (µg/g)	
	(a) Mg alone (Table 6.6)	(b) Mg + Mn (Table 6.8)	(c) Mn alone (Table 6.7)	(d) Mn + Mg (Table 6.8)
1000	*	198	379	680
100	*	103	153	622
10	701	31	182	268
1	492	36	152	131
0.1	368	41	114	160
Control	16		< 1	

6.7 Conclusions

The results suggest that the major cation pumps Na and Mg may play an important role in the uptake of Mn. The link between the low concentration of Mn and Mg in *P. nigroculus* suggests strong correlations in the uptake of the two metals.

- The quantity of Mg in the body of *P. nigroculus* increases with availability far above concentration 'in nature';
- Mn seems to be regulated (but at a much higher concentration than in nature) over the range of 0.1 - 100 mg/L.
- The toxic effects of Mg are mitigated by Mn.
- The concentration of Mg in the body of *P. nigroculus* is significantly ($p < 0.002$) lower in the presence of Mn than when no Mn is present. Mn therefore reduces the toxicity of Mg in *P. nigroculus*.
- I conclude that Mn is not imported via the Mg pump. There may be interference between the uptake of both metals as indicated by the high r values in the correlation analyses between Na/Mg and Na/Mn (0.7194 for Na/Mg and 0.6253 for Na/Mn).

CHAPTER 7

Statistical analysis of survival, growth and reproduction

7.1 Analysis of survival (Tables 3 and 4 in Chapter 2 - the paper)

In addition to the brief statistical analyses discussed in Chapters 3, 4 and 5, this chapter provides statistical support to my thesis, discussing differences in survival and growth rates between and within concentrations of the mixtures of Al, Cu and Mn. The first part of the chapter deals with the analysis of variance while the second part deals with paired tests of comparison in those cases where the null hypothesis was rejected (i.e. where variations are not attributable to chance).

Statistical analyses of survival of juvenile and mature amphipods were performed to ascertain whether or not differences in survival rates in different concentrations were significant. When mean values were not statistically significant, the null hypothesis (H_o) was accepted but where differences appeared to be statistically significant, the null hypothesis was rejected.

Analysis of variance (ANOVA) was used to describe differences in mean values for survival and growth in all concentrations of the mixtures of Al, Cu and Mn used. I wanted to know if the observed differences between the mean numbers of animals surviving at different concentrations were due to chance (F value not statistically significant) or to effects of metals (F value statistically significant). When the F value for the analyses of variance was significant at $p < 0.05$, I used the Newman-Keuls test for comparison of survival and growth in different concentrations (Clarke 1980, Zar 1984, Bonhivers & De Ketele 1986, USEPA 1991, Kooijman & Bedaux 1996).

For the data on reproduction, I used the non-parametric Mann-Whitney U-test. I wanted to know if mixtures of metals (Al + Cu or Al + Cu + Mn), or the type dilution water used significantly affected reproduction in *P. nigroculus*.

7.1.1 Survival: analysis of variance

Data on survival at different concentrations are repeated for convenience in Table 7.0. Concentrations of metals used in the experiments and code numbers assigned to each as in Table 1.3.

Table 7.0: Concentrations of metals used in the experiments and code numbers assigned to each as in Table 1.3.

Solution codes	*Conc. of Al (mg/L)*	*Conc. of Cu (mg/L)*	*Conc. of Mn (mg/L)*
C1 (Control)	0	0	0
C2 (Ca1)	0.030	0.0025	0
C3 (Aa1)	0.062	0.001	0
C4 (Ba1)	0.0925	0.0025	0
C5 (Cb1)	0.0925	0.0175	0
C6 (AEV2a)	0.1225	0.005	0
C7 (Ab1)	0.185	0.004	0
C8 (Bb1)	0.2775	0.005	0
C9 (Cc1)	0.4325	0.004	0
C10 (Ac1)	0.926	0.008	0
C11 (Bc1)	1.388	0.0175	0
C12 (Ca2)	0.030	0.0025	0.6075
C13 (Aa2)	0.062	0.001	1.217
C14 (Cb2)	0.0925	0.0175	1.218
C15 (Ba2)	0.0925	0.0025	1.825
C16 (Ab2)	0.185	0.004	2.434
C17 (Bb2)	0.2775	0.005	3.650
C18 (Cc2)	0.4325	0.004	4.665
C19 (AEV2b)	0.1225	0.005	5.273
C20 (Ac2)	0.926	0.008	9.328
C21 (Bc1)	1.388	0.0175	13.993

Tables 7.1 - 7.8 show the effects of the Al + Cu or Al + Cu + Mn mixtures in 'artificial' or stream water on the survival of mature or juvenile *P. nigroculus* after 21-day exposures (data derived from Table 3 in the paper presented as chapter 2). Concentrations C1...C21 correspond with concentrations in Table 7.0.

Table 7.1 analyses the effects of Al + Cu mixtures in 'artificial' water on the survival of mature *P. nigroculus* after 21-day exposures. Table 7.2 analyses the effects of Al + Cu + Mn mixtures in 'artificial' water on the survival of *mature P. nigroculus* after 21 days of exposure; Table 7.3 analyses the effects of Al + Cu mixtures

in stream water on the survival of mature *P. nigroculus* after 21 days of exposure; Table 7.4 analyses the effects of Al + Cu + Mn mixtures in stream water on the survival of mature *P. nigroculus* after 21 days of exposure; Table 7.5 analyses the effects of Al + Cu mixtures in 'artificial' water on the survival of juvenile *P. nigroculus* after 21 days of exposure; Table 7.6 analyses the effects of Al + Cu + Mn mixtures in 'artificial' water on the survival of juvenile *P. nigroculus* after 21 days of exposure; Table 7.7 analyses the effects of Al + Cu mixtures in stream water on the survival of juvenile *P. nigroculus* after 21 days of exposure; Table 7.8 analyses the effects of Al + Cu + Mn mixtures in stream water on the survival of juvenile *P. nigroculus* after 21 days of exposure.

Table 7.9 indicates that differences in mortality of mature amphipods in 'artificial' water mixtures containing different concentrations of Al + Cu (Table 7.1) and in stream water mixtures containing different concentrations of Al + Cu (Table 7.3) or Al + Cu + Mn (Table 7.4) may be attributed to the effects of metals. It also shows that differences in mortality of juvenile amphipods in stream water containing different concentrations of Al + Cu (Table 7.5) and in 'artificial' water containing different concentrations of Al + Cu (Table 7.7) or Al + Cu + Mn (Table 7.8) may be attributed to the effects of metals. Differences in mortality in mixtures of Al + Cu + Mn in 'artificial' water for mature amphipods are not statistically significant and may be attributed to chance.

7.1.2 Survival: paired tests of comparison

Where the calculated F value > than that from the statistical Table at $p < 0.05$ for the data sets described above, the Newman-Keuls test was performed in order to provide paired analyses of comparison for the significance ($p < 0.05$) of variation in survival between different concentrations. Calculations were done according to Zar (1984) and Reish and Oshida (1986) and results are presented in Tables 7.10 - 7.15. They report significant results for each concentration compared to the controls and also to each other.

For each case, A & B = two samples ranks with B the larger number; q = difference between the means of B & A as XB - XA, divided by the standard error; p = number of means in the range of means being tested; df = degrees of freedom for group. "*" indicates that the difference between the two means is statistically significant at the 95% level. Only statistically significant results are presented in Tables 7.10 - 7.15.

Table 7.10 summarises the results of survival for mature *P. nigroculus* in 'artificial' water with mixtures of Al + Cu (data from Table 7.1).

Table 7.10 indicates that number of individuals surviving in the control was significantly different from the number surviving in mixtures of Al + Cu at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu (Bc1); 0.926 mg/L Al + 0.008 mg/L Cu (Ac1); 0.925 mg/L Al + 0.0025 mg/L Cu (Ba1); and 0.4325 mg/L Al + 0.004

mg/L Cu (Cc1). Number of individuals surviving was not different from the number surviving in the control at any other concentrations.

Table 7.11 indicates that number of individuals surviving in the control or in Ca1 (0.030 mg/L Al + 0.0025 mg/L Cu) was significantly different from the number surviving in stream water mixtures of Al + Cu at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu (Bc1), 0.185 mg/L Al + 0.004 mg/L Cu (Ab1) and 0.2775 mg/L Al + 0.005 mg/L Cu (Bb1) may be attributed to the effects of metals.

Tables 7.12 - 7.15 summarise the results of paired analysis on survival of mature or juvenile amphipods at different concentrations of mixtures of metals in either type of water.

Table 7.12 indicates that only two concentrations showed significantly difference in survival of mature amphipods exposed in stream water mixtures of Al + Cu + Mn (i.e. 1.388 mg/L Al + 0.0175 mg/L Cu, and 0.926 mg/L Al + 0.008 mg/L Cu). All other variations observed in survival may be attributed to chance.

Table 7.13 shows that diffrences in number of juvenile amphipods surviving in the control or in Ca1 and Bc1, Ac1, Cc1, Aa1 and Ba1 may be attributed to the effects of metals (i.e. Al + Cu); that differences in survival between Cb1 and Bc1, Cc1 and Ac1; and differences between AEV2a (0.1225 mg/L Al + 0.005 mg/L Cu) and Bc1 and Ac1 may be attributed to the effects of Al + Cu.

Table 7.14 indicates that differences (*) in survival were significantly different and observed variations in mortalities of juvenile amphipods in stream water mixtures of Al + Cu may be attributed to the effects of Al and Cu.

Table 7.15 indicates that variations in survival of juvenile amphipods in stream water mixtures of Al + Cu + Mn may be attributed to the effects of Al + Cu + Mn for the paired-treatments (*).

Table 7.1: Number of mature amphipods surviving after 21 days of exposure to test concentrations C1 - C11 of Al + Cu mixtures in 'artificial' water. Number of replicates = 3; SD = standard deviation of the mean; SSg = the square of the summation of the number of survivors divided by the sample size (3)

Replicate	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C7*	*C8*	*C9*	*C10*	*C11*
1	9	8	6	5	5	6	5	5	4	4	4
2	8	8	4	5	5	5	5	5	5	4	4
3	8	8	5	4	5	5	5	5	5	4	4
Total no. of survivors	25	24	15	14	15	16	15	15	14	12	12
Mean	8.3	8	5	4.7	5	5.3	5	5	4.7	4	4
SD of mean	0.6	0	1.0	0.6	0	0.5	0	0	0.6	0	0
SSg	208.3	192	75	65.3	75	85.3	75	75	65.3	48	48

Table 7.2: Number of mature individuals surviving after 21 days of exposure to mixtures of Al + Cu + Mn in 'artificial' water

Replicate	*C1*	*C12*	*C13*	*C14*	*C15*	*C16*	*C17*	*C18*	*C19*	*C20*	*C21*
1	9	9	6	8	6	7	6	9	7	7	6
2	8	7	7	8	6	6	6	8	8	6	6
3	8	8	7	9	6	6	5	8	7	6	7
Total no. of survivors	25	24	20	25	18	19	17	25	22	19	19
Mean	8.3	8	6.7	8.3	6	6.3	5.7	8.3	7.3	6.3	6.3
SD of mean	0.6	1.0	0.6	0.6	0	0.6	0.6	0.6	0.6	0.6	0.6
SSg	208.3	192	133.3	208.3	108	120.3	96.3	208.3	161.3	120.3	120.3

Table 7.3: Number of mature amphipods P. nigroculus surviving after 21 days of exposure to Al + Cu mixtures in stream water

Replicate	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C7*	*C8*	*C9*	*C10*	*C11*
1	9	8	6	5	5	6	5	5	6	4	4
2	9	9	5	6	5	6	5	5	5	5	4
3	8	8	5	5	6	4	5	5	5	4	4
Total no. of survivors	26	25	16	16	16	16	15	15	16	13	12
Mean	8.7	8.3	5.3	5.3	5.3	5.3	5	5	5.3	4.3	4
SD of mean	0.6	0.6	0.6	0.6	0.6	1.2	0	0	0.6	0.6	0
SSg	225.3	208.3	85.3	85.3	85.3	85.3	75	75	85.3	56.3	48

Table 7.4: Number of mature amphipods surviving after 21 days of exposure to Al, Cu and Mn mixtures in stream water.

Replicate	*C1*	*C12*	*C13*	*C14*	*C15*	*C16*	*C17*	*C18*	*C19*	*C20*	*C21*
1	9	8	7	8	6	7	8	8	8	7	7
2	9	8	6	8	6	7	6	8	8	7	5
3	8	9	7	8	7	6	6	8	8	5	6
Total no. of survivors	26	25	20	24	19	20	20	24	24	19	18
Mean	8.7	8.3	6.7	8	6.3	6.7	6.7	8	8	6.3	6
SD of mean	0.6	0.6	0.6	0	0.6	0.6	1.2	0	0	1.2	1.0
SSg	225.3	208.3	133.3	192	120.3	133.3	133.3	192	192	120.3	108

Table 7.5: Number of juvenile amphipods surviving after 21 days of exposure to Al + Cu mixtures in 'artificial' water

Replicate	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C7*	*C8*	*C9*	*C10*	*C11*
1	8	7	5	5	6	6	4	5	4	4	3
2	8	7	5	5	6	5	5	4	4	4	3
3	8	8	4	4	6	6	5	5	4	4	3
Total no. of survivors	24	22	14	14	18	17	14	14	12	12	9
Mean	8	7.3	4.7	4.7	6	5.7	4.7	4.7	4	4	3
SD of mean	0	0.6	0.6	0.6	0	0.6	0.6	0.6	0	0	0
SSg	192	161.3	65.3	65.3	108	96.3	65.3	65.3	48	48	27

Table 7.6: Number of juvenile amphipods surviving after 21 days of exposure to Al + Cu + Mn mixtures in 'artificial' water

Replicate	*C1*	*C12*	*C13*	*C14*	*C15*	*C16*	*C17*	*C18*	*C19*	*C20*	*C21*
1	8	8	6	8	8	6	6	7	8	6	5
2	8	7	5	7	5	6	6	6	6	6	5
3	8	8	5	7	5	6	5	6	6	5	6
Total no. of survivors	24	23	16	22	18	18	17	19	20	17	16
Mean	8	7.7	5.3	7.3	6	6	5.7	6.3	6.7	5.7	5.3
SD of mean	0	0.6	0.6	0.6	1.4	0	0.6	0.6	1.2	0.6	0.6
SSg	192	176.3	85.3	161.3	108	108	96.3	120.3	133.3	96.3	85.3

Table 7.7: Number of juvenile amphipods surviving after 21 days of exposure to Al + Cu mixtures in stream water

Replicate	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C7*	*C8*	*C9*	*C10*	*C11*
1	8	7	5	4	7	7	4	3	4	4	3
2	7	7	4	5	8	7	4	3	5	4	4
3	8	7	4	4	7	6	4	4	5	3	3
Total no. of survivors	23	21	13	13	22	20	12	10	14	11	10
Mean	7.7	7	4.3	4.3	7.3	6.7	4	3.3	4.7	3.7	3.3
SD of mean	0.6	0	0.6	0.6	0.6	0.6	0	0.6	0.6	0.6	0.6
SSg	176.3	147	56.3	56.3	161.3	133.3	48	33.3	65.3	40.3	33.3

Table 7.8: Number of juvenile amphipods surviving after 21 days of exposure to Al, Cu and Mn mixtures in stream water

Replicate	*C1*	*C12*	*C13*	*C14*	*C15*	*C16*	*C17*	*C18*	*C19*	*C20*	*C21*
1	8	8	6	7	6	5	6	7	8	6	6
2	7	7	6	8	6	5	6	6	6	4	6
3	8	7	6	8	5	4	5	7	7	5	5
Total no. of survivors	23	22	18	23	17	14	17	20	21	15	17
Mean	7.7	7.3	6	7.7	5.7	4.7	5.7	6.7	7	5	5.7
SD of mean	0.6	0.6	0	0.6	0.6	0.6	0.6	0.6	1.0	1.0	0.6
SSg	176.3	161.3	108	176.3	96.3	65.3	96.3	133.3	147	75	96.3

Table 7.9: *Summary of the analysis of variance for surviving P. nigroculus after 21-day exposures to different concentrations; a.w. = 'artificial' water; n.w. = stream water; s.v. = source of variation; s.s. = sums of squared means; df = degrees of freedom; m.s. = mean squared. * = variation statistically significant at $p<0.05$; N.S. = not significant at $p<0.05$.*

	Water	*Metal Mixture*	*s.v.*	*s.s*	*df*	*m.s.*	*F value*
Adults	a.w.	Al + Cu	total	67.6	32	-	10.5*
			group	62.8	10	2.1	
			error	4.8	22	0.2	
		Al + Cu + Mn	total	25.7	32	-	2 N.S.
			group	17.4	10	0.8	
			error	8.3	22	0.4	
	n.w.	Al + Cu	total	73.6	32	-	7.7*
			group	66	10	2.3	
			error	7.6	22	0.3	
		Al + Cu + Mn	total	52.5	32	-	3.2*
			group	41.6	10	1.6	
			error	10.9	22	0.5	
Juveniles	n.w.	Al + Cu	total	70.2	32	-	11*
			group	66	10	2.2	
			error	4.2	22	0.2	
		Al + Cu + Mn	total	39.6	32	-	2 N.S.
			group	26	10	1.2	
			error	13.6	22	0.6	
	a.w.	Al + Cu	total	48.1	32	-	7.5*
			group	43.8	10	1.5	
			error	4.3	22	0.2	
		Al + Cu + Mn	total	42.5	32	-	3.3*
			group	32.9	10	1.3	
			error	9.6	22	0.4	

Table 7.10: Summary of results on survival of mature P. nigroculus in 'artificial' water containing mixtures of Al + Cu.

Concentration B (mg/L)	*Concentration A (mg/L)*	*q calc.*	*q table*	*p*	** at 0.005*
Control: 0	Bc1: Al = 1.388; Cu = 0.0175	16.540	5.722	11	*
	Ac1: Al = 0.926; Cu = 0.008	13.850	5.461	9	*
	Ba1: Al = 0.925; Cu = 0.0025	12.69	5.124	7	*
	Cc1: Al = 0.4325; Cu = 0.004	11.54	3.877	3	*
Ca1: Al = 0.030; Cu =0.0025	Bc1: Al = 1.388; Cu = 0.0175	15.38	5.599	10	*
	Ac1: Al = 0.926; Cu = 0.008	12.69	5.305	8	*
	Ba1: Al = 0.0925; Cu = 0.0025	11.54	4.912	6	*
	Cc1: Al = 0.4325; Cu = 0.004	10.38	3.151	2	*

Table 7.11: Summary of the results for survival of mature P. nigroculus in stream water with mixtures of Al + Cu (data of Table 7.3)

Concentration B (mg/L)	*Concentration A (mg/L)*	*q calc.*	*q table*	*P*	*0.05 level of difference*
Control: 0	Bc1: Al = 1.388 & Cu = 0.0175	14.69	5.722	11	*
	Ac1: Al = 0.926 & Cu = 0.008	13.75	5.599	10	*
	Ab1: Al = 0.185 & Cu = 0.004	11.56	5.461	9	*
	Bb1: Al = 0.2775 Cu = 0.005	10.63	5.124	7	*
Ca1: Al = 0.030; Cu = 0.0025	Bc1: Al = 1.388; Cu = 0.0175	13.44	5.599	10	*
	Ac1:Al = 0.926; Cu = 0.008	12.50	3.151	9	*
	Ab1: Al = 0.185; Cu = 0.004	10.31	5.305	8	*
	Bb1: Al = 0.2775; Cu = 0.005	9.38	4.912	6	*

Table 7.12: Summary of the results for survival of mature P. nigroculus in stream water for the mixtures of Al + Cu + Mn (data from Table 7.4)

Concentration B (mg/L)	*Concentration A (mg/L)*	*q calc.*	*q Table*	*p*	** at 0.05*
Control: 0	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	6.59	5.722	11	*
	Ac2:Al = 0.926; Cu = 0.008; Mn = 9.328	5.85	5.599	10	*

Table 7.13: Summary of the results for survival of the juvenile P. nigroculus in 'artificial' water for the mixtures of Al + Cu (data from Table 7.5)

Concentration B (mg/L)	*Concentration A (mg/L)*	*q calc.*	*q Table*	*p*	** at 0.05*
Control: 0	Bc1: Al = 1.388; Cu = 0.0175	19.13	5.722	11	*
	Ac1:Al = 0.926; Cu = 0.008	15.38	5.599	10	*
	Cc1: Al = 0.4325; Cu = 0.004	12.69	5.305	8	*
	Aa1:Al = 0.062; Cu = 0.001	8.85	4.327	4	*
	Ba1:Al = 0.0925; Cu = 0.0025	7.69	3.877	3	*
Ca1: Al = 0.030; Cu = 0.0025	Bc1: Al = 1.388; Cu = 0.0175	16.54	5.599	10	*
	Ac1:Al = 0.926; Cu = 0.008	12.69	5.461	9	*
	Cc1: Al = 0.4325; Cu = 0.004	10	5.124	7	*
	Aa1: Al = 0.062; Cu = 0.001	6.15	3.877	3	*
	Ba1:Al = 0.0925; Cu = 0.0025	5	3.151	2	*
Cb1: Al = 0.0925; Cu = 0.0175	Bc1: Al = 1.388; Cu = 0.0175	11.54	5.461	9	*
	Cc1:Al = 0.4325; Cu = 0.004	7.69	5.305	8	*
	Ac1:Al = 0.926; Cu = 0.008	5	4.912	6	*
AEV2a: Al = 0.1225; Cu = 0.005	Bc1: Al = 1.388; Cu = 0.0175	10.38	5.305	8	*
	Ac1: Al = 0.926; Cu = 0.008	6.54	5.124	7	*

Table 7.14: Summary of the results for survival of juvenile P. nigroculus in stream water for mixtures of Al + Cu (data from Table 7.7)

Concentration B (mg/L)	*Concentration A (mg/L)*	*q calc.*	*q Table*	*p*	** at 0.05*
Control: 0	Bc1: Al = 1.388; Cu = 0.0175	16.92	5.722	11	*
	Bb1: Al = 0.2775; Cu = 0.005	15.38	5.461	9	*
	Ac1: Al = 0.926; Cu = 0.008	14.23	5.305	8	*
	Ab1: Al = 0.185; Cu = 0.004	13.08	5.124	7	*
	Cb1: Al = 0.0925; Cu = 0.0175	11.54	4.654	5	*
Ca1: Al = 0.030; Cu = 0.0025	Bc1: Al = 1.388; Cu = 0.0175	15.38	5.599	10	*
	Ac1: Al = 0.926; Cu = 0.008	13.85	5.305	8	*
	Ab1: Al = 0.185; Cu = 0.004	12.69	5.124	7	*
	Cc1: Al = 0.4325; Cu = 0.004	11.54	4.912	6	*
	Ba1: Al = 0.0925; Cu = 0.0025	10	4.327	4	*
AEV2a: Al = 0.1225; Cu = 0.005	Bc1: Al = 1.388; Cu = 0.0175	14.23	5.461	9	*
	Ac1: Al = 0.926; Cu = 0.008	12.69	5.124	7	*
	Ab1: Al = 0.185; Cu = 0.004	11.54	4.912	6	*
	Cc1: Al = 0.4325; Cu = 0.004	10.38	4.654	5	*
	Ba1: Al = 0.0925; Cu = 0.0025	8.85	3.877	3	*

Table 7.15: Summary of the results for survival of juvenile P. nigroculus in stream water for mixtures of Al + Cu + Mn (data from Table 7.8)

Concentration B (mg/L)	*Concentration A (mg/L)*	*q calc.*	*q Table*	*p*	** at 0.05*
Control: 0	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	8.11	5.599	10	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	7.30	5.461	9	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	5.41	5.305	8	*
Ca2: Al = 0.030; Cu = 0.0025; Mn = 0.6075	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	7.03	5.4	9	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	6.22	5.305	8	*
AEV2b: Al = 0.1225; Cu = 0.005; Mn = 5.273	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	6.22	5.305	8	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	5.41	5.124	7	*
Cc2: Al = 0.4325; Cu = 0.004; Mn = 4.665	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	5.41	5.124	7	*

*Table 7.17: Summary of paired-analysis for growth of juveniles in stream water in mixtures of Al + Cu + Mn after 21-day exposures (data from Table 5 in the paper as Chapter 2 and Table 7.16); * = statistically significant at $p<0.05$*

Concentration B (mg/L)	*Concentration B (mg/L)*	*q calc.*	*q Table*	*p*	** at 0.05*
Ca2: Al = 0.030; Cu = 0.0025; Mn = 0.6075	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	43.306	5.722	11	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	43.152	5.599	10	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	34.146	5.461	9	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	28.030	5.305	8	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	24.916	5.124	7	*
	Aa2: Al = 0.030; Cu = 0.001; Mn = 1.217	16.023	4.912	6	*
	Cc2: Al = 0.4325; Cu = 0.004; Mn = 4.665	14.522	4.654	5	*
	Cb2: Al = 0.0925; Cu = 0.0175; Mn = 1.218	13.133	4.327	4	*
	AEV2b: Al = 0.1225; Cu = 0.005; Mn = 5.273	10.657	3.877	3	*
	Control: Al = 0; Cu = 0; Mn = 0	6.266	3.151	2	*
Control: 0	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	43.304	5.599	10	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	36.886	5.461	9	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	27.880	5.305	8	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	21.764	5.124	7	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 9.238	18.649	4.912	6	*
	Aa2: Al = 0.062; Cu = 0.001; Mn = 1.217	9.756	4.654	5	*
	Cc2: Al = 0.4325; Cu = 0.004; Mn = 4.665	8.255	4.327	4	*
	Cb2: Al = 0.0925; Cu = 0.0175; Mn = 1.218	6.866	3.877	3	*
	AEV2b: Al = 0.1225; Cu = 0.004; Mn = 4.665	4.415	3.151	2	*
AEV2b: Al = 0.1225; Cu = 0.005; Mn = 5.273	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	38.649	5.461	9	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	32.495	5.305	8	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	23.490	5.124	7	*

*Table 7.17(continued): Summary of paired-analysis for growth of juveniles in stream water in mixtures of Al + Cu + Mn after 21-day exposures (data from Table 5 in the paper as Chapter 2 and Table 7.16); * = statistically significant at $p<0.05$*

Concentration B (mg/L)	*Concentration B (mg/L)*	*q calc.*	*q Table*	*p*	** at 0.5*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	17.373	4.912	6	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	14.259	4.654	5	*
	Aa2: Al = 0.030; Cu = 0.001; Mn = 1.217	5.366	4.327	4	*
Cb2: Al = 0.0925; Cu = 0.005; Mn = 3.65	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	36.173	5.305	8	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	30.019	5.124	7	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	21.013	4.912	6	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.238	14.897	4.654	5	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	11.782	4.327	4	*
Cc2: Al = 0.4325; Cu = 0.004; Mn = 4.665	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	34.784	5.124	7	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	28.630	4.912	6	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	19.625	4.654	5	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	13.508	4.327	4	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	10.394	3.877	3	*
Aa2: Al = 0.030; Cu = 0.001; Mn = 1.217	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	33.283	4.912	6	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	27.129	4.654	5	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	18.124	4.327	4	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	12.008	3.877	3	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	8.893	3.151	2	*
Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	24.390	4.654	5	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	18.236	4.327	4	*

*Table 7.17(continued): Summary of paired-analysis for growth of juveniles in stream water in mixtures of Al + Cu + Mn after 21-day exposures (data from Table 5 in the paper as Chapter 2 and Table 7.16); * = statistically significant at $p < 0.05$*

Concentration B (mg/L)	*Concentration B (mg/L)*	*q. calc.*	*q. table*	*p*	** at 0.05*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	9.231	3.877	3	*
Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	21.276	4.327	4	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	15.122	3.877	3	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	6.116	3.151	2	*
Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	15.159	3.877	3	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	9.006	3.151	2	*
Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	6.154	3.151	2	*

*Table 7.18: Summary of the results of paired-analysis for growth of juvenile amphipods in 'artificial' water for mixtures of Al + Cu + Mn after 21-day exposures (data from Table 5 in paper as Chapter 2 and Table 7.16 in this chapter); * = variations observed in growth of P. nigroculus may be attributed to the combined effects of Al + Cu + Mn*

Concentration B (mg/L)	*Concentration A (mg/L)*	*q calc.*	*q Table*	*p*	** at 0.05*
Ca2: Al = 0.030; Cu = 0.0025; Mn = 0.6075	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	228.995	5.722	11	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	226.712	5.599	10	*
	Bb2: Al = 0.2775; Cu = 0.005	222.146	5.461	9	*
	Ab1: Al = 0.185; Cu = 0.004; Mn = 2.434	198.630	5.305	8	*
	Cc2: Al = 0.4325; Al = 0.004; Mn = 4.665	196.347	5.124	7	*
	AEV2b: Al = 0.1225; Cu = 0.005; Mn = 5.273	194.064	4.912	6	*
	Cb2: Al = 0.0925; Cu = 0.0175; Mn = 1.218	192.466	4.327	4	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	101.826	3.877	3	*
	Aa2: Al = 0.062; Cu = 0.001; Mn = 1.217	67.580	3.151	2	*
Control: Al = 0; Cu = 0; Mn = 0	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	161.416	5.599	10	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	159.132	5.461	9	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	154.566	5.305	8	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	131.050	5.124	7	*
	Cc2: Al = 0.4325; Cu = 0.004; Mn = 4.665	128.767	4.912	6	*
	AEV2a: Al = 0.1225; Cu = 0.005; Mn = 5.273	126.484	4.654	5	*
	Cb2: Al = 0.0925; Cu = 0.0175; Mn = 1.218	124.886	3.877	3	*
	Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	34.247	3.151	2	*
Aa2: Al = 0.062; Cu = 0.001; Mn = 2.434	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	127.169	5.461	9	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	124.886	5.305	8	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	120.320	5.124	7	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	96.804	4.912	6	*

*Table 7.18(continued): Summary of the results of paired-analysis for growth of juvenile amphipods in 'artificial' water for mixtures of Al + Cu + Mn after 21-day exposures (data from Table 5 in paper as Chapter 2 and Table 7.16 in this chapter); * = variations observed in growth of P. nigroculus may be attributed to the combined effects of Al + Cu + Mn*

Concentration B (in mg/L)	*Concentration B (mg/L)*	*q.calc.*	*q Table*	*p*	** at 0.05*
	Cc2: Al = 0.4325; Cu = 0.004; Mn = 4.665	94.521	4.654	5	*
	AEV2b: Al = 0.1225; Cu = 0.005; Mn = 5.273	92.237	4.327	4	*
	Cb2: Al = 0.0925; Cu = 0.0175; Mn = 1.218	90.639	3.151	2	*
Ba2: Al = 0.0925; Cu = 0.0025; Mn = 1.825	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	36.530	5.305	8	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	34.247	5.124	7	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	29.680	4.912	6	*
	Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	6.164	4.654	5	*
Cb2: Al = 0.0925; Cu = 0.0175; Mn = 1.218	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	34.932	4.912	6	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	32.648	4.654	5	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	28.082	4.327	4	*
Ab2: Al = 0.185; Cu = 0.004; Mn = 2.434	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	32.648	4.654	5	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	30.365	4.327	4	*
	Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	25.799	3.877	3	*
Bb2: Al = 0.2775; Cu = 0.005; Mn = 3.65	Bc2: Al = 1.388; Cu = 0.0175; Mn = 13.993	30.365	4.654	4	*
	Ac2: Al = 0.926; Cu = 0.008; Mn = 9.328	28.082	3.877	3	*

7.2 Statistical analyses of growth

7.2.1 Growth: analysis of variance

Data for growth as changes in body weight of juveniles after 21-day exposures to test solutions (Table 5 in paper as Chapter 2) were subjected to ANOVA. Where F values were significantly different $p<0.05$, the Newman-Keuls test was used to ascertain the effects of mixtures of Al + Cu and Al + Cu + Mn on changes in the body weight of amphipods and to determine which sets of results differed significantly from the controls (Clarke 1980, Zar 1984, Bonhivers & De Ketele 1986, Reish & Oshida 1986, Kooijman & Bedaux 1996).

Four cases derived from Table 2.8 were examined, as follows:

- Case 1: growth of juvenile *P. nigroculus* in stream water with mixtures of Al + Cu;
- Case 2: growth of juvenile *P. nigroculus* in stream water with mixtures of Al + Cu + Mn;
- Case 3: growth of juvenile *P. nigroculus* in 'artificial' water with mixtures of Al + Cu, and
- Case 4: Growth of juvenile *P. nigroculus* in 'artificial' water for mixtures of Al + Cu + Mn.

*Table 7.16: Summary of the analysis of variance for growth of P. nigroculus in mixtures of Al + Cu or Al + Cu + Mn in either water (H_o: null hypothesis accepted and variations in growth are attributed to chance; * = statistically significant: differences in growth at different concentrations of metal mixtures may be attributed to the effects of metals)*

Mixture	*Source of variation*	*Sums of squares*	*df*	*Means squares*	*F* at 0.05*
Al + Cu in stream water	Total	15926497	32	-	0.94 H_o
	group	4297278	10	497703	
	error	11629219	22	528601	
Al + Cu + Mn in stream water	total	5192824.2	32	-	76.17*
	group	5145955.2	10	162275.76	
	error	46869	22	2130.41	
Al + Cu in 'artif.' water	total	33086290.91	32	-	0.75 H_o
	group	2943821.9	10	1033946.59	
	error	301442469.01	22	1370112.23	
Al + Cu + Mn in 'artif.' water	total	3247854.55	32	-	1759.63*
	group	3246585.5	10	101495.45	
	error	1269.05	22	57.68	

Table 7.16 indicates that variations in growth of *P. nigroculus* in mixtures of Al + Cu + Mn in either stream or 'artificial' water may be attributed to the combined effects of Al + Cu + Mn, while observed variations in growth in mixtures of Al + Cu in either water may be attributed to chance (H_o).

7.2.2 Growth: paired-tests of comparison

As for data on survival of *P. nigroculus*, where $F \geq 0.05$ for the data sets described above, the Newman-Keuls test was performed to provide paired-analyses of comparison of variation in growth for individual concentrations with each other and with the control.

Table 7.17 indicates that for all treatments, growth of juvenile *P. nigroculus* in stream water with Al + Cu + Mn differed significantly from that in the control at the concentrations specified in same table.

Statistical analyses of growth of juvenile amphipods in 'artificial' water with mixtures of Al + Cu + Mn after 21 days of exposure are given in Table 7.18.

Table 7.18 indicates that rates of growth of *P. nigroculus* after 21 days significantly differed from the control in 'artificial' water mixtures of Al + Cu + Mn at concentrations specified in the table. This suggests that all these variations in growth may be attributed to the effects of Al + Cu + Mn. However the fact that significant growth was reported only from mixtures of Al + Cu + Mn in either water suggests that Mn may increase the growth rates of *P. nigroculus* to a certain extent by reducing the toxicity of solutions containing mixtures of Al + Cu.

7.3 Statistical analyses of data on reproduction

I wanted to know if mixtures of metals had affected reproductive rates in *P. nigroculus*. Because reproduction was taken as the number of amphipods present in the test containers after 45-day exposures, the Mann-Whitney U-test was used (Zar 1984). For this non-parametric test, ranks are used instead of actual measurements (Zar 1984, Reish & Oshida 1986, Bonhivers & De Ketele 1986). Data from Tables 3.1 and 3.2 were ranked from the lowest number to the highest number of amphipods recorded after 45-day exposures, giving rank 1 to the lowest values for numbers of amphipods in each set of data to be compared (regardless of the original treatment) and rank N to the highest value for numbers of individuals.

The formula to calculate the Mann-Whitney statistic (U) is then

$$U = n1n2 + [n1\ (n1 + 1)/2] - R1$$

where n1, n2 = number of different concentrations used for each mixture of metals (i.e. Al + Cu or Al + Cu + Mn); R1, R2 = sum of rank for each group of data; the lowest sum is selected as R for comparison. When the U-value is < R1, or when the calculated U-value is < U-value from the table at a given level for n1 = x1 and n2 = x2, the null hypothesis is accepted and variations in number of amphipods after 45-day exposures to metal mixtures may be attributed to chance; when the U-value > R1, the null hypothesis is rejected and variations may be attributed to the effects of metal mixtures on reproductive rates of *P. nigroculus*.

Using the data from Tables 3.1 and 3.2 on reproduction (change in numbers of individuals after 45 days of exposure), results are given in Table 7.19.

Table 7.19: Number of individuals of P. nigroculus in mixtures of Al + Cu and Al + Cu + Mn in either water after 45-day exposures starting with 10 amphipods per replicate (initial data from Tables 3.1 and 3.2; number of replicates = 3)

Concentrations of metals (mg/L)	*No. of amphipods in stream water*	*No. of amphipods in 'artificial' water*
Control: 0	257	193
Al 0.030 + Cu 0.0025	267	248
Al 0.062 + Cu 0.001	189	167
Al 0.0925 + Cu 0.0025	149	150
Al 0.0925 + Cu 0.0175	152	145
Al 0.1225 + Cu 0.005	145	143
Al 0.185 + Cu 0.004	131	119
Al 0.2775 + Cu 0.005	120	116
Al 0.4325 + Cu 0.004	122	118
Al 0.926 + Cu 0.008	127	113
Al 1.388 + Cu 0.0175	83	61
Al 0.030 + Cu 0.0025 + Mn 0.6075	260	251
Al 0.062 + Cu 0.001 + Mn 1.217	237	243
Al 0.0925 + Cu 0.0175 + Mn 1.218	250	247
Al 0.0925 + Cu 0.0025 + Mn 1.825	247	234
Al 0.185 + Cu 0.004 + Mn 2.434	269	259
Al 0.2775 + Cu 0.005 + Mn 3.65	239	244
Al 0.4325 + Cu 0.004 + Mn 4.665	240	231
Al 0.1227 + Cu 0.005 + Mn 5.273	241	237
Al 0.926 + Cu 0.008 + Mn 9.328	250	233
Al 1.388 + Cu 0.0175 + Mn 13.993	224	224

7.3.1 Discussion

From Table 7.19, the calculated U-value for mixtures of Al + Cu and Al + Cu + Mn in stream water = 91 and the U-value from the table at the level of significance $\alpha = 0.05$ is 78. There is thus a significant difference in reproductive rates of *P. nigroculus* in Al + Cu and Al + Cu + Mn mixtures in stream waters, reproductive rate being greater in mixtures of Al + Cu + Mn than in mixtures of Al + Cu.

From the same table, the calculated U-value for mixtures of Al + Cu and Al + Cu + Mn in 'artificial' waters is 92 and the U-value from the Table at the level $\alpha = 0.05$ is 78. There is thus a significant difference in reproductive rates of *P. nigroculus* in Al + Cu and Al + Cu + Mn mixtures in 'artificial' water, reproductive rate being greater in mixtures of Al + Cu + Mn than in mixtures of Al + Cu.

7.3.2 Conclusions

I conclude that, in the presence of Al + Cu, Mn increases the reproductive rate, in the sense of the number of individuals alive after 45-day exposures, in *P. nigroculus*. Since reproduction of mature *P. nigroculus* after 45-day exposure to Al + Cu or Al + Cu + Mn mixtures in either stream water or 'artificial' water is higher in the Al + Cu + Mn mixtures than in the Al + Cu mixtures, the combination of Al + Cu + Mn is less toxic than the combination of Al + Cu. No significant differences were found in number of offspring produced in stream water and 'artificial' water. This result confirms the previous results on survival, on growth as changes in body weight, on interactions between elements and on bioaccumulation. Mn, being an antagonist of the combination of Al and Cu, reduces the toxicity of these elements, and also their uptake (they become biologically unavailable)as shown in Chapter 4.

All these results indicate that *P. nigroculus* may be suitable for use as a test organism because it is sensitive to changes in water chemistry. More information on the reproductive biology of this amphipod is urgently needed, however.

CHAPTER 8

Regression analyses on survival, growth, bioaccumulation and reproduction

In this chapter, results of experiments on growth, reproduction and bioaccumulation are statistically analysed to determine possible interactions between Al, Cu and Mn, and the dilution water effects and finally to find simple fitted models. As stated before, this chapter gives an additional statistical support to my thesis.

8. 1 Risk analysis of survival times of *P. nigroculus* exposed to mixtures of Al + Cu or Al + Cu + Mn

The risk of death for a given individual amphipod exposed to mixtures of Al + Cu or Al + Cu + Mn is assumed to be proportional to the toxic effects of Al, Cu and Mn, which are concentration-dependent. This analysis provides more information on the interactions between toxicants in combination than does the standard analysis based on the death records. It allows one to predict the risk of death before exposed organisms die and predictions are useful in ecotoxicology more likely to allow rational assessment of the likely effects of pollutants.

Table 8.1: *Variables analysed and their combinations (metals = Al, Cu and Mn; life history stage of amphipod = mature, juvenile and moulting individuals; concentration in µg/L refers to Table 1.3; type of water refers to stream water and 'artificial' water; 'baseline' refers to juveniles in stream water without metal added)*

Variables	*Specification*	*Used as*
Metals	None	Baseline (reference) condition
	AlCu	Al + Cu
	AlCuMn	Al + Cu + Mn
Life history stage of amphipod	Juvenile	Baseline
	Adult	Mature amphipod
	Moult	Moulting amphipod
Concentration	µg/L	micrograms per litre
Type of water used	Stream	Baseline
	Water	'Artificial'

For the analysis of risk of death, I used the proportional hazards model (Cox 1987, Collett 1994). In this model, the immediate risk of death for an amphipod alive at time t is

$$h_{(t)} = l_{(t)}*\exp(b_1x_1 + b_2x_2 + \ldots b_px_p),$$

where $h_{(t)}$ represents the risk of death at time t; $l_{(t)}$ represents the baseline risk of death (i.e that for a juvenile in uncontaminated stream water); x_1, x_2,...x_p represent the effects of metals and b_1, b_2,...b_p are constants representing the proportions of metals (i.e. concentrations of Al, Cu and Mn in Table 1.3). The function $h_{(t)}$ is called the hazard function. Cumulative proportions of amphipods (mature, juvenile and moulting individuals) surviving after 21 days of exposure to mixtures of metals (Al + Cu and Al + Cu + Mn) in either dilution water (stream and 'artificial') are shown in Figures 8.1 - 8.6.

The risk of death at any time t, compared to the defined baseline (i.e. juvenile *P. nigroculus* in stream water without Al, Cu or Mn added), is called the relative risk and is adjusted for the presence of all the other variables in $h_{(t)}$. The relative risk for a unit change in variable x_i is given by

$$r = \exp(b_i).$$

From the hazard function, $h_{(t)}$, one can derive the survival curve, $S_{(t)}$, which shows at each time-point for all individuals alive at time t, the probabilty of surviving longer than t. Figures 8.1-8.6 show cumulative proportions of mature amphipods after 21-day exposure in stream-water mixtures of Al + Cu and Al + Cu + Mn (Figure 8.1) or in 'artificial' water mixtures of Al + Cu and Al + Cu + Mn (Figure 8.2) containing 1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn. Figure 8.3 gives cumulative proportion of juveniles surviving after 21-day exposure in stream water mixtures of Al + Cu and Al + Cu + Mn containing above amounts of Al,Cu and Mn; Figure 8.4 gives the same information for juveniles in 'artificial' water mixtures of Al + Cu and Al + Cu + Mn. Figures 8.5 and 8.6 report on moulting individuals.

FITTING THE PROPORTIONAL HAZARDS MODEL

Initially, the model was fitted using the variables in Table 8.1 above and also included two-way interactions between them as reported in Table 8.2, in which columns represent variables and their combinations, changes for variable, standard error, t-value, risk beta exponent and p level.

Table 8.2 *Results of the analysis of survival times of P. nigroculus using data from Tables 3-4 in the paper (all data and their combinations at concentration of 1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn) after 21 days of exposure. N = 3782; dependent variable: days; censoring variable: censor; AD: adult; MO: moult; 1 ppb: µg/L; AlCuMn = effect of mixture of Al + Cu + Mn; AlCu = effect of mixture of Al + Cu; MnAD = effect of Mn on mature amphipods; AlCuAD = effect of mixture of Al + Cu on mature amphipods; MnMO = effect of Mn on moults; AlCuMO = effect of mixture of Al + Cu on moults; Adult = mature amphipods; moult = moulting individuals; water = 'artificial' water)*

N = 3782	*variation*	*Standard error*	*t-value*	*exponent beta*	*p*
µg/L	0.000090	0.000030	3.01394	1.000090	0.002581
AlCuMn	0.564921	0.287263	1.96656	1.75931	0.04924
AlCu	1.10038	0.282893	3.88973	3.00529	0.0001
MnAD	0.064690	0.444934	0.14539	1.06683	0.88440
AlCuAD	0.183715	0.441162	0.41643	1.20167	0.6771
MnMO	-0.29931	0.318580	-0.93951	0.741331	0.347481
AlCuMO	-0.71761	0.316608	-2.2666	0.487917	0.02342
Adult	-0.39597	0.43363	-0.91315	0.67303	0.36117
Moult	1.92189	0.30913	-6.2171	6.83383	0.0000
Water	-0.10286	0.042003	2.4489	0.902251	0.014334

These analyses showed that there were no significant ($p < 0.884$ for mature amphipods in Al + Cu + Mn and $p < 0.677$ in Al + Cu) two-way interactions between the presence of metals and adult (mature amphipods) in all data at the highest concentrations for combinations Al + Cu or Al + Cu + Mn (i.e 1.388 mg/L Al + 0.0175 mg/L Cu for Al + Cu or 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn for Al + Cu + Mn), but there was significant interaction between Al + Cu and moulting status. This interaction (Al + Cu + Moult) was included in the model, and the non-significant interactions between the presence of metals and adult status were excluded. The results of this analysis are shown in table 8.3.

Table 8.3: Survival times and relative risk for mature, juvenile and moulting amphipods exposed to mixtures of Al + Cu or Al + Cu + Mn after 21 days (dependent variable: days; censoring variable: censor; Chi^2 = 1286.81; df = 7; n = 3782) (data from Tables 2.3-2.8)

	Variation	*Std. error*	*t-value*	*Relative risk*	*p*
1µg/L	0.00009	0.00003	3.01795	1.00009	0.0025
AlCuMn	0.37208	0.12397	3.00147	1.45075	0.0027
AlCu	0.97403	0.12819	7.5985	2.64859	0
AlCuM0	-0.4931	0.08491	-5.808	0.610709	0
ADult	-0.2625	0.06227	-4.2152	0.769144	0
MOult	1.67436	0.06978	23.994	5.33535	0
Water	-0.1027	0.042	-2.4462	0.90235	0.0144

In Table 8.3 the baseline conditions are those of juveniles in stream (natural) water without Al, Cu and Mn added after 21 days of exposure. All comparisons of risks are made relative to these. Relative risks together with their 95% confidence intervals, are given in Table 8.4. Note that:

- Compared to the baseline (i.e. juveniles in stream water), addition of Al and Cu at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu increases the risk of death 2.6 times.
- Compared to the baseline (i.e juveniles in stream water), the addition of Al, Cu and Mn at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn increases the risk of death 1.5 times. Thus the presence of manganese reduces the risk of death somewhat.
- The risk of death for an adult is 0.8 or 80% of the risk for a juvenile.
- The risk of death for a moulting creature is 5.3 times that of a juvenile giving an over all increase in risk in the presence of these metals of 9.8 times.
- When Al and Cu are present the risk for a moulting amphipod increases a further 1.8%.
- The risk of death in artifical water in only 90% of the risk in natural water.
- If the concentration of Al,Cu or Mn increases by 1 µg/L) the risk of death for any amphipod (juvenile, mature or moulting individual) increases by a minute fraction (1.000086).

Table 8.4: Relative risk of death at 95% confidence intervals for concentrations of 1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn in mixtures of Al + Cu or Al + Cu + Mn. AlCuMn = interactions in mixture of Al + Cu + Mn; AlCu = interactions in mixture of Al + Cu; AlCuMo = interactions between Al, Cu and moulting amphipods; Adult = mature amphipods; Moult = moulting amphipods; Water = 'artificial' water (data from Tables 2.3-2.8)

Variable	*Variation*	*Std. error*	*Relative risk*	*Lower limit*	*Upper limit*
1 µg/L	0.000090	0.000030	1.000090	1.000031	1.000149
AlCuMn	0.37	0.12	1.45	1.14	1.85
AlCu	0.97	0.13	2.65	2.06	3.41
AlCuMo	-0.494	0.085	0.61	0.52	0.72
Adult	-0.262	0.062	0.77	0.68	0.87
Moult	1.674	0.070	5.34	4.65	6.12
Water	-0.103	0.042	0.90	0.83	0.98

Plots of the survival function for various combinations of the risk factors are shown in Figures 8.1-8.6.

Figure 8.1 shows that the percentage survival of mature amphipods in stream water at a combined concentration of 1.388 mg/L Al and 0.0175 mg/L Cu was 62% after 21-day exposures, and that the percentage of survival was 70% after 21-day exposures when 13.993 mg/L Mn were added to the mixture of Al + Cu. This means that in both cases, median survival times (LT_{50}) were greater than 21 days. In terms of toxicity, no combination was acutely toxic to mature *P. nigroculus*.

Figure 8.2 shows that the percentage survival of mature amphipods in 'artificial' water with mixtures of Al + Cu at concentration of 1.388 mg/L Al + 0.0175 mg/L Cu was 60% and that the percentage of survival is 71% when 13.993 mg/L Mn are added.

This indicates that the risk of death for amphipod in either water decreases with the addition of Mn and thus that combination of Al + Cu is more toxic than combination of Al + Cu + Mn.

Figure 8.3 shows that 53% of juveniles survived after 21-day exposures to mixtures of Al + Cu in stream water at concentration of 1.388 mg/L Al + 0.0175 mg/L Cu and that 60 % survived when 13.993 mg/L Mn were added.

Figure 8.4 shows that 55% of juveniles survived after 21-day exposures to mixtures of Al + Cu in 'artificial' water at concentration of 1.388 mg/L Al + 0.0175 mg/L Cu and that the percentage of survival was 60% when 13.993 mg/L Mn were added to above mixture of Al + Cu.

For all data shown in Figures 8.1-8.4 LT_{50} values were greater than 21 days, and therefore combinations of metals (Al + Cu or Al + Cu + Mn) may be assumed not acutely toxic to *P. nigroculus* at the highest concentrations tested, which represent at least 139 times (for Al) or 10 times (for Cu and Mn) the actual South African interim guidelines for aquatic ecosystems.

Figures 8.5 and 8.6 show that fewer than 10% of moulting individuals of *P. nigroculus* survived after 21-day exposures to mixtures of Al + Cu or Al + Cu + Mn in either stream or 'artificial' waters using the same concentrations as for figures 8.1-8.4. This result is valueless because of the high mortality (> 73%) recorded in the controls, which suggests that moulting individuals are susceptible to culture conditions as well as to the toxic effects of the metals. Anyway, results on moults are disregarded as stated in the paper (presented as Chapter 2 of this thesis).

The combinations of Al + Cu and Al + Cu + Mn at the highest concentrations tested (1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn) are the main cause of death of *P. nigroculus*. From toxicological point of view, they may be considered as not acutely toxic since LT_{50} > 21 days, and the increase in relative risk reported in Table 8.4 may be neglected.

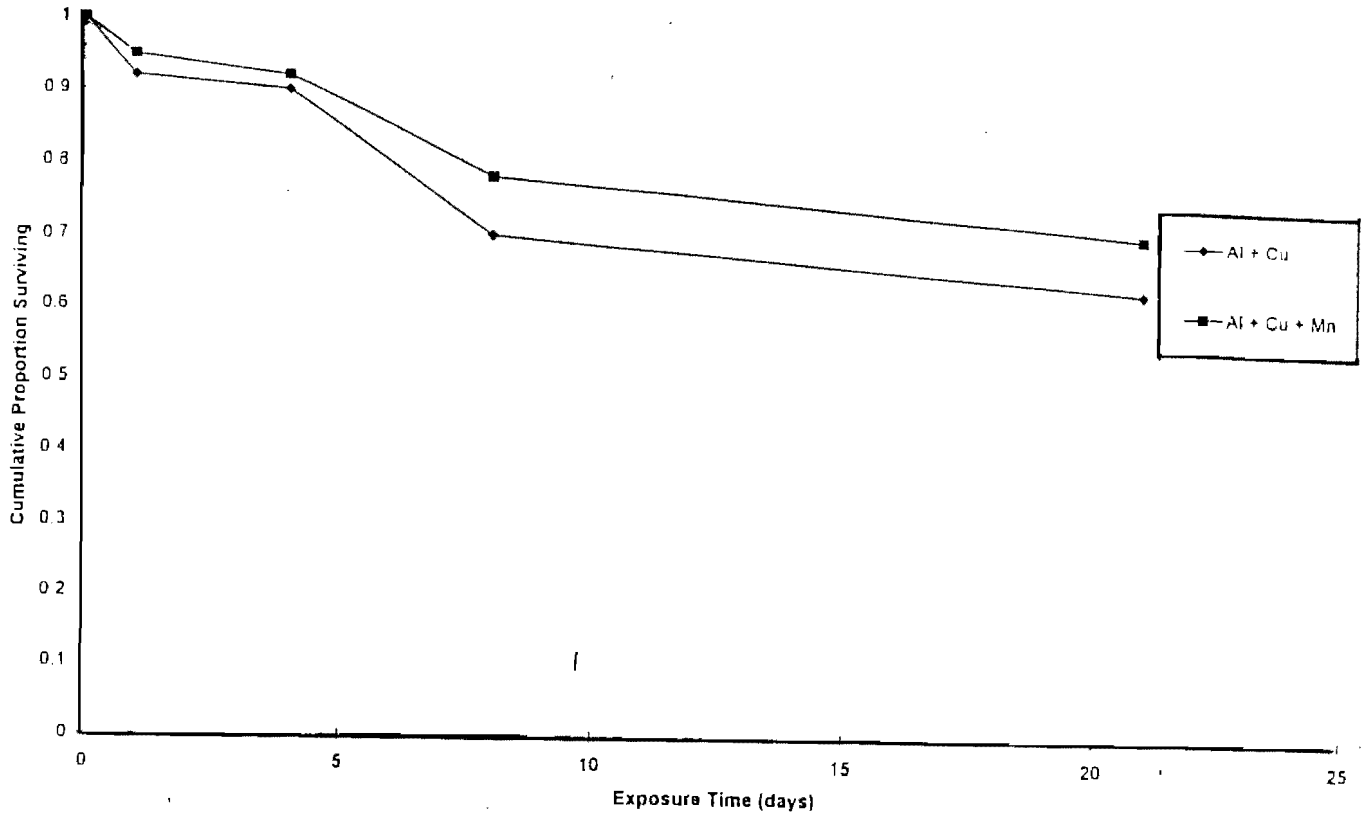


Figure 8.1 Cumulative proportion of mature *P. nigroculus* surviving after 21 days of exposure in stream water mixtures of Al+Cu and Al+Cu+Mn at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu and of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn respectively

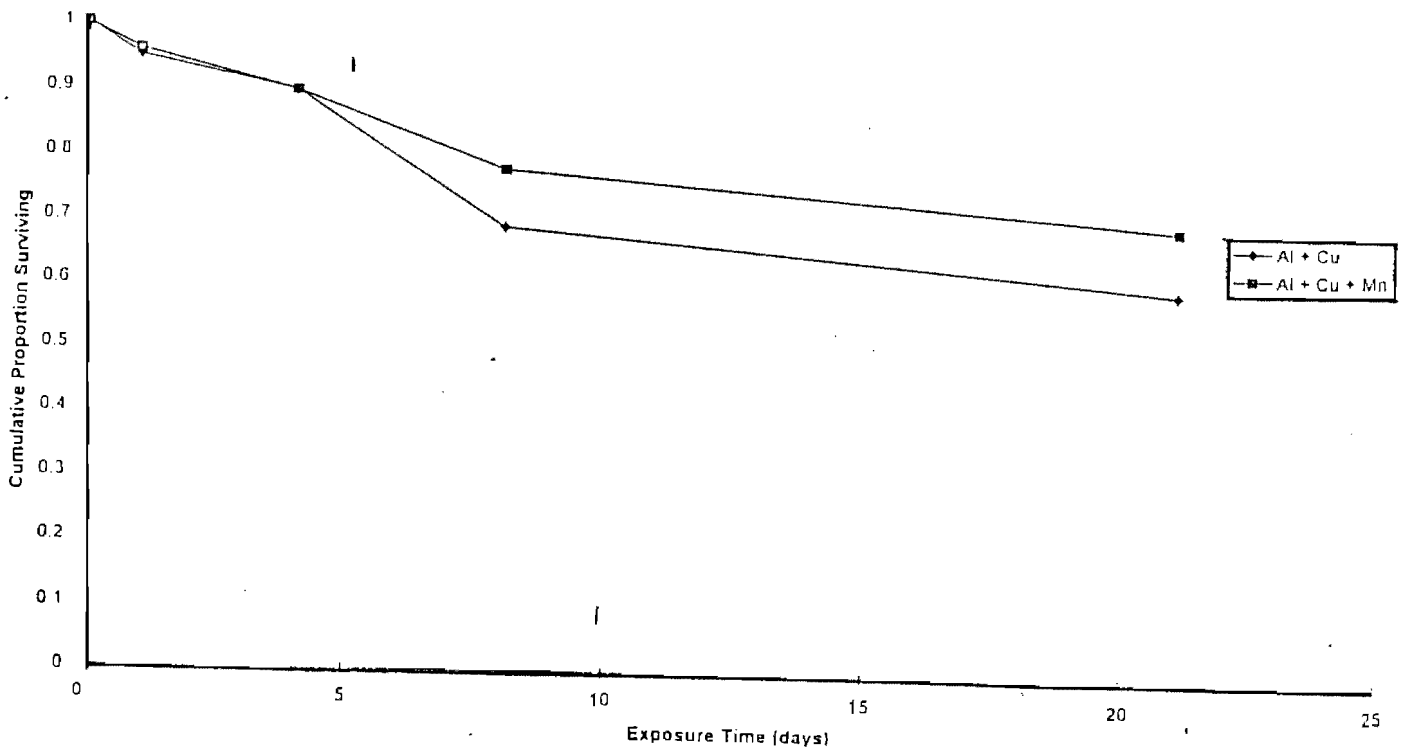


Figure 8.2 Cumulative proportion of mature *P. nigroculus* surviving after 21 days of exposure in 'artificial' water mixtures of Al+Cu and Al+Cu+Mn at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu and of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn, respectively.

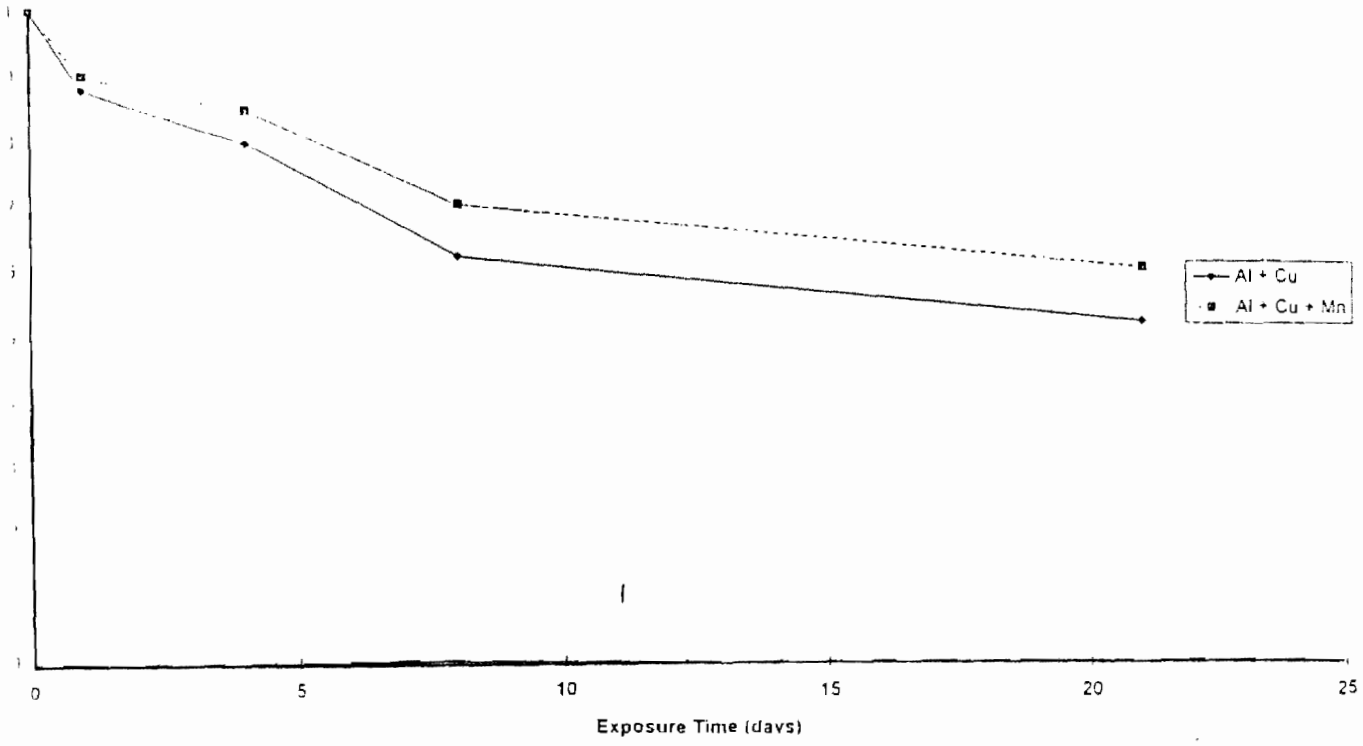


Figure 8.3 Cumulative proportion of juvenile *P. nigroculus* surviving after 21 days of exposure in stream water mixtures of Al+Cu and Al+Cu+Mn at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu and of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn

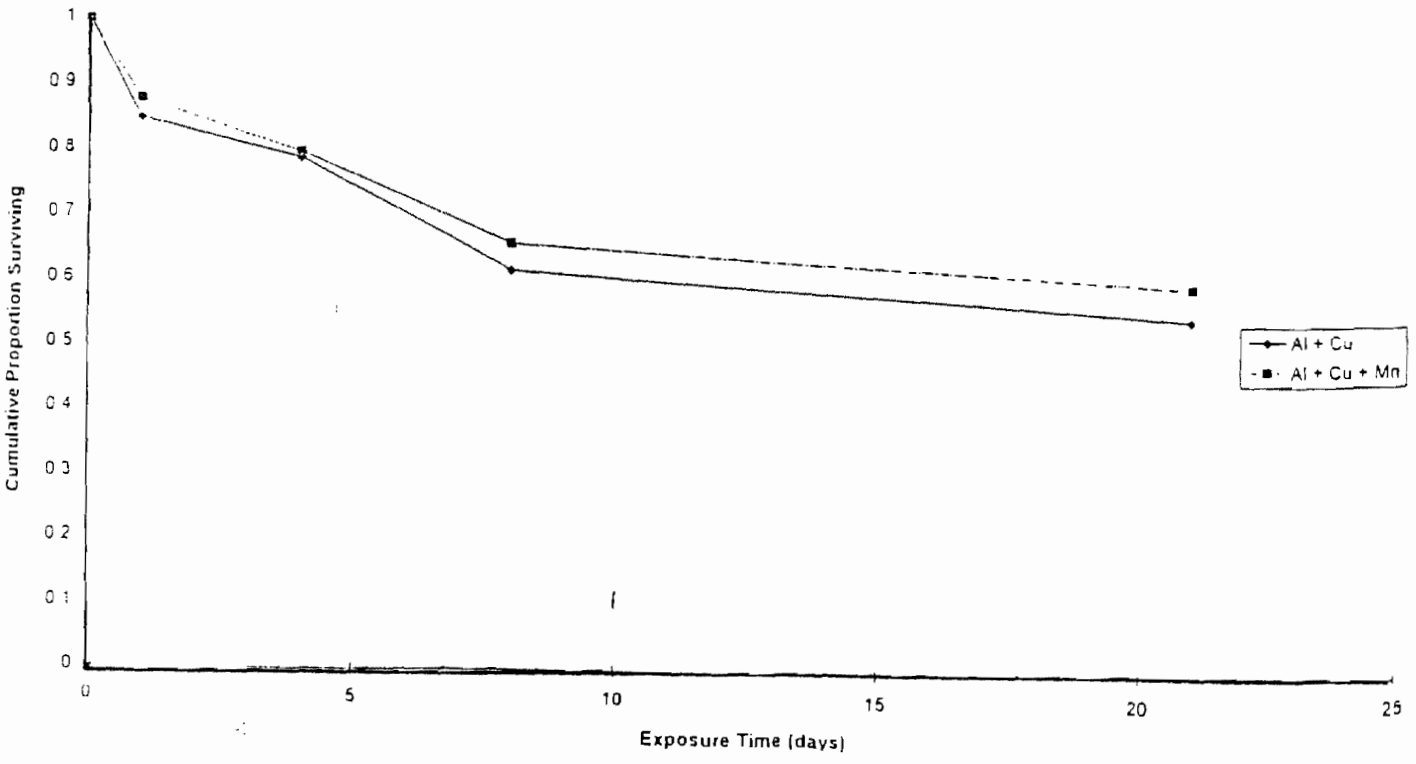


Figure 8.4 Cumulative proportion of juvenile *P. nigroculus* surviving after 21 days of exposure in 'artificial' water mixtures of Al+Cu and Al+Cu+Mn at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu and of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn, respectively.

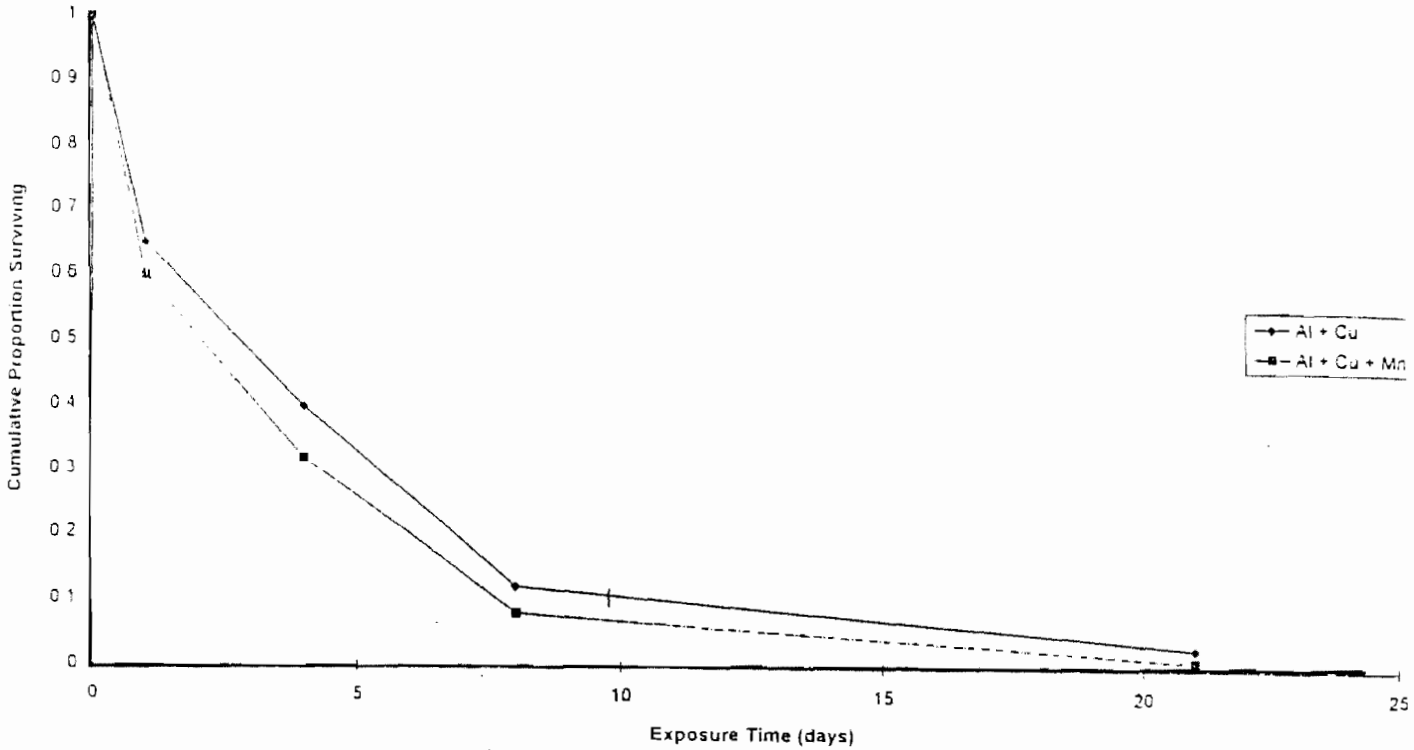


Figure 8.5 Cumulative proportion of moulting *P. nigroculus* surviving after 21 days of exposure in stream water mixtures of Al+Cu and Al+Cu+Mn at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu and of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn.

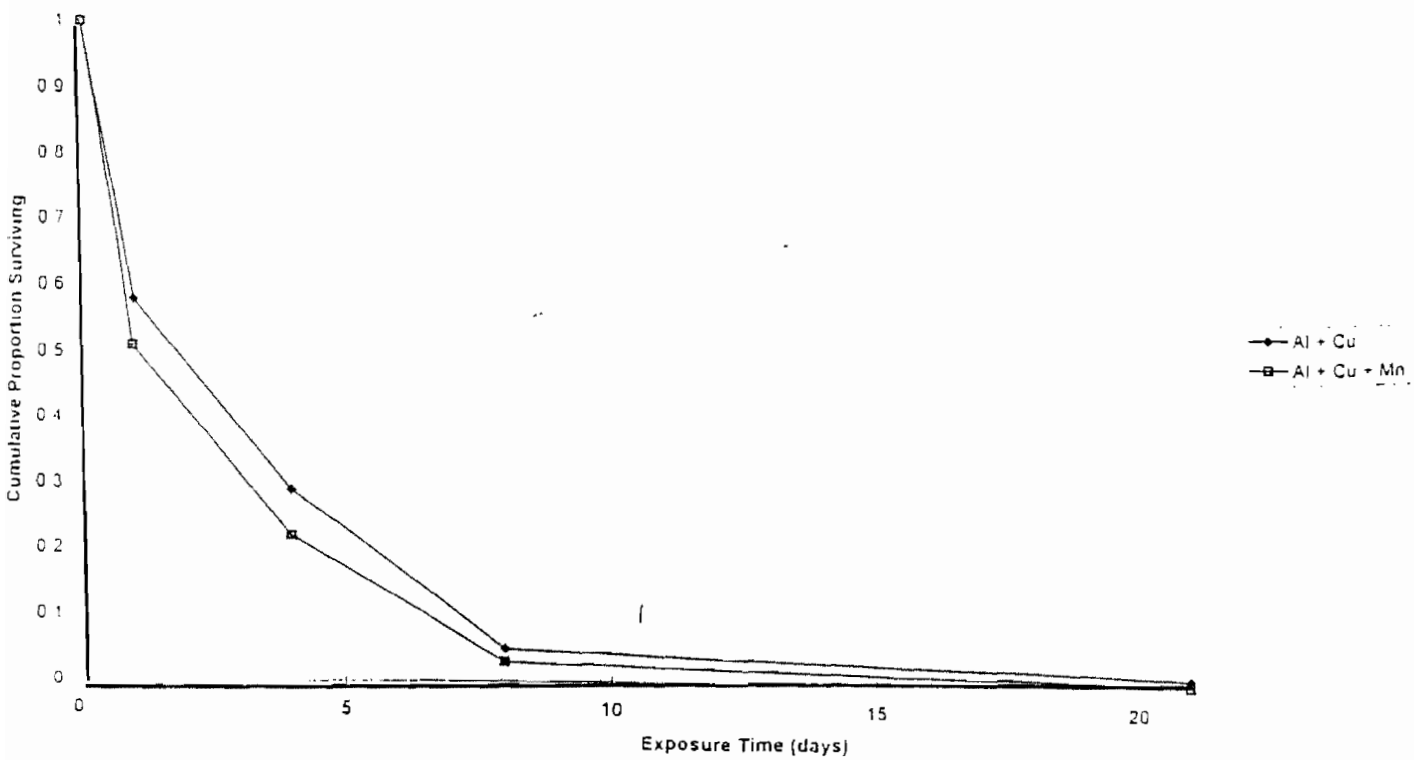
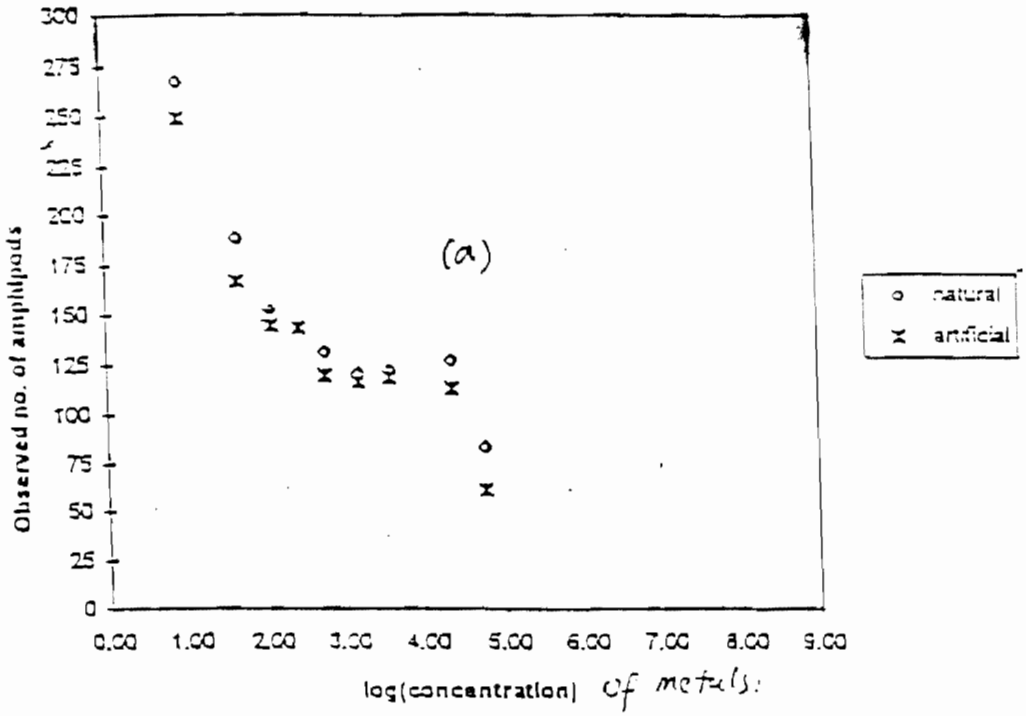


Figure 8.6 Cumulative proportion of moulting *P. nigroculus* surviving after 21 days of exposure in 'artificial' water mixtures of Al+Cu and Al+Cu+Mn at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu and of 1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn, respectively.



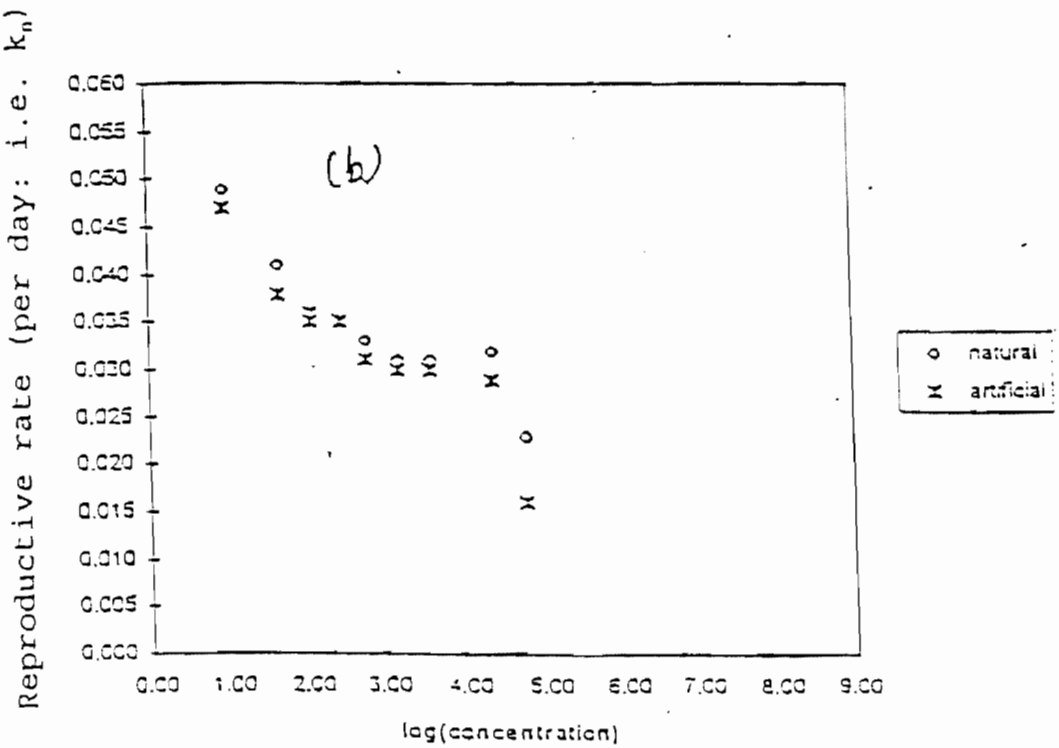


Figure 8.7 Observed numbers of amphipods (a) and observed reproductive rates (b) per day vs log-concentration of Al+Cu+Mn in natural (stream) and 'artificial' water after 45 days of exposure

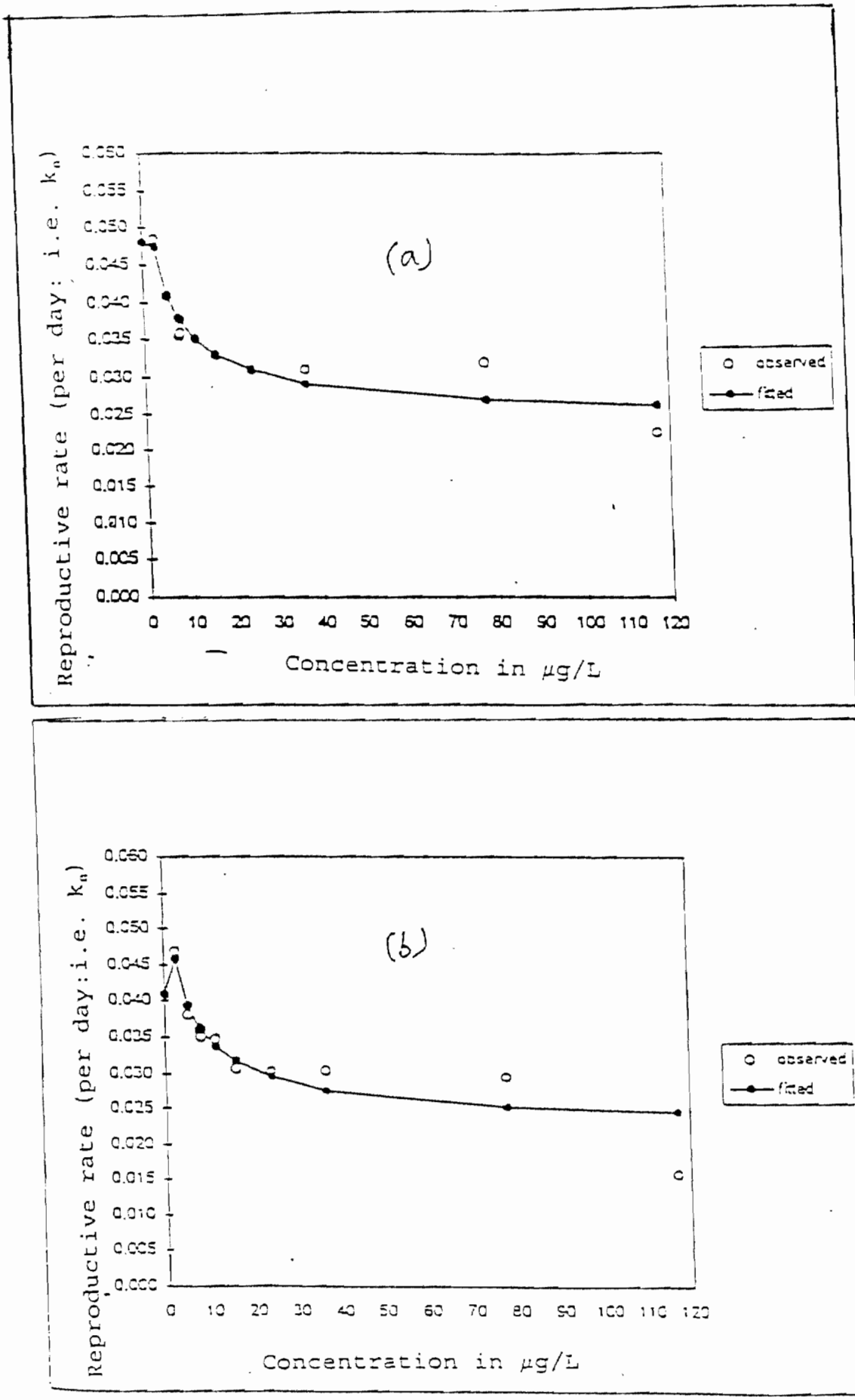


Figure 8.8 Observed and fitted reproductive rates per day vs concentration of mixtures of Al+Cu in stream (a) or 'artificial' (b) water after 45-day exposure

8.2 Regression analysis applied to growth data

After the analyses of variance and the paired-tests of comparison described in Chapter 7, regression analyses on the growth of *P. nigroculus* were performed using the Genstat package and Statgraphics. The aim was to investigate interactions between concentrations of different mixtures of metals, and exposure times, on the growth rates of the amphipods.

Table 5 in the paper (as Chapter 2) gives the average growth rates as changes in dry body mass after 21 days of exposure to mixtures of Al, Cu and Mn. Values for both growth rate and concentrations of test solutions (Table 1.3) were transformed by $\log_e$ (USEPA 1991, Collett 1994). These transformed data were used throught out the analyses and back-transformed at the end by means of Statgraphics (Statistical Graphics Corporation, Maryland, USA, 1985) and Genstat (DEC OpenVMS, Lawes Agricultural Trust at Rothamsted Experimental Station, 1994).

Assuming that log-growth was approximately normally distributed, I used multiple linear regression to assess the effects on growth of concentration of Al + Cu, with or without Mn and with each water-type (stream or 'artificial' water). Interactions (i.e. combined toxicity) between these variables were also investigated. Variables were fitted step by step and the effect of each term on growth rates was tested, adjusting for all the variables at 95% level of significance. The toxic effects of the mixtures of Al + Cu and Al + Cu + Mn on growth were analysed. The results of this analysis are shown in Table 8.5.

*Table 8.5: Accumulated analysis of variance for regression analysis of growth (df = degrees of freedom; s.s. = sum square; m.s. = mean squared; v.r. = variation; * = significant); +lconc = log(concentration) of Al + Cu without Mn; +Mn = Mn added; +water = 'artificial' water; +lMn = log(concentration) of Al + Cu + Mn; +Mnwater = effect of mixtures of Al + Cu + Mn in 'artificial' water) (data from Tables 2.3-2.8)*

Variation	*df*	*s.s.*	*m.s.*	*v.r.*
+lconc	1	0.046471	0.046471	5.45*
+Mn	1	2.734905	2.734905	320.65*
+Water	1	5.637345	5.637345	660.94*
+lMn	1	0.034859	0.034859	4.09*
+lwater	1	0.001918	0.001918	0.22
+MnWater	1	0.201472	0.201472	23.62*
Residual	113	0.963803	0.008529	
Total	119	9.620773	0.080847	

From Table 8.5, it is clear that all the variables except the interaction between log-concentration of Mn and type of water (i.e. 'artificial' water and stream water) significantly affected growth at the 5% level. This means that the growth of the organisms was affected by water type (bigger in stream water without Mn added than in stream water with Mn added), by the presence of Mn (better in mixtures of Al + Cu + Mn than in mixtures of Al + Cu) and by the concentration of Al, Cu and Mn. So the effect of Mn on the growth of *P. nigroculus* depended on the water type ('artificial' or stream water with or without Mn added) and also on the concentration of Al, Cu and Mn.

The multiple regression model using all the variables above explained 89.5% of the differences in growth between treatments. However, because of the interactions and because the residual analysis showed that the residual tended to be larger that the observed values for high concentrations and smaller than the observed values for low concentrations, the decision was taken to fit separate models for each water type (stream or 'artificial' waters) and chemical combination (Al + Cu or Al + Cu + Mn) at concentrations of 1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn.

The equation linking log-growth to log-concentration for each combination of manganese and water type has the form

$$\log(y) = a+b*\log(x).$$

In terms of growth and concentration, the equation has the form

$$y = cx^b$$

where y = growth; x = concentration; c = log(a); a,b are constants.

All separate models are shown in Table 8.6

Table 8.6: Summary of regression analyses for separate models fitted for growth of P. nigroculus after 21 days of exposure to mixtures of Al + Cu or Al+ Cu + Mn in stream or 'artificial' water (with df = 1 for regression; df = 28 for residual; total df = 29 and change = -1) (data from Table 2.9)

Model	*Specification*	*Sum square*	*Means squared*	*Variation and (%) explained*
Stream water, no Mn	regression	1.4497	1.449667	145.70 (83.3%)
	residual	0.2786	0.009950	
	total	1.7283	0.059595	
	change	-1.4497	1.449667	145.70
Stream water with Mn	regression	0.2068	0.206833	26.53 (47%)
	residual	0.2183	0.007795	
	total	0.4251	0.014659	
	change	-0.2068	0.206833	26.53
'Artificial' water, with Mn	regression	0.3095	0.309453	52.35 (64%)
	residual	0.1655	0.005912	
	total	0.4750	0.016379	
	change	-0.3095	0.309453	52.35
'Artificial' water, no Mn	regression	0.1899	0.189901	37.84 (56%)
	residual	0.1405	0.005018	
	total	0.3304	0.011393	
	change	-0.1899	0.189901	37.84

According to the results in Table 8.6, the best-fitting model was obtained with stream water with Al + Cu but without Mn added (which explained 83.3% of difference in growth), followed by the model of 'artificial' water with Al + Cu + Mn (which explained 64% of difference), followed by the model of 'artificial' water with Al + Cu but without Mn (which explained 56% of variation). The least useful model was obtained with stream

water with Mn added (which explained 47%). This last model did not explain thedifference in growth very well. Other factors must explain the difference in growth instead of Mn.

Different equations of the relationship between log-concentration and log-growth or in terms of growth and concentration are summarised in Table 8.7 (as in Table 6 of the paper: Chapter 2).

Table 8.7: Terms in the regression equations for growth as change in body weight of P. nigroculus (data from Table 2.9)

	$log\ Y = a + b\ logx$		$Y = cx^b$	*% of difference in growth explained*
	a	*b*	*c*	
Stream water (Al + Cu, no Mn)	6.1510	-0.194	468.7	83.3
Stream water (Al + Cu + Mn)	6.421	-0.0888	614.6	47
'Artificial' water (Al + Cu, no Mn)	6.0721	0.1086	433.6	64
'Artificial' water (Al + Cu + Mn)	5.4182	-0.0702	225.5	56

Table 8.7 shows that in stream water with Al + Cu but without Mn, the model explained the difference in growth of amphipods very well (83.3%), while the addition of Mn explained the difference in growth poorly (47%). This suggests that added Mn is not the only factor that explains the diffrence in growth of *P. nigroculus*. However, the fact that in natural conditions stream water contained 323 μg/L Mn (Table 2 in the paper) may explain why additional Mn reduced growth. High mortality was also recorded with juveniles in stream water containing additional Mn after 24 hours of exposure (Table 4 in the paper). So additional Mn at concentrations of 13.993 mg/L (to stream water containing high amount of Mn naturally) might be toxic to amphipods. The main difficulty is to know the real biological needs with regards to Mn for *P. nigroculus*, and at which extent an excess of Mn becomes toxic. In 'artificial' water with Mn added, the model fitted well and explained 64% of variation. This showed the important role that this metal may play in the growth of *P. nigroculus*.

8.3 Regression analyses: bioaccumulation data

Regression analysis was used to assess the joint effects of the type of metal, the concentrations of the metals (Table 5.3), and exposure times (Table 5.1) on the bioaccumulation process. The log of the BCF (bioconcentration factor) was regressed on log-concentration of metal and on exposure time. The effects of Al + Cu on log(BCF) were compared initially with the additional effects of Mn (Al + Cu + Mn). The possibility of interaction (i.e. synergism, antagonism) between the combined effects was also investigated. The results of this initial analysis are shown in Table 8.8.

Table 8.8: *Regression analysis of bioaccumulation of Al, Cu and Mn in P. nigroculus. (df = degrees of freedom; s.s. = sum square; m.s = mean squared; v.r = variation; F pr = probability for variation as F value; +time = effect of exposure time on bioaccumulation; +lconc = effect of log-concentration of Al + Cu; +metal = effect of Al + Cu + Mn; +lconc.metal = combined effect of log-concentration of Al + Cu + Mn; +time.metal = combined effect of exposure time and Al + Cu + Mn; +timesq = effect of time squared) (data from Tables 5.3-5.8)*

Change	*df*	*s.s.*	*m.s.*	*v.r.*	*F pr.*
+lconc	1	0.7261	0.7261	2.83	0.099
+time	1	83.4042	83.4042	325.54	<.001
+metal	2	3.4568	1.7284	6.75	0.003
+lconc.metal	2	1.0448	0.5224	2.04	0.141
+time.metal	2	0.1291	0.0645	0.25	0.778
+timesq	1	25.6999	25.6999	100.31	<.001
Residual	50	12.8100	0.2562		
regression	3	112.50	37.5014	142.22	
change	-1	-3.40	3.4002	12.89	
Total	59	127.2710	2.1571		

Table 8.8 shows that the rate of uptake of Al and Cu at different concentrations of Mn did not correlate with concentrations of Mn (F = 0.141). This may be because values used in this study are low (criteria levels). Because Mn is regulated (see Chapter 5) its concentration in the external medium may not play an important role in bioaccumulation of Al and Cu. Indeed, in mixtures, an given element can reduce or increase the uptake of another element, as explained in Chapter 4.

Bioaccumulation of Al, Cu and Mn depended upon the length of exposure time and the metal concerned. This is also understandable because at low concentrations, and using a static-renewal system (test containers not cleaned), exposure time must play a key role in the bioaccumulation during chronic toxicity tests because of the possible increase of residual metal concentration bioavailable.

There is a quadratic effect of time on log(BCF) of Mn. A regression model was fitted with the variable time and Mn effect (Al + Cu+ Mn) on BCF of Al and Cu. Regression coefficients shown in Table 8.9 indicate that the rate of bioaccumulation of Al and Cu differs from that of Mn. BCFs of Al and Cu tend to increase with time and are not significantly different from each other, while Mn tends to lower the rate of bioaccumulation of Al

and Cu and therefore reduces their toxic effects. This means that BCF values for Al and Cu are higher when Mn is absent than when it is present in water. This may explain why mortality of *P. nigroculus* is reduced (Tables 3 - 4 in the paper) and growth and reproduction are increased (Tables 5 in the paper, and in Tables 3.1 and 3.2) when Mn is added to test solutions containing combinations of Al + Cu.

Table 8.9: Regression coefficients of BCFs for Al, Cu and Mn vs exposure time (time = exposure time, timesq = time squared, estimate = value of regression cefficients, s.e. = standard error, t(22) = t value for 21-day exposure) (data from Table 5.3)

	Estimate	*s.e.*	*t(22)*
Constant	4.954	0.182	27.18
Time	0.5630	0.0426	13.22
Timesq	-0.01762	0.00180	-9.80
Al	0.543	0.164	3.32
Mn	-0.505	0.141	-3.59
Cu	0.467	0.164	2.86

Al showed a larger estimate (0.543) than Cu (0.467), which means that more Al is taken up and accumulated than Cu. Negative value of the estimate for Mn (i.e. -0.505) means that Mn decreases rate of bioaccumulation when presented together with Al + Cu.

Discussion

The model for regression analysis applied to bioaccumulation of Al, Cu and Mn in *P. nigroculus* explained 88% of the difference in log(BCF) and the effect of the Mn was to lower the BCF of Al and Cu. In the other words, Mn reduces the uptake and accumulation of Al and Cu in *P. nigroculus*, and therefore their toxicity, as stated before. The effect of exposure time on BCF was the same in water with and without Mn.

The model equations are:

$$\log(BCF) = 5.459 + (0.5630)t - 0.1762\ t^2$$

for solutions containing Al + Cu, and

$$\log(BCF) = 4.954 + (0.5630)t - 0.1752\ t^2$$

for solutions containing Mn as well as Al and Cu.

8.4 Analysis of data on reproduction in P. nigroculus after 45-day exposures

8.4.1 Statistical theory

For each concentration of mixtures of metals (Al + Cu or Al+ Cu + Mn) and for each water type (natural or artificial), the data (Tables 3.1 - 3.2) were the number of amphipods found after forty-five days, denoted by n_{45}, starting from an initial population of 30 individuals. I wanted to know if variations in reproductive rates of *P. nigroculus* were affected by mixtures of Al + Cu and Al + Cu + Mn. As 45 is a count of amphipods, n_{45} has a Poisson distribution. This takes care of the natural variability found with count data where it is known that the variance increases as the mean count increases (Zar 1984, Reish & Oshida 1986, Collett 1994, Kooijman 1996). Let u_{45} be the true mean count after 45 days exposure. I assumed that $\log(u_{45})$ depended on water-type (natural or artificial); log(concentration) of Al + Cu; and whether or not Mn was present. I investigated the possibility of two-way interactions between these variables. Having found a suitable simple model, the maximum likely estimate of mean count at 45 days could be found and the reproductive rate, k_n, calculated using the formula given by Hyne *et al.* (1993), who assume that growth rate is exponential and that at time t, the mean count is

$$u(t) = n_o \exp(k_{nt}).$$

From this, it follows that the reproductive rate at 45 days is given by

$$k_{nt} = \{\log(u_{45})-\log(n_o)\}/45.$$

This means that the mean reproductive rate per day for amphipods after 45 days of exposure (k_{nt}) is obtained from the difference of log mean count after 45 days (u_{45}) and log mean count at the beginning of the experiment (n_o), divided by 45 days. In this application, the reproductive rate will depend upon the variables that significantly affect the count at 45 days. As I have estimated the mean count at 45 days by maximum likelihood, the values of k_n are also maximum likely estimates of the reproductive rates. Further, approximate standard errors can be found, and 95% confidence intervals for them. The method also allowed me to isolate outlying values that were poorly fitted by the model. If the variation of the count was greater than that of a Poisson distribution, the modelling procedure could be adapted to allow for this.

The Genstat VMS package was used for all calculations and data were taken from Tables 3.1 and 3.2.

The model employed for the counts of amphipods is somewhat mechanistic in the sense that it is based upon the assumption that the count at any time is governed by a Poisson distribution (J.Juritz: pers.com.,1997) and

that the growth rate of this mean count is exponential. The concept of a generalised line model allows one to make statistically valid comparisons of the growth rate under number of conditions, which was the aim of this investigation. Moreover, this method allows to decide if these assumptions were reasonable. The model used for the growth as change in body mass was not mechanistic. The aim of this thesis, as stated in section 1.5 (Chapter 1), was not to find a mechanistic model for growth, but rather to compare the growth of the amphipods under different experimental conditions. The multiple regression models used allow valid statistical comparisons to be made of the growth of the amphipods both under different chemical regimes and at different concentrations.Importantly, the method allows us to test for the presence of interactions between chemical and water types, showing for instance that the effect of Mn depends upon the water type and upon its concentration. This is a simple model rather than a naive one (J. Juritz :pers. com., D. Musibono: pers.obs., 1997).

8.4.2 Poisson Regression

The accumulated analysis of deviance is shown in Table 8.10. Each line of this table shows the effect of that variable on the count at 45 days adjusted for all the variables above it in the table.

Table 8.10 *Accumulated analysis of deviance of reproductive rates of P. nigroculus after 45-day exposure (+lconc = effect of log-concentration of Al + Cu; +Mn = effect of Mn + Al + Cu; +water = effect of stream or 'artificial' water; +lconc.Mn = effect of log-concentration of Al + Cu + Mn; +lconc.water = combined effect of log-concentration of Al + Cu and water-type; +Mn.water = combined effect of Al + Cu + Mn and water-type; df = degrees of freedom) (data from Tables 3.1, 3.2)*

Change	*df*	*Deviance*	*Mean deviance*	*Deviance ratio*	*p*
+lconc	1	215.974	215.974	215.97	< 0.001
+Mn	1	444.321	444.321	444.32	< 0.001
+water	1	3.693	3.693	3.69	< 0.063
+lconc.Mn	1	106.077	106.077	106.08	< 0.001
+lconc.wate r	1	0.331	0.331	0.33	0.569
+Mn.water	1	1.181	1.181	1.18	0.285
Residual	33	51.353	1.556		
Total	39	822.929	21.101		

From Table 8.10, I concluded that the number of individuals present at day 45 varied depending on the presence or absence of Mn and that the effect of the concentrations of Al and Cu on the counts at day 45 depended on

whether or not Mn was present but not on the concentration of Mn. There is weak but statistically insignificant ($p > 0.05 < 0.063$) evidence for interaction with water type, but more for interactions between water type and log concentration or between water type and the presence of Mn. I then fitted separate models to the data with Mn and to the data without Mn and I tested for the effect of water type in both cases.

These models are illustrated in Figures 8.7-8.9 in which X-axis represents either log(concentration) or concentration (in μg/L) of Al, Cu and Mn, and Y-axis represents either observed number of amphipods or reproductive rates. Two cases are discussed below.

Case 1:

Poisson distribution different concentrations of Al + Cu but no Mn

As the plot of the observed numbers of amphipods present after 45 days (i.e. n_{45}) against log(concentration) in natural and artificial water showed a distinct curve, I added a term, lsq, the log concentration squared. The effect of artificial water was significant ($p < 0.067$). Thus the evidence for a difference in the effects of water types was not strong.

The results of the accumulated analysis of deviance for the effects of Al + Cu and water type on reproductive rates in *P. nigroculus* after 45 days of exposure are shown in Table 8.11, which indicates that there is a significant ($p < 0.001$) effect of log-concentration of Al + Cu on reproductive rates of *P. nigroculus*.

Table 8.11: *Accumulated analysis of deviance for the effects of Al + Cu in stream or 'artificial' water on reproductive rates in P. nigroculus (+lconc = effect of log-concentration of Al + Cu; +water = effect of stream or 'artificial' water; +lsq = effect of log-squared; df = degrees of freedom) (data from Tables 3.1, 3.2)*

Variation	*df*	*Deviance*	*Mean deviance*	*Deviance ratio*	*p*
+lconc	1	237.881	237.881	237.88	<0.001
+water	1	3.849	3.849	3.85	0.067
+lsq	1	14.248	14.248	14.25	0.002
Residual	16	31.954	1.997		
Total	19	287.933	15.154		

The estimates of the regression coefficients are given in Table 8.12.

Table 8.12: Estimates of regression coefficients for reproductive rates of P. nigroculus in 'artificial' water mixtures of Al + Cu (data from Tables 3.1, 3.2)

Parameter	*Estimate*	*s.e.*	*t(*)*	*tpr.*
Constant	6.033	0.101	59.48	<0.001
lconc	-0.5587	0.0793	-0.704	<0.000
wat. 'artif.'	-0.0733	0.0374	-1.96	<0.025
lsq	0.0534	0.0140	3.81	<0.001

The estimates of the reproduction rates from this model are given in Table 8.13, together with standard errors at 95% confidence intervals. The observations indicated by asterisks fitted the model poorly. In particular observation 20, for the highest concentrations of Al + Cu in artificial water, has a very large standardised residual of -4.07, suggesting that it might be an outlier.

Table 8.13 *Estimates of reproduction of P. nigroculus in stream or 'artificial' water mixtures of Al + Cu at concentrations in Table 1.3. [kn = reproductive rate observed (kn obs.) or fitted (kn fit.); lower and upper values of kn; s.e. = standard error]* *(data from Tables 3.1, 3.2)*

No.	*Conc. (mg/L)*	*lconc*	*water*	*kn obs*	*kn fit.*	*s.e.*	*Lower*	*Upper*
1	0.030 Al + 0.0025 Cu	0.97	nat.	0.04858	0.04752	0.00100	0.04556	0.04947
2	0.062 Al + 0.001 Cu	1.67	nat.	0.04090	0.04108	0.00065	0.03981	0.04236
3	0.0925 Al + 0.0025 Cu	2.07	nat.	0.03562	0.03785	0.00065	0.03657	0.03912
4	0.0925 Al + 0.0175	2.08	nat.	0.03606	0.03780	0.00065	0.03653	0.03908
5	0.1225 Al + 0.005 Cu	2.45	nat.	0.03501	0.03518	0.00069	0.03383	0.03654
6	0.185 Al + 0.004 Cu	2.77	nat.	0.03276	0.03318	0.00072	0.03178	0.03459
7	0.2775 Al + 0.005 Cu	3.18	nat.	0.03081	0.03101	0.00073	0.02958	0.03244
8	0.4325 Al + 0.004 Cu	3.6	nat.	0.03117	0.02917	0.00074	0.02772	0.03063
*9	0.926 Al + 0.008 Cu	4.36	nat.	0.03207	0.02691	0.00099	0.02498	0.02884
*10	1.388 Al + 0.0175 Cu	4.76	nat.	0.02261	0.02626	0.00132	0.02367	0.02885
11	0.030 Al + 0.0025 Cu	0.97	art.	0.04694	0.04589	0.00101	0.04391	0.04787
12	0.062 Al + 0.001 Cu	1.67	art.	0.03815	0.03945	0.00067	0.03814	0.04077
13	0.0925 Al + 0.0025 Cu	2.07	art.	0.03577	0.03622	0.00067	0.03491	0.03753
14	0.0925 Al + 0.0175 Cu	2.08	art.	0.03501	0.03617	0.00067	0.03486	0.03748
15	0.1225 Al + 0.005 Cu	2.45	art.	0.03470	0.03355	0.00071	0.03217	0.03494
16	0.185 Al + 0.004 Cu	2.77	art.	0.03062	0.03155	0.00073	0.03011	0.03299
17	0.2775 Al + 0.005 Cu	3.18	art.	0.03005	0.02938	0.00075	0.02792	0.03084
18	0.4325Al + 0.004 Cu	3.60	art.	0.03043	0.02754	0.00076	0.02606	0.02903
19	0.926 Al + 0.008 Cu	4.36	art.	0.02947	0.02528	0.00100	0.02332	0.02724
*20	1.388 Al + 0.0175 Cu	4.76	art.	0.1577	0.2463	0.00133	0.02202	0.02724

Plots of the observed and fitted reproduction rate per day against log (concentration) and concentrations are in Figure 8.8

Case 2: Poisson Regression: differences in reproductive rates of amphipods in relation to different concentrations of Al+ Cu and Mn

As there was also a possibility of extra-Poisson variation, I investigated the effects allowing for this, as follows:

Table 8.16 Accumulated analysis of deviance

Change	*d.f.*	*Deviance*	*Mean deviance*	*Deviance ratio*	*Tpr.or significance*
+ lconc	1	237.881	237.881	121.37	<0.001
+ water	1	3.849	3.849	1.96	0.180
+ lsq	1	14.248	14.248	7.27	0.016
Residual	16	31.954	1.997		
Total	19	287.933	15.154		

Message: ratios are based on dispersion parameter with value 1.96

Table 8.17 gives the estimates of regression coefficients

Table 8.17: Estimates of regression coefficients

Parameter	*Estimate*	*s.e.*	*t(*)*	*tpr.*
constant	6.033	0.142	42.49	<0.001
lconc	-0.559	0.111	-5.03	<0.000
wat.art.	-0.0733	0.0523	-1.40	0.081
lsq	0.0534	0.0196	2.76	0.003

Message: standard errors are based on dispersion parameter with value 1.96

The accumulated analysis of deviance shows that the effect of artificial water is no longer significant when I allow for over dispersion but the quadratic effect of log concentration remains strong. Observations 9 and 20,

the counts at the highest concentrations (1.388 mg/L Al + 0.0175 mg/L Cu), remain badly fitted by the model, but the standardised residual of observation 20 drops to -2.90 from -4.07.

Conclusion: With the increasing concentration of aluminium and copper, the amphipods under stress show a reduced reproduction rate greater that would be predicted by the model. I give the confidence intervals and standard errors for this model. They are naturally larger than in the model with no over dispersion (see the following Table 8.17).

Table 8.17 Estimates of reproduction rates (with over dispersion) (data from Tables 3.1, 3.2)

No.	*Conc.(mg/L)*	*lconc*	*Water*	*Kn obs.*	*Kn fit.*	*s.e.*	*Lower*	*Upper*
1	0.030 Al+ 0.0025 Cu	0.97	nat.	0.04858	0.04752	0.00140	0.04478	0.05025
2	0.062 Al+0.001 Cu	1.67	nat.	0.04090	0.04108	0.00091	0.03930	0.04287
3	0.0925 Al+0.0025 Cu	2.07	nat.	0.03562	0.03785	0.00091	0.03606	0.03963
4	0.0925 Al+0.0175 Cu	2.08	nat.	0.03606	0.03780	0.00091	0.03602	0.03959
5	0.1225 Al+0.005 Cu	2.45	nat.	0.03501	0.03518	0.00097	0.03329	0.03708
6	0.185 Al+0.004 Cu	2.77	nat.	0.03276	0.03318	0.00100	0.03122	0.03515
7	0.2775 Al+0.005 Cu	3.18	nat.	0.03081	0.03101	0.00102	0.02901	0.03301
8	0.4325 Al+0.004 Cu	3.60	nat.	0.03117	0.02917	0.00104	0.02714	0.03121
9	0.926 Al+0.008 Cu	4.36	nat.	0.03207	0.02691	0.00138	0.02421	0.02961
10	1.388 Al+0.0175 Cu	4.76	nat.	0.02261	0.02626	0.00185	0.02263	0.02989
11	as for 1	0.97	art.	0.04694	0.04589	0.00141	0.04312	0.04866
12	as for2	1.67	art.	0.03815	0.03945	0.00094	0.03761	0.04129
13	as for 3	2.07	art.	0.03577	0.03622	0.00094	0.03438	0.03805
14	as for 4	2.08	art.	0.03501	0.03617	0.00094	0.03433	0.03801
15	as for 5	2.45	art.	0.03470	0.03355	0.00099	0.03161	0.03550
16	as for 6	2.77	art.	0.03062	0.03155	0.00103	0.02954	0.03357
17	as for 7	3.18	art.	0.03005	0.02938	0.00104	0.02733	0.03143
18	as for 8	3.60	art.	0.03043	0.02754	0.00106	0.02547	0.02962
19	as for 9	4.36	art.	0.02947	0.02528	0.00140	0.02254	0.02802
20	as for 10	4.76	art.	0.01577	0.02463	0.00187	0.02098	0.02829

Case 3: Excluding the Water Effect

As there was no significant effect of water type, I fitted a final model with overdispersion, and excluding water. The regression analysis using the response variate = n45; Poisson distribution; link function =Log; and fitted terms = constant, +lconc, lsq gave the results summarised in the following table 8.18.

Table 8.18: Summary of the regression analysis

Parameter	*d.f.*	*Deviance*	*Mean deviance*	*Deviance ratio*
Regression	2	252.13	126.065	64.32
Residual	17	35.80	2.106	
Total	19	287.93	15.154	
Change	-2	-252.13	126.065	64.32

Message 1: ratios are based on dispersion parameter with value 1.96;

Message 2: The following units have large standardized residuals:

9 --- 2.25; and 20 --- -3.14;

Message 3: The following units have high leverage:

1 --- 0.41; 10 -- 0.30; 11 --- 0.41; and 20 --- 0.30.

The estimates of regression coefficients are reported in the following table 8.19.

Table 8.19: Estimates of regression coefficients

Parameter	*Estimate*	*s.e.*	*t(*)*
Constant	5.997	0.140	42.92
lconc	-0.559	0.111	-5.03
lsq	0.0534	0.0196	2.72

Message 4: standard errors are based on dispersion parameter with value 1.96.

The confidence intervals and standard errors for this model are given in the following table 8.4.10.

Table 8.20: Confidence intervals and standard errors (excluding the water effect and with over-dispersion)

No.	*Conc (mg/L)*	*lconc*	*Water*	*Kn. obs*	*Kn fit.*	*s.e.*	*Lower*	*Upper*
1	0.030 Al+ 0.0025 Cu	0.97	nat.	0.04858	0.04672	0.00128	0.04421	0.04922
2	0.062 Al + 0.001 Cu	1.67	nat.	0.04090	0.04028	0.00072	0.03887	0.04169
3	0.0925 Al+ 0.0025 Cu	2.07	nat.	0.03562	0.03705	0.00072	0.03564	0.03845
4	0.0925 Al + 0.0175 Cu	2.08	nat.	0.03606	0.03700	0.00072	0.03559	0.03841
5	0.1225 Al + 0.005 Cu	2.45	nat.	0.03501	0.03438	0.00079	0.03284	0.03593
6	0.185 Al + 0.004 Cu	2.77	nat.	0.03276	0.03238	0.00083	0.03075	0.03402
7	0.2775 Al + 0.005 Cu	3.18	nat.	0.03081	0.03021	0.00085	0.02854	0.03188
8	0.4325 Al+ 0.004 Cu	3.60	nat.	0.03117	0.02837	0.00087	0.02666	0.03009
9	0.926 Al + 0.008 Cu	4.36	nat.	0.03207	0.02611	0.00126	0.02364	0.02858
10	1.388 Al + 0.0175 Cu	4.76	nat.	0.02261	0.02546	0.00177	0.02200	0.02892
11	as 1	0.97	art.	0.04694	0.04672	0.00128	0.04421	0.04922
12	as 2	1.67	art.	0.03815	0.04028	0.00072	0.03887	0.04169
13	as 3	2.07	art.	0.03577	0.03705	0.00072	0.03564	0.03845
14	as 4	2.08	art.	0.03501	0.03700	0.00072	0.03559	0.03841
15	as 5	2.45	art.	0.03470	0.03438	0.00079	0.03284	0.03593
16	as 6	2.77	art.	0.03062	0.03238	0.00083	0.03075	0.03402
17	as 7	3.18	art.	0.03005	0.03021	0.00085	0.02854	0.03188
18	as 8	3.60	art.	0.03043	0.02837	0.00087	0.02666	0.03009
19	as 9	4.36	art.	0.02947	0.02611	0.00126	0.02364	0.02858
20	as 10	4.76	art.	0.01577	0.02546	0.00177	0.02200	0.02892

Plots of the observed and fitted reproduction rate against log(concentration) and concentrations are, as follows (Fig.8.10).

CHAPTER 9

General discussion and conclusions

9.1 Discussion

9.1.1 The combined effects of Al, Cu and Mn on the survival of amphipods

The mortality of mature amphipods after 24-hour exposure to mixtures of Al + Cu + Mn in either stream water (mortality 16.6%) or 'artificial' water (mortality 15.7%) is lower than the mortality of juveniles under similar conditions in either stream water (mortality 29.2%) or 'artificial' water (mortality 32.1%). Table 2.2 shows that under natural conditions in stream water, concentrations of Al (221 μg/L) and Cu (9 μg/L) are appreciable compared to AEV values (10 μg/L for Al and 1.5 μg/L for Cu) as defined by DWAF (1995). The actual concentrations at the end of the experiments in stream water of 342 μg/L for Al and 14 μg/L for Cu are high compared to AEV values. This may explain the lower risk of death for a mature amphipod (77%) compared to a juvenile used as a baseline (100%) after addition of Al, Cu and Mn because of possible hormesis due to pre-exposure. The added metals have increased the risk of death, as shown in section 8.1. The fact that LT_{50} is greater than 21 days shows that high concentrations used in the my experiments are not acutely dangerous from toxicological point of view. These concentrations (of 1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn) are at least 10 times (for Cu and Mn) and 140 times (for Al) greater than the South African AEV levels (DWAF 1995 as published in 1996: Document 7) for these elements (e.g. AEV for Al = 10 μg/L; Cu = 1.6 μg/L and Mn = 1300 μg/L). My experiments support the assumption that South African criteria regarding the combined toxicity of Al, Cu and Mn are adequate to protect some aquatic organisms (e.g. *P. nigroculus*) but studies on reproductive biology, ethology and on other sensitive organisms still need to be done. But also the fact that *P. nigroculus* is quite resistant at low pH, sensitive organisms to low pH may not be protected by criteria based only on *Paramelita* responses.

Tian (1996), reporting on a first attempt to use *P. nigroculus* for toxicity testing, studied the combined effects of Al and H^+ at low pH in acute toxicity tests. This study showed that *P. nigroculus* is Al-tolerant (e.g. a nominal concentration of 211 mg/L Al at pH 5.0 - 5.4 killed only 45% of exposed individuals after 96 hours while a nominal concentration of 500 mg/L Al at pH 6.4 - 6.7 killed only 40% of individuals after 96 hours of exposures). These results showed that the highest criterion value for Al (i.e. 0.01 mg/L Al) is not a problem for *P. nigroculus*. Nevertheless these nominal concentrations might not correspond the bioavailable concentrations. Tian's results also showed how an increase in pH reduces the toxicity of Al.

The fact that mature amphipods were collected from a stream containing appreciable levels of Al (221 μg/L), Cu (9 μg/L) and Mn (323 μg/L) means that they must have been pre-exposed to small doses of these metals. Such beneficial effects, also called hormesis, may explain the lower mortalities recorded for mature amphipods

after 24 hours of exposure than for juveniles. Similar results have been reported by Welton (1979), Roesijadi *et al.* (1987), Klerks *et al.* (1993), Roux *et al.* (1993) and Pynnonen (1995) for *Daphnia magna*. The size of amphipods may have also played an important role in their tolerance of pollutants. Small individuals, being generally more sensitive than bigger ones, are at higher risk of death (Green *et al.* 1986, Naylor *et al.* 1990, Kooijman 1996). Hallam (1996), for example, showed how large individuals of *D. magna* tolerate narcotics better than small ones do. He suggested that the quantity of lipids in an organism may play a buffering role when metals are taken up and accumulated in the body and he concluded that while fat (big) individuals can tolerate certain levels of toxins, only genetically fortunate ones can tolerate long effects. I am tempted to consider the same mechanism for heavy metals. However, additional study is needed. Some heavy metals (e.g. Cu) may accumulate in lipid.

Tian has shown that growth rates as change in body mass were higher in juvenile than in mature *P. nigroculus*. This means that when juvenile and mature individuals of *P. nigroculus* are exposed to metals in solution, juveniles might accumulate metals faster than mature ones because juveniles have higher metabolic rates. This may lead to their high mortality. However, 8-day mortalities of mature amphipods were higher than those of juveniles in both natural (52.5 % > 35.5%) and 'artificial' water (51.7 % > 34.7 %). This may be due to the decrease in tolerance as mature amphipods became older, whereas juveniles became bigger and tolerance increases. Of course, these are tests using mortality as the criterion for toxicity evaluation. Other chronic effects such as reproductive rate can only be seen after much more larger periods.That mortality after 24 hours of exposure was higher than that after 96 hours, and that mortality after 8 days was higher than after 21 days in all tests, seemed to be linked to the bioaccumulation process (as BCF per day : Table 5.2). Indeed, the bioconcentration factor per day was highest after 24-hour and 8-day exposures, which correspond to highest daily mortality rates. This result is particularly interesting for routine work on environmental quality control since it indicates that the shortening of test duration (i.e. to 24-h from 96-h and to 8-d from 21-d) may be appropriate under certain circumstances. Similar results have been found by Geiger (1980), Horning and Weber (1985), Munzinger and Monicelli (1991) and Santojanni *et al.* (1995) working on *Daphnia magna*.

The high mortality rates (>73%) of moulting individuals even in controls for these tests suggests that mortalities are largely due to physiological circumstances rendering moulting individuals fragile. Similar results are reported by Green *et al.* (1986), Reish and Oshida (1986), Kooijman *et al.* (1989) and USEPA (1991) and indicate that moulting individuals should not be used in standard toxicity tests.

9.1.2 Effect of Mn on the toxicity of mixtures of Al + Cu

The fact that mortality in solutions containing mixtures of Al + Cu is higher than in solutions containing mixtures of Al + Cu + Mn suggests that Mn (II) reduces the toxicity of Al + Cu by increasing precipitation

of Al in acidic waters (Chapter 4). Similar results on the effects of Mn (II) in metal mixtures are reported by Pergusson (1990), Shuttleworth and Unz (1991) and Driehaus *et al.* (1995). It has been reported that Mn (II) also increases the toxicity of Cr (III) by converting it to Cr (VI), which is very toxic; and Mn (II) reduces the toxicity of As (III) by converting it to non-toxic As (V). Al, by adsorbing cations (such as Cd (II)), reduces the availability of these cations and therefore reduces related toxicity. The modelling of chemical speciation and assessment of toxicity (Chapter 4) confirms this behaviour of Al. Indeed, since the chemical speciation of the metals exhibited no changes in respect of ion species when different combinations of metals were simulated, the observed changes in toxicity in respect of mortalities in the metal mixtures (mortality higher in Cu + Mn > Al + Cu > Al + Mn > Al + Cu + Mn) cannot be explained on the basis of speciation of the metals in aqueous solutions. However, the fact that some of the mixtures were supersaturated with respect to solid hydroxides of Al may significantly have affected metal speciation, and therefore toxicity. Firstly, precipitation of Al reduces its own bioavailability (about 85% of Al removed) and therefore toxicity. Secondly, solid hydroxides of Al adsorb other metal ions from solution. Sorbed metals become unavailable biologically and therefore their toxicities are reduced. The fact that the addition of Mn (II) to Al + Cu mixtures renders this combination less toxic suggest that hydroxides of Al may not be toxic to *P. nigroculus*. Increases in the Al hydroxide concentrations during experiments may not increase the pH value since Al and its salts form a very stable buffering system around pH 4.5 (J. Pretorius: pers. com. 1997) and therefore toxicity due to Al^{3+} may not be affected. Unfortunately, as the metals were measured and modelled at the end of the experiments, when precipitation have been already occurred, I cannot talk about the toxicity of Al hydroxides. However, Al behaviour in metal mixtures may play a regulatory role where Al ions are allowed to precipitate.

Finally, no 50% mortality was recorded within 21 days of exposure for animals in mixtures of Al + Cu at high concentrations (1.388 mg/L Al + 0.0175 mg/L) and Al + Cu + Mn (1.388 mg/L Al + 0.0175 mg/L Cu + 13.993 mg/L Mn) corresponding to concentrations 50% above the acute effect values given in the South African guidelines (DWAF 1995). These are 10 times higher for Cu and Mn and about 140 times higher for Al than acute effect values (AEV) in the actual guidelines, namely 10 μg/L Al + 1.6 μg/L Cu for the combination of Al + Cu and 10 μg/L Al + 1.6 μg/L Cu + 1300 μg/L Mn for the combination of Al + Cu + Mn. These concentrations did not cause statistically significant mortalities, allow me to conclude that S.A. criteria for the three metals are adequate for protecting *P. nigroculus* under acidic conditions. Indeed, as the toxicity of many metals (e.g. Al) is greater in acidic conditions (pH < 5) than at high pH values, and since the above quantities did not cause significant mortalities after 21 days of exposure in acidic conditions, my results provide some tentative evidence that these criteria are adequate for protecting aquatic life even at higher pH values. Tian (1996), for example, showed that concentrations of 1000 mg/L Al killed only 40% of individuals of *P. nigroculus* after 96-hour exposure at pH 6.4 - 6.7 and 560 mg/L Al killed 80% of individuals after 96-hour exposure at pH 5.0 - 5.4, while 902 mg/L Al at pH 3.5 - 3.7 killed 95% of individuals after 96 hours of

exposure. This shows how the toxicity of Al is reduced with an increase in pH. But also, the fact that in their natural environment, where I collected them, the amphipods were abundant despite the fact that this stream water contains high concentrations of these metals according to South African criteria for aquatic ecosystems (concentrations found 323 μg/L Mn, 221 μg/L Al and 9 μg/L Cu) confirms that South African criteria are adequate with regard to these three metals and *P. nigroculus* at least for the acidic waters of the south-western Cape.

9.1.3 Risk analysis

As discussed above (Chapter 8), risk analysis showed that mortality and survival depend on the type of water used for dilution (stream water or 'artificial' water), the concentration of the metal mixtures, and the life history stage of the amphipods. So, taking juveniles in stream water solutions as a baseline, and using Cox's regression equation (1987), risk analysis showed that:

- when the concentration of Al, Cu or Mn increases by 1 μg/L, the risk of death increases by the minute factor of 1.000086;
- compared to stream water, the risk of death in Al + Cu + Mn mixtures (at concentrations of 1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn) was 1.45 times as great;
- compared to stream water, the risk of death in Al + Cu mixtures (at concentrations of 1.388 mg/L Al + 0.0175 mg/L Cu) was 2.64 times as great;
- the risk of death for an adult in either mixtures was only 77% of the risk for a juvenile (100%);
- compared to stream water, the risk of death in 'artificial' without metals water was 90% [probably due to the presence of Al (221 μg/L), Cu (μg/L) and Mn (μg/L) and other elements in stream water: Table 2 in the paper as Chapter 2]. Juveniles, being very sensitive to increases in the concentrations of metals, are at greater risk of death than are adults, which develop hormesis.

9.1.4 Growth and reproduction

Growth in individuals, and the production of eggs and juveniles, occurred in all treatments. Growth, as change in dry mass, was assessed after 21 days of exposure, while reproduction, as production of eggs and juveniles, was assessed after 45 days of exposure (Chapters 2 and 3 respectively). Variations in growth and reproductive rates within treatments may be interpreted as resulting from the bioaccumulation of the metals in the amphipods. Indeed, the fact that BCFs differed even through I was using the same concentrations, and the same test organisms of the same species and the same sizes (i.e. juveniles), suggests individual differences in the ability to take up and accumulate metals (Green *et al.* 1986, Naylor *et al.* 1990).

9.1.5 Interactions between Al, Cu and Mn

As discussed in Chapter 4, the results reported in Chapters 2 and 3 showed mixtures of Al + Cu to be more toxic than those of Al + Cu + Mn with regard to mortality and growth of amphipods. In Chapter 4, I focused on the types of interactions occurring in different mixtures of metals using the MINTEQA2/PRODEFA computer package for chemical speciation and Gaddum diagrams, which define interactions between metals. Results showed the following toxicity profile (from the most to the least toxic): Cu + Mn > Al + Cu > Al + Mn > Al + Cu + Mn, corresponding to median survival times (LT_{50}) of 73, 107, 139 and 147 hours respectively.

According to the Gaddum diagrams, the interaction between Cu + Mn was supra-additive (synergistic), the combined action of Cu and Mn giving higher mortality rates than if each metals were acting alone; the interaction between Al + Cu was infra-additive (mitigated synergism) and the interactions for Al + Mn and Al + Cu + Mn were antagonistic. Chemical speciation based on the MINTEQA2/PRODEFA computer simulation showed that Mn (II) allowed the precipitation of Al (III) as the mineral diaspore (aluminium monohydrate), which adsorbed free metals ions Cu (II) and Mn (II), thus reducing the bioavailability of all three ions and therefore decreasing toxicity.

9.1.6 The Bioaccumulation process and regulation of metals

Many factors may play key roles in the bioaccumulation process, which comprises the uptake, transport, distribution and storage, and excretion, of metals and other substances. These factors include the bioavailability and concentration of a metal, exposure time, and the species of test organisms, as reported in Chapter 5. It is well-known that animals living in fresh water are osmoregulators and have to regulate throughout life; that most of freshwater animals including amphipods regulate by having an epidermis of low permeability and by producing copious amounts of urine (for example, if urine production per day is expressed as a percentage of the body weight of the animal, amphipods of the genus *Gammarus* produce around 40% of their body weight per day, cladocerans like *Daphnia* spp. > 200% and the freshwater bivalve *Anodonta* > 400%). Useful ions such as Na^+, K^+, Cl^-, Ca^{2+} and essential metals (such as Mn, Cu, Zn) are selectively removed in the excretory system, while unimportant or non-essential ions such as Al^{3+} may be taken up, stored and/or excreted in various ways, usually in relation to their mimicking of other essential elements. As far as I know, most toxic elements are not specifically excreted but some metals (in some mammals at least) are taken up by specific proteins (e.g. metallo-thiamine). The fact that the BCFs for Al changed significantly with changes in the concentration of Al in water allows me to conclude that *P. nigroculus* is an accumulator of this element. The fact that the BCFs did not change significantly with changes in concentration of Cu and Mn in water for most treatments allows me to conclude that *P. nigroculus* is a weak accumulator and a regulator for these two metals.

Of course, regulation and accumulation are not mutually exclusive. Regulation may be explained through metabolic requirements for essential elements such as Cu and Mn. After a certain level of accumulation when a metal is required in the body, regulation may occur. Another method of regulation may be the reduction of uptake by closing gills or shell for a given time. This mechanism, however cannot be the only one because organisms needing food and nutrients to survive (e.g. Waterman 1960, Russel-Hunter 1979, Silby & Calow 1989, Rainbow & Dallinger 1993, Timmermans 1993, Barnes *et al.* 1995). I do not think that amphipods can do this. *P. nigroculus* may have an epidermis of low permeability and may also produce copious urine to regulate Mn and Cu ions. This needs to be verified with radiotracers. However the fact that regression factors showed significant correlations (Chapter 6) in *P. nigroculus* between the amount of Ca vs Al ($r = 0.5626$), Ca vs Mg ($r = 0.3927$), Na vs Mg ($r = 0.7194$), Na vs Mn ($r = 0.6253$), Al vs Mg ($r = 0.4264$), Al vs Cu ($r = 0.5129$), and Mg vs Mn ($r = 0.3676$) suggests that the uptake of Al, Cu and Mn by *P. nigroculus* seemed to follow the uptake of Na, Ca and Mg (Table 6.5). Al transport may also use the Ca pump ($r = 0.5626$). There may be interferences between the uptake of Al and Cu ($r = 0.5129$) and between Mn and Mg ($r = 0.3676$). Ca and Na pumps worked independently and no significant correlation was shown at 0.05 level ($r = 0.2396$). In short, *P. nigroculus* uses the active pump of Ca for Al and Na for the selective reabsorption and active uptake of Al and Cu, and Mn. Low concentrations of Mn and low concentrations of Mg may signify the regulation of Mn, but also that Mn lowers the uptake of Mg (see Table 6.9). It has been known for a long time that crustaceans, including amphipods, do no tolerate high concentrations of Mg, which may act as anaesthetic. This may also explain the link between low concentrations of Mn and Mg in *P. nigroculus* as both metals significantly correlated each other and with Na pump.

9.1.7 Statistical analyses

Using ANOVA, Student's t and Newman-Keuls test for survival and growth, the Mann-Witney U-test for reproduction (Chapter 7) and regression analysis (Chapter 8) for survival, growth, reproduction and bioaccumulation, allowed me to compare differences within treatments and controls, as well as differences between organisms of different sizes, and the effects of the two different types of dilution water. The regression analysis for growth, for example, showed that growth of the amphipods is affected by water type (individuals grow faster in stream water than in 'artificial' water due to the presence of nutrients obviously), presence of Mn and the concentration of the chemicals (individuals are bigger in mixtures of Al + Cu+ Mn than in mixtures of Al+Cu in 'artificial' water). The effect of Mn on growth depends on the water type and also on its concentration. A multiple regression model using all the variables explained 89.5% of the variation in growth. However, because of the interactions and because the residual analyses showed that the residuals tended to be larger than the observed values at high concentrations (i.e. 1.388 mg/L Al, 0.0175 mg/L Cu and 13.993 mg/L Mn), I decided to fit separate models for each water type and chemical combination. In stream water without Mn added, the model fitted very well and explained 83.3% of the variation in body mass. In stream

water with Mn added, the model did not fit well and explained only 47% of the variation in body mass. This may be due to the fact that in stream water, Mn is not the only chemical that affects growth, but also the fact that Mn was present in stream water (323 μg/L), additional Mn (i.e. 13.993 mg/L) may disturb the natural equilibrium regarding growth.

In 'artificial' water with Mn, the model explained the variation in body mass better (64%) than in stream water with Mn (47%), while in 'artificial' water without Mn, the model explained only 56% of the variation (less than with Mn).

The models for reproduction (using Poisson distribution) and the bioaccumulation are also described in the Chapter 8. For bioaccumulation for example, the model explained 88% of the variation in log(BCF) and the effect of Mn was to lower the BCFs for Al and Cu (for example by allowing the formation of solid hydroxides of Al). There is therefore an obvious link between mortality/survival, bioaccumulation, growth, reproduction and toxicity.

9.2 Conclusions

The combined effects of Al, Cu and Mn on *Paramelita nigroculus* in acidic waters allowed me to conclude that:

- the South African criteria (DWAF 1995, Document 7 as published in 1996) are adequate for protecting *P. nigroculus* with respect to mixtures of Al, Cu and Mn because LT_{50} was > 21 days at concentrations at least 10 times (for Cu and Mn) and 140 times (for Al) higher than the guidelines values. Indeed, survival after 21-day exposures was 70% for mature amphipods in the stream water mixture of Al+Cu+Mn (at concentration of 1.388 mg/L Al + 0.0175 mg/L Cu and 13.993 mg/L Mn) and 62% at concentration of 1.388 mg/L Al + 0.0175 mg/L Cu for combination Al + Cu; survival after 21 days of exposure 71% and 60 % for adults in the artificial water mixtures of Al + Cu + Mn and Al + Cu at the same concentrations as above, respectively.For juveniles in the stream water mixtures of Al + Cu + Mn and Al + Mn, survival was 69 and 62% respectively for the same concentrations as above. In the artificial water mixtures of Al + Cu + Mn and Al + Cu, survival for juveniles was 68 and 62% respectively. For moulting amphipods, survival after 21-day exposures was below 10% and due to high mortality in controls (>73%), I rejected the results using the moulting individuals. Another fact in favour of the adequacy of South African interim guidelines for aquatic ecosystems is that high concentrations of Al (221 μg/L), Cu (9 μg/L) and Mn (323 μg/L) were recorded in Skeleton Gorge stream from which amphipods were collected in abundance. The increase

of pH and alkalinity and the presence of organics and suspended solids in natural conditions will reduce the bioavailability of these metals in water and thus the toxicity.

- the mixtures of Al + Cu are more toxic than those of Al + Cu + Mn; Mn reduces the toxicity of Al and Cu. However, the behaviour of Al (i.e. precipitation into $Al(OH)_n$) also plays an important role in reduction in toxicity. The effects of Cu and Mn are supra-additive; Al and Cu are additive; Al + Mn and Al + Cu + Mn are antagonistic. So, from the most to the least toxic combinations, Cu + Mn > Al + Cu > Al + Mn > Al + Cu + Mn, with the 96-h LC_{50} values corresponding to 0.5, 0.75, 1.25 and 1.5 toxic units, while the percentage of animals surviving after 96-hour exposures to $0.50LC_{50}$ proportions were 40.0%, 54.4%, 61.1% and 86.7% respectively; and the median survival times or LT_{50} in hours were 73, 107, 139 and 147 respectively; this confirmed that the toxicity of Al + Cu was higher than that of Al + Cu + Mn;

- mortalities were high after 24-hour and 8-day exposures in both types of dilution water. These intervals of exposure times (24 hours and 8 days) showed highest BCFs. Death may be therefore attributed to bioaccumulation in sublethal toxicity tests (i.e. at low concentrations of pollutants);

- *Paramelita nigroculus* is an accumulator of Al, and a weak accumulator/regulator of both Cu and Mn. Indeed, the BCF values changed significantly with the changes in treatments in almost cases for Al, but not for Cu and especially not for Mn.

- Growth and reproduction occurred in all treatments but some significant differences were reported within treatments. I cannot attribute these variations only to the effects of metals since more information on the reproductive biology of *P. nigroculus* is still needed (e.g. number of offspring and range of body weight in natural conditions).

- Juveniles of *P. nigroculus* seem to be good test organisms because they have not yet developed hormesis. But further information is needed on the reproductive biology and ethology of this amphipod before deciding on its daily use for monitoring water quality. Because *P. nigroculus* is so tough, it is an ideal animal for looking for example at bioaccumulation and combined toxicities, but the some toughness means that it is not a good bioindicator for field conditions. However, *P. nigroculus* might be useful in chronic testing of acidic waters (e.g. mining areas where concentrations of metals are high) because of its ability to accumulate metals and to tolerate acidic waters. Further, because it is local, and (unlike *Daphnia magna*) is well adapted to south-western Cape rivers, it provides a useful addition to the suite of species used in ecotoxicology. It is premature to put *P. nigroculus* and *D. magna* at the same level at this stage. I do not think they should be directly compared since they have different uses. It will seem like comparing a baby who tries to stand up and an adult.

May I suggest additional studies on the ethology, histology, genetics and reproductive biology of *P. nigroculus* for a better use of this promising test organism!

Thank you.

Moral: " DO NOT DESTROY EVEN A VERY SMALL LIFE FORM (i.e. *P. nigroculus*); HUMANITY CAN BE SAVED THROUGH IT".

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Appendix 1:

Table 5.12 shows an example on the calculations of BCFs after 21-day exposure of *P. nigroculus* to some test solutions using the 2-compartment model at the steady state equilibrium

Treatment and initial metal concentrations in mg/L	Metal	concent. in mg/g amphipod	concent. in mg/L in water after 21 days	BCF
B c2a: Al=1.388; Cu=0.0175; Mn=13.993	Al	25.909	1.42	18,246
	Cu	2.734	0.201	13,600
	Mn	2.499	0.351	7,120
B c2: as above	Al	11.524	0.701	16,440
	Cu	1.500	0.120	12,500
	Mn	1.350	0.210	6,430
B c2b: as above	Al	27.855	1.450	19,210
	Cu	11.056	0.830	13,320
	Mn	49.224	6.13	8,030
B b2: Al=0.2775; Cu=0.005; Mn=3.65	Al	6.834	0.408	16,750
	Cu	2.460	0.200	12,300
	Mn	2.706	0.308	7,120
B b2a: as above	Al	13.246	0.788	16,810
	Cu	1.340	0.113	11,860
	Mn	9.340	1.310	7,130

C b2: Al=0.0925; Cu=0.0175; Mn=1.218	Al	10.730	0.761	14,100
	Cu	3.762	0.330	11,400
	Mn	34.387	4.850	7,090
C b2a: as above	Al	3.790	0.268	14,140
	Cu	3.798	0.334	11,370
	Mn	3.320	0.475	6,990
C b2b: as above	Al	10.639	0.761	13,980
	Cu	3.904	0.330	11,830
	Mn	5.959	0.850	7,010
C c2: Al=0.4325; Cu=0.004; Mn=4.665	Al	2.296	0.205	11,200
	Cu	0.520	0.090	5,780
	Mn	4.314	0.605	7,130
AEV2b: Al=0.1225; Cu=0.005; Mn=5.273	Al	10.128	0.633	16,000
	Cu	3.539	0.325	10,890
	Mn	6.376	0.907	7,030
AEV2b': as above	Al	9.932	0.618	15,100
	Cu	4.031	0.337	11,960
	Mn	5.876	0.839	7,004