TOXICITY OF METALS IN BIOLOGICAL SYSTEMS

by

Jennifer L. Stauber MSc

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Declaration

This thesis contains no material that has been accepted for the award of a higher degree or graduate diploma in any tertiary institution To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due acknowledgement is made in the text of the thesis

J L. Stauber

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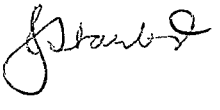

J L Stauber

Abstract

This thesis incorporates reports of research on the development and application of innovative chemical and biochemical techniques to the study of mechanisms of metal toxicity in simple single-celled organisms and more complex multicellular organisms including humans. The thesis is based on research undertaken over the last 10 years and published in 26 refereed scientific papers

Single-celled algae are the foundation of most aquatic food chains and account for much of the productivity of aquatic ecosystems Marine and freshwater algae are particularly sensitive to a wide range of metals and organic compounds, and their viability can be used as an indicator of environmental change. Chronic toxicity tests, based on algal growth and enzyme inhibition, have been developed and applied to assessments of the bioavailability and toxicity of metals in aquatic systems Specific applications of the algal bioassays for assessing mine-derived contaminants in natural systems has been described

Knowledge of modes of action of different metal species at a sub-cellular level is essential to understanding and predicting the bioavailability and toxicity of metals in natural waters. Research on the mechanisms of toxicity of copper and zinc to microalgae has been described, with particular emphasis on the link between chemical speciation and toxicity

Research on the mechanisms of toxicity of metals was extended from the study of simple unicellular organisms to investigating metal toxicity in humans. Novel methods for analysing metals in human sweat, urine and hair were developed, with particular emphasis on lead and manganese These techniques were used to study skin absorption of lead in occupationally-exposed workers and in the study of manganese toxicity in Australian aborigines from Groote Eylandt, Northern Territory

Skin absorption was identified as a new route of exposure to inorganic lead in humans The ability of lead salts to enter the body through the skin without significantly raising blood lead levels has important implications for occupational health control because measurement of lead in blood is the main criterion which has previously been used to assess exposure Additional biological monitoring techniques that can detect skin-absorbed lead are now required by occupational health and safety authorities

The research described in this thesis has shown that the mechanisms of toxicity of metals is largely dependant on the chemical form of the metal By taking into account the speciation of the metal and its mode of action within the cell, it is now possible to predict the toxicity of metals and to identify those factors which ameliorate toxicity in natural waters and biological systems, thus facilitating timely and cost-effective remediation.

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Introduction - Thesis Outline and Scope

This thesis describes research into metal toxicity in biological systems, evolving from early studies on whole organism effects, such as mortality and physiological change, to biochemical studies at the cellular and sub-cellular level. It incorporates the development and application of innovative chemical and biochemical techniques to the study of mechanisms of metal toxicity in simple single-celled organisms and more complex multicellular organisms including humans.

The research has been undertaken at CSIRO over the 10 years prior to enrolment at the University of Tasmania. It has led to the publication of 82 journal papers and reports, of which 26 journal papers are submitted in this thesis to the University of Tasmania, under the rules for Research Higher Degrees based on published work.

Chapter 1 is a general overview of metal toxicity, including a brief description of metal uptake, partitioning, mode of action and detoxification in organisms. In Chapter 2, the biochemical mechanisms of toxicity of copper and zinc to microalgae are explored, incorporating my research from 1985-1990 (10 papers). The innovative techniques developed to study the toxicity of metals to algae were then more generally applied to establishing bioassays to assess the bioavailability and toxicity of metals in aquatic systems Research on the development of these rapid toxicity tests based on algal growth and enzyme activity is discussed in Chapter 3 (8 papers).

This research on metal toxicity in single-celled organisms was then extended to more complex organisms. Development of new biological monitoring techniques for metals in humans are described in Chapter 4 (13 papers). Applications of these techniques to the study of lead absorption through human skin, and manganese toxicity in Groote Eylandt aborigines, are given. Implications for the protection of occupationally-exposed workers, the public and the environment are discussed.

Chapter 5 outlines my contribution to collaborative research and joint authorship of the papers submitted as part of this thesis.

The text of the thesis summarises the work in each section and refers the reader to the relevant publications, it does not attempt to present the work in each section in full detail where this is present in the published work. The 26 principal publications have

been incorporated in full into Appendix 2, which constitutes an important part of the thesis

Chapter 1

Toxicity of Heavy Metals - An Overview

1.1 Introduction

Ecotoxicologists generally use the term heavy metals to refer to metals that have been shown to cause environmental problems (Depledge *et al.*, 1993). These include Cd, Hg, Zn, Cu, Ni, Cr, Pb, Co, V, Ti, Fe, Mn, Ag and Sn, together with the metalloids As and Se. These metals are continually released into the environment by natural sources, such as volcanoes and weathering of rocks, and from anthropogenic inputs including mining activities, sewage/stormwater, land application of fertilizers and pesticides, and combustion of fossil fuels. Human activities are increasingly disturbing the natural biogeochemical cycling of metals e.g. Schindler (1991) estimated that anthropogenic releases of Hg, Pb, Zn, Cd and Cu into the oceans are one-to-three orders of magnitude higher than natural fluxes

Many metals such as zinc, copper and manganese, are essential components of enzymes involved in transfer, redox and hydrolytic reactions in cells, respiratory proteins and structural elements of organisms. However, at concentrations above a certain threshold, essential metals can disrupt cell metabolic processes leading to toxic effects in whole organisms. Non-essential metals such as mercury, have no biological role and may be toxic even at low concentrations. Animals and plants must therefore regulate their uptake of these metals. There is an extensive literature on the accumulation and toxicity of metals in plants and animals in both aquatic and terrestrial ecosystems (Fowler, 1990; Bryan and Langston, 1992)

In order to understand the interactions of metals with biota, it is important to take into account metal speciation in the external environment, metal interactions and uptake at the biomembrane, metal bioaccumulation and partitioning in the organism, and mechanisms of toxicity.

1.2 Metal Speciation

It is now well established that the physico-chemical form of a metal (its speciation) is a critical factor controlling metal bioavailability In natural waters, factors such as pH, hardness, dissolved organic mater and the presence of inorganic colloids, all control metal speciation and, consequently, metal toxicity. Similarly, in sediments and

soils, organic matter, clay content, pH, cation exchange capacity and particle size all determine metal bioaccumulation and toxicity.

In aquatic systems, it is generally accepted that the free metal ion is the most bioavailable form of a metal (Sunda and Guillard, 1976; Anderson and Morel, 1978). The free metal ion concentration is determined not only by the total dissolved metal concentration, but also by the concentration and nature of the ligands present In natural waters, however, knowledge of the free-ion activity is not sufficient to predict biological response. In addition, competition for metal binding sites by H^+, and hardness cations Ca^{2+} and Mg^{2+} must be considered.

In human toxicology, the form of the metal has important consequences for absorption, transport, distribution, accumulation and toxicity of the metal. For example, organic forms of arsenic and mercury may be more easily absorbed in the gastrointestinal tract than inorganic forms (Vercruysse, 1984). Similarly, aluminium as an aluminium citrate complex, is more bioavailable than other species of aluminium in the gut

1.3 Metal Uptake

Metal accumulation in biota can occur by either direct uptake from the surroundings across the body wall or respiratory surfaces, or via food.

At the surfaces of living organisms, a metal will first encounter a protective polysaccharide or glycoprotein layer such as a plant cell wall or mucus for animal cells This external layer consists of a number of macromolecules dominated by oxygen-containing donor groups which are negatively-charged at neutral pH. Metals first have to migrate through this negatively charged matrix to the plasma membrane, which in all organisms consists of a hydrophobic, phospholipid bilayer traversed by proteins and ion channels. The membrane, therefore, contains a wide variety of binding sites including active sites at which metal binding can affect metabolic function (Campbell, 1995).

Some organisms such as algae and fish are actively able to exclude metals by excreting polysaccharides or mucus which bind the metals and prevent their uptake into cells. In general, the amount of exudate increases with increasing metal concentration (Zhou *et al.*, 1989)

Dissolved metals in aquatic systems interact at the surface of the cell membrane as a free ion (M^{z+}) or as a metal complex (ML) to form metal-ligand surface complexes. Morel (1983) formulated the free-ion-activity model (FIAM) which described the importance of free metal ion activities in determining the uptake, nutrition and toxicity of cationic metals. The model assumes that the metal uptake and consequent biological response is strictly dependant on the concentration of the metal surface complex and the stability of the complex.

A number of exceptions to the FIAM have been noted by Florence *et al.* (1984) and Campbell (1995). These include lipophilic metal complexes which can diffuse directly through the cell membrane, some inorganic anions of silver and aluminium which can form ternary surface complexes, and low molecular weight organic ligands forming hydrophilic metal complexes (Cu-glycine, Cu-citrate) which are accidentally transported through the cell. In natural waters in particular, the FIAM is not always applicable. It appears that dissolved organic matter can sorb to biological surfaces and its surfactive properties may modify metal-organism interactions in natural waters (Campbell, 1995)

In aquatic organisms, it was generally assumed that the predominant route of uptake of metals was via passive diffusion across the body surface, gills or lungs or by active transport via calcium pumps. However, Bryan (1984) concluded that for many molluscs, crustaceans and annelids, metal uptake via food was more important. Benthic organisms that ingest particles tend to select the smaller particles to maximise the ingestion of organic carbon in the form of attached bacteria or surface bound organic coatings. In so doing, they also ingest fine-grained sediment particles rich in metals. Assimilation of particle-bound metals involves their conversion to dissolved forms in the gut and facilitated diffusion across the intestinal membrane (Luoma, 1983) Thus, the amount of metal taken up is dependant on the pH, digestion time, and redox status in the gut (Campbell and Tessier, 1996). Metals are not only absorbed through the intestine, but in some bivalves, a fraction of food is processed by a slower intracellular glandular digestion, during which metals can be assimilated. Uptake from solution can also take place in the alimentary tract, when water is taken in during food ingestion. Teleosts in the sea maintain their water balance by drinking seawater and excreting Na^+ and Cl^- across the gills. This represents a source of dissolved toxicants additional to food and absorption across the gills.

In some invertebrates, solution and particles may also be absorbed through membranes by the process of endocytosis Metal-bearing particles are engulfed by the epithelial membrane which then pinches off and forms a membrane-limited vesicle within the cell. Such processes have also been shown to be involved in the uptake of metal rich particles in the gills and pharynx of molluscs and ascidians (Kalk, 1963; Hobden, 1967). Although this may be a major digestive pathway for some benthic invertebrates, its significance as a route of metal uptake is unknown.

In terrestrial organisms, uptake via food is generally the predominant route, with the exception of earthworms which take up a proportion of metals directly from the soil and pore waters through their moist, permeable body surface. Very few studies have investigated the species of metal that is taken up across the lining of the digestive system after ingestion of soil particles.

In humans, metals are taken up by resorption in the gastrointestinal tract, the lungs and the skin. The exact mechanisms of uptake are not fully understood, although it is believed that lead in the gut is absorbed by means of a calcium-binding protein (Barton, 1978). About 10% of lead in the gut is absorbed in adults, whereas children absorb up to 50% of lead ingested. Resorption of lead in the lungs depends on particle size, but is typically 1-50 μg/day in urban populations. Apart from quantitative studies with lead and mercury, there is very little information on resorption mechanisms for other metals

1.4 Metal Bioaccumulation and Excretion

Tissue concentrations of metals in biota depend on the amount of metal taken up, its distribution between tissues, metabolic requirements for that metal and detoxification/excretion mechanisms Most of our information on heavy metal concentrations in aquatic organisms comes from studies with fish, molluscs and crustaceans, particularly edible species due to concerns about metal transfer to humans through ingestion of seafood (Furnass and Rainbow, 1990). However, data on bioaccumulation of metals in polychaete worms, coelenterates, echinoderms and algae have also been published (Hellawell, 1986; Depledge *et al.*, 1993). Studies on terrestrial animals have focused on a few species of annelids (earthworms), arthropods and molluscs (Hopkin, 1989). Mammalian data are largely restricted to small rodents, bats, birds and domestic animals (Zook *et al.*, 1979; Samiullah, 1990)

Some organisms have the ability to tolerate high metal concentrations in their tissues. Barnacles in the Thames estuary have been found to contain zinc concentrations of 153,000 μg Zn/g dry weight, equivalent to 15% of their dry weight (Rainbow, 1987). Metal hyperaccumulation (particularly Cu, Cr, Pb and Ni) is also found in plants e.g. concentrations of up to 230,000μg Ni/g ash weight have been found in *Aeolanthus floribundus* (Severne and Brooks, 1972).

Excretion (depuration) of metals from the bodies of invertebrates takes place by a variety of routes. Passive desorption can occur if external concentrations change and defecation will also remove the non-available fraction of metal in the gut. Metals may also be excreted through permeable surfaces including the gills and in urine, in both soluble and particulate form (Florence and Stauber, 1991).

In humans, following resorption from the lungs, skin or gut, metals are transported in the blood stream bound to plasma proteins or low molecular weight compounds. Metals such as mercury and arsenic undergo biotransformation reactions including methylation, oxidation/reduction and binding with macromolecules. From the blood, metals partition into cellular fluids and tissues, with different metals and metal species having different affinities for particular organs. Inorganic mercury accumulates in the kidney, whereas methylmercury accumulates in the kidney, liver and brain (Vercruysse, 1984). Lead is retained in three exchangeable pools, including blood and readily exchangeable soft tissue, soft tissue and readily exchangeable bone, and bone (Rabinowitz *et al*, 1974)

In common with other vertebrates, humans excrete metals via the kidneys (urine) and the bile and gastrointestinal tract (faeces) Approximately 75% of absorbed lead is excreted in urine, with 16% of absorbed lead and all non-absorbed lead excreted via faeces. Smaller amounts of metals are also excreted in sweat, nails and hair.

1.5 Detoxification

Apart from excretion, ingested metals may be detoxified in the liver, kidney and spleen of vertebrates, invertebrates and procaryotes, by binding to specific proteins called metallothioneins. Metallothioneins are cysteine-rich low molecular weight proteins (6000-10000 daltons) which are synthesised in response to specific metals, particularly Cd, Zn, Hg and Cu. These metals trigger a specific messenger RNA for thionein synthesis (Morrison *et al* , 1989). They bind metals in the cytoplasm as

mercaptide complexes and, although they have a half life of 4-5 days, continued synthesis allows long-term metal retention.

Metallothioneins also play a role in funnelling metals to other complementary sinks such as lysosomes and granules. They may also be involved in homeostatic control of copper and zinc e.g they play a central role in the moulting cycle of marine crustaceans (Engel and Brouwer, 1987). It is possible that metallothionein binding of metals may not be purely protective - it could be a deleterious event if a toxic metal displaces an essential metal in the metallothionein, leading to disruption of the protein's homeostatic function (Campbell and Tessier, 1996).

Like metallothioneins in animals and procaryotes, phytochelatins are metal-binding proteins produced enzymatically by higher plants, fungi and algae in response to many metals particularly cadmium (Ahner *et al.*, 1995). They are polypeptides with the amino acid structure (gamma-glu-cys)$_n$-gly, where n ranges from 2-11 (Grill *et al.*, 1985) An enzyme, phytochelatin synthase (isolated from higher plants), catalyses phytochelatin formation by cleaving the terminal glycine from one glutathione (gamma-glu-cys)-gly and joining the gamma-glu-cys to the amino terminus of another glutathione. Phytochelatins detoxify metals by chelation through co-ordination with the sulfhydryl group in cysteine Ahner *et al.* (1995) have recently detected low background levels of phytochelatins in algae, even before induction by the addition of cadmium at environmentally relevant concentrations.

Other mechanisms for detoxification of metals in algae have been described. It has been suggested that the production of dimethylsulfide by the marine alga *Phaeodactylum tricornutum* confers copper tolerance on this alga by intracellular copper sulfide precipitation (Armstrong and Boalch, 1960).

1.6 Mechanisms of Toxicity

Much of the early work on the mechanisms of toxicity of metals explored growth effects and related these to elemental composition of the cells such as total cellular phosphorus and sulphur. More recently, toxic effects of metals have been studied at the biochemical and molecular levels to understand the specific modes of action of different metals on enzyme pathways and cell membrane function

Metals exert their toxicity by a diverse array of mechanisms (Goyer, 1991). If the metal is excluded from the cell and retained in the cell membrane, it may deactivate membrane-bound enzymes essential to the functioning of the cell or interfere with membrane structural integrity, leading to leaky membranes and cell lysis. The metal can also catalyse the production of free radicals and activated oxygen species which can cause membrane lipid peroxidation (Florence, 1984).

Many theories of metal toxicity to algae propose that the toxic metal competitively inhibits the uptake of an essential trace metal at the membrane. Harrison and Morel (1983) proposed that cadmium displaced iron uptake at low iron concentrations, leading to displacement of iron in the algal cell Similarly, Jones (1986) proposed that metals interfere with the ability of algae to utilise organophosphate, so that metal-treated algal cells are phosphorous limited. Reduction in algal cell growth rates due to inhibition by the toxic metal of the uptake and/or metabolism of a nutrient metal has been proposed for copper and zinc (Rueter and Morel, 1981), cadmium and zinc (Braek *et al.*, 1980), cadmium and manganese (Van Duijvendijk-Matteoli and Desmat, 1975) and copper and manganese (Sunda and Huntsman, 1983)

When heavy metals are transported into the cell by facilitated diffusion, intracellular toxic effects can occur. When the capacity of a cell to detoxify accumulated metal is exceeded, damage to the cell and consequently the whole organism may occur. Signs of intracellular damage include morphological and ultrastructural deformities, as well as reduction in cell division rates, respiration, motility and enzyme activity (Morrison *et al*, 1989).

In plants and algae, metals at low concentrations can inhibit cell division without seriously affecting other cell processes such as photosynthesis, so the cells continue to grow without dividing. This has been noted for Cu, Co, Hg, Zn, Pb and Se (Davies, 1974, Fisher *et al.*, 1981). At higher concentrations, metals inhibit other metabolic processes such as ATP production, enzyme activity, photosynthesis, pigment production and membrane structure and integrity

Effects on invertebrates include inhibition of growth, reproduction and oxygen consumption, damage to gills, and impairment of tissue repair processes. At a molecular level, damage to plasma membranes, DNA synthesis and repair, inhibition of ATPases and other enzymes, lipid peroxidation and depletion of reduced

glutathione may occur (Viarengo, 1989). Lysosomes when ruptured can also release hydrolytic enzymes such as trypsin which rapidly destroy the cell.

In humans, the mechanisms of toxicity of metals, particularly lead and mercury, have been well studied. Mercury toxicity is due largely to binding of the Hg^{2+} to enzyme thiol groups and macromolecules. Lead at low levels of exposure affects the biosynthesis of haem (the iron porphyrin component of haemoglobin). Specifically, lead interferes at 5 different sites in the biosynthetic pathway from glycine to succinyl CoA. The enzyme delta-aminolevulinic acid dehydrase is severely inhibited by lead, leading to delta-aminolevulinic acid build up in urine and plasma. In the renal system, lead damages the peripheral tubular system, resulting in reduced re-absorption of glucose and amino acids and with chronic exposure, glomerular atrophy and renal failure (Fergusson, 1990). The exact biochemical basis of lead toxicity to human reproductive and central nervous system functions has not been established.

1.7 Conclusions

Research on metal toxicity suggests that the biochemical mode of action of a particular metal is similar in many cells, whether they be simple microorganisms or more complex multicellular organisms. Similarly, metal detoxification mechanisms such as the induction of metal binding proteins, share common characteristics amongst plants and animals.

In the following chapter, specific toxic mechanisms of two metals, copper and zinc, to microalgae are explored in detail. This research has yielded new insights into the chemistry of the toxic process at the cellular level.

Chapter 2

Mechanisms of Toxicity of Metals to Unicellular Algae

2.1 Introduction

Single-celled algae are the foundation of most aquatic food chains and account for much of the productivity of aquatic ecosystems Any adverse impact on algae may directly or indirectly affect organisms at higher trophic levels. Marine and freshwater algae are particularly sensitive to a wide range of metals and organic compounds, and their viability can be used as an indicator of environmental change. Diatoms, which are abundant and widely distributed, are particularly useful for detecting toxic impacts and low levels of environmental stress.

It is now well established that the physico-chemical form of a metal (its speciation) is a critical factor controlling metal bioavailability in natural waters. Natural waters contain a variety of organic (e.g. humic, fulvic, tannic acids, lignin) and inorganic (iron and manganese oxides) compounds which complex or adsorb metals and reduce metal bioavailability and toxicity.

Generally, the free metal ion (e.g. Cu^{2+}) is the most bioavailable and toxic form of a metal (Sunda and Guillard, 1976, Anderson and Morel, 1978). Exceptions to the free-ion activity model include hydrophilic metal-citrate complexes (Guy and Kean, 1980) and the highly toxic lipid-soluble copper complexes that can diffuse rapidly into cells, transporting both the ligand and copper into the cell (Florence and Stauber, 1986). Whether these complexes are significant in natural waters is still unknown.

The toxicity of free metal ion towards phytoplankton is usually found to decrease in the order $Hg^{2+}>Ag^{+}>Cu^{2+}>Pb^{2+}>Cd^{2+}>Zn^{2+}$, which is approximately the order of the decreasing solubility product of the metal sulfides, or the stability constants of the metals with thiol ligands (Canterford and Canterford, 1980). Because mercury, silver and copper are strongly bound by organic matter in natural waters, and lead is largely hydrolysed, cadmium is often found to be the metal of most concern in natural systems

Metal speciation measurements have been made by a variety of techniques including electroanalysis, ion exchange, dialysis, ultrafiltration, solvent extraction and computer modelling. Many attempts have been made to chemically determine the bioavailable

or toxic fraction of the metal i.e. the fraction of its total concentration that is toxic to aquatic organisms (**Paper 25**; Zhang and Florence, 1987; Florence, 1983). However, many of these chemical methods seriously underestimate the toxic fraction. On the other hand, bioassays with single-celled algae give a direct measure of bioavailability of metals in polluted waters

Knowledge of modes of action of different metal species at a sub-cellular level is essential to understanding and predicting the bioavailability and toxicity of metals in natural waters. This chapter describes research on the mechanisms of toxicity of copper and zinc to microalgae, with particular emphasis on the link between speciation and toxicity.

2.2 Ecotoxicology of Copper and Zinc to Algae

Large quantities of copper and zinc from mining processes and industrial/domestic sewage are released annually into coastal marine waters. Zinc in seawater is present partly as inorganic species (Zn^{2+}, $ZnOH^{+}$ and $ZnCO_3^{o}$), in contrast to copper which is present almost entirely in the organically-bound form. The Australian and New Zealand Environment Conservation Council (ANZECC) water quality guidelines for copper and zinc for the protection of marine and estuarine life are 5 µg Cu/L and 86 µg Zn/L (ANZECC, 1992).

Although zinc is an essential element for almost all forms of life, it can, like other essential elements, show toxic effects if present in excess. Acute 48-96 h LC50 values for zinc for a variety of marine organisms range from 0.046 to 55 mg/L, with an acute to chronic ratio of 100-200 (USEPA,1987). The most sensitive group are the crustaceans, particularly early life stages of brine shrimp (*Artemia salina*), crab zoeae (*Cancer magister*) and juvenile shrimp (*Mysidopsis bahia*). Sublethal effects of zinc on marine organisms, however, may occur at much lower zinc concentrations. For freshwater species, the acute toxicity of zinc is slightly higher, with LC50 values in the range 0.051-81 mg/L (Vaughan and Lim 1996).

Some species of algae are particularly sensitive to copper and both marine and freshwater algae vary considerably in their sensitivity. The concentration of copper reported in the literature to cause a 50% decrease in algal growth (EC50) ranges from 7 µg/L to 58,000 ug/L (Fisher *et al.*, 1981; Gavis *et al* 1981). Most of these data, however, were obtained from bioassays in culture media, which can seriously

underestimate the toxicity of copper. Culture media contain phosphate, silicate, EDTA, manganese and iron, which complex or adsorb copper and reduce its bioaccumulation and toxicity (**Paper 19**) For this reason all of my research on the toxicity of copper to algae used either seawater for marine algae or synthetic softwater for freshwater algae, with no additional nutrients (**Paper 19**). In the case of the marine alga, the EC50 for copper was 10 μg Cu/L in unsupplemented seawater, whereas a value of 200 μg Cu/L was found when the assay was carried out in medium f (Guillard and Ryther, 1962). (The lower the EC50, the more toxic the copper). Thus most of the literature data available for copper toxicity to algae is not very useful because chelation by culture media itself was not taken into account e.g. Bentley-Mowatt and Reid (1977), Overnell (1975).

The toxicity of copper to algae in well-defined culture media containing synthetic chelators such ethylene diamine tetra-acetic acid (EDTA) or nitrilotriacetic acid (NTA) to control the free metal ion concentration, has been repeatedly studied. This enables computation of the free copper(II) ion activity from thermodynamic equilibrium constants. Such studies show that the toxicity of copper is related to the copper activity rather than to total copper concentration (Sunda and Guillard, 1976) However, whether these studies are applicable to natural waters is debatable because natural waters contain a wide range of ligands, the stability constants of which are unknown. Furthermore, kinetic factors are not taken into account in the equilibrium calculations.

Apart from factors such as water hardness and pH which affect metal speciation, the toxicity of copper and zinc to algae is also dependant on seasonal variability, synergistic interaction of metals, algal biomass and algal exudates. The toxicity of copper and zinc can be ameliorated by exudates such as glycollic acid, oxalic acid, pyruvic acid, amino acids and carbohydrates produced by algal cells (Zhou *et al* , 1989) In general, the amount of exudate produced by the cells increases with increasing copper concentration. Obernosterer and Herndl (1995) showed that the marine alga *Chaetoceros affinis,* grown under nitrogen- (N:P=5) or phosphorus- (N:P=100) limiting conditions, exhibited higher rates of photosynthetic exudate release during exponential phase growth (40% and 93% respectively) than when grown under balanced nutrient conditions (N:P=16). Thus the nutrient status of the algae could also have a profound effect on the toxicity of metals observed

2.3 Mechanisms of Toxicity of Copper to Algae

2.3.1 Ionic copper

Ionic copper may exert its toxic effect at the cell membrane, by interfering with cell permeability or the binding of essential metals (Sunda and Huntsman, 1983) or after transport into the cell where it can affect cell division, photosynthesis or other metabolic processes.

All algae have the ability to accumulate copper, with ranges from 1 μg Cu/g dry weight for algae in pristine areas (Denton and Burdon-Jones, 1986) to 300 μg Cu/g in polluted waters. Copper is adsorbed rapidly at the algal cell surface. Gonzalez-Davila *et al* (1995) found that copper was bound to the green alga *Dunaliella tertiolecta* by two functional groups, one with a high affinity for copper (possible algal exudate) and another with low affinity for copper. Florence *et al.* (1983) proposed that initial copper binding to the alga *Nitzschia closterium* may be to membrane protein, carboxylic and amino acid groups, rather than thiol groups because the Cu-algae stability constant was orders of magnitude lower than the thiol-Cu binding constant

If the copper is retained in the cell membrane, it may deactivate membrane-bound enzymes essential to the functioning of the cell or it may interfere with membrane structural integrity, leading to leaky membranes or cell lysis.

The rapid binding of copper to algal cell walls is followed by a much slower active uptake in which copper is transported across the cell membrane by a carrier protein. This process of facilitated diffusion removes copper from its hydrophilic complex in solution and transports it as a more hydrophobic complex through the membrane (Gonzalez-Davilla *et al.*, 1995) Once inside the cell, copper is released while the ligand to which it was bound remains in solution

Following copper transport into the cell, copper may react with -SH enzyme groups in the cytoplasm, disrupting enzyme active sites and cell division, or it may exert its toxicity in subcellular organelles, interfering with mitochondrial electron transport, respiration (Cedono-Maldonado and Swader, 1974), ATP production (Viarengo *et al.*, 1981) and photosynthesis in the chloroplast (Overnell, 1975)

To obtain more direct evidence of the mechanisms of copper toxicity in algae, I investigated the effect of copper on the growth, photosynthesis, respiration, ATP

production and mitochondrial electron-transport chain activity in two marine diatoms and one freshwater green alga (**Paper 6**). Copper ions depressed both cell division and photosynthesis in *Asterionella glacialis* and *Chlorella pyrenoidosa*, whereas ionic copper concentrations which were inhibitory to cell division in *Nitzschia closterium* had no effect on photosynthesis, respiration, ATP production, electron transport or membrane ultrastructure. Photosynthetic products accumulated, leading to enlarged cells which were unable to divide. A similar uncoupling of cell division and photosynthesis has also been observed in *Asterionella japonica* (Fisher and Jones, 1981). My results suggested that in *Nitzschia closterium*, copper does not act on the chloroplast, the mitochondrion or the cell membrane.

Using a lipid-soluble thiol reagent, I showed that the treatment of *Nitzschia* with copper ions significantly reduced the concentration of intracellular thiol groups (**Paper 5**). Compounds such as cysteine, glutathione (GSH), EDTA and bathocuproine disulfonate could reverse copper toxicity, whereas oxidised glutathione (GSSG), cystine and glycine had no effect. It was proposed that copper(II) adsorbed to algae is reduced to copper(I) by -SH groups and bound as a Cu(I)-S complex. A large proportion of intracellular -SH groups would be present as free glutathione (GSH). Copper toxicity may principally be due to copper binding to intracellular rather than membrane thiols, since protein thiol-Cu binding would completely disrupt membrane functions. In addition, sulfhydryl compounds such as cysteine, which penetrate the cell, could reverse toxicity through thiol-disulfide interchange reactions, allowing algal growth to recommence. Powerful copper chelating agents produced the same effect by withdrawing copper from the cell.

This work was extended and it was proposed that ionic copper toxicity in this species may result from an intracellular reaction between copper and reduced glutathione in the cytoplasm, leading to a lowering of the reduced to oxidised glutathione ratio and suppression of mitosis (**Paper 6**). The ratio of GSH.GSSG has been found by other workers to be important in cell division, a high GSH concentration favouring cell division in sea urchin eggs and algae (Kosower and Kosower, 1978; Hare and Schmidt, 1969). Nath and Rebhun (1976) suggested several possible mechanisms by which GSH acts on target systems involved in mitosis. Both regulation of calcium uptake (via Ca^{2+}-activated ATPase) necessary for cell division, and the tubulin molecule itself, may be regulated by reduction and oxidation of sulfhydryls. In addition, accumulation of GSSG, when glutathione reductase is inhibited by copper,

may shut off protein synthesis which in turn inhibits cell division. The concentration of copper in algal cells was found to be more than sufficient to oxidise all the intracellular GSH to GSSG and that compounds which oxidised GSH also inhibited cell division in this alga (**Paper 6**).

It is also possible that copper toxicity may be due to its ability to reduce cell defence mechanisms against hydrogen peroxide and oxygen free radicals, which can inhibit algal growth (Florence *et al.*, 1984) or that copper catalyses the oxidation of membrane lipids (Hochstein *et al.*, 1980). Copper was shown to inhibit the H_2O_2-dissociating enzyme catalase, possibly by displacing iron from the active centre (**Paper 6**). Copper also has superoxide dismutase activity, leading to the formation of more H_2O_2 from the superoxide radical. Copper can also catalyse the decomposition of H_2O_2 to produce hydroxyl radical which may accelerate oxidative deterioration of membrane lipids.

Recent collaborative work with Dr Elizabetta Morelli (CNR Instituto di Biophysica, Pisa, Italy) has shown that copper in *Nitzschia closterium* induces the formation of phytochelatins, small cysteine-rich peptides involved in detoxification of copper in the cell. These peptides, formed from glutathione or its precursor gamma-glutamyl cysteine, are similar to metallothioneins induced by cadmium, copper and zinc in animals.

2.3.2 Copper complexes

Most studies of copper toxicity to aquatic organisms have assumed that the free hydrated Cu^{2+} is the most toxic physicochemical form of copper Water-soluble copper complexes including Cu-nitrilotriacetic acid have been shown to be less toxic to the marine diatom *Nitzschia closterium* than ionic copper, whereas lipid-soluble organo-copper complexes were much more toxic (Florence *et al.*, 1983). Lipid-soluble copper complexes, which occur in industrial effluents, can diffuse directly through the cell membrane into the cell, where both copper and the ligand may exert separate toxicity. Prior to our work, very little was known about the mechanism of toxicity of these lipid-soluble copper complexes

The toxicity of a wide range of lipid-soluble copper complexes to *Nitzschia closterium* was investigated (**Paper 4**). The most toxic copper complexes tested were those of the phenanthrolines, oxine, and pyridyl- and thiazolyl- hydroxyazo

compounds, possibly due to intracellular generation of hydrogen peroxide. Lipid solubility and strong chelation with copper were known to be essential for high toxicity. For example, copper(II) complexes of oxine, 4-(2-pyridylazo) naphthol (PAN) and 1-(2-thiazolylazo)-2-naphthol (TAN), were exceptionally toxic to this alga, whereas the non-chelating isomers of these ligands (4-hydroxyquinoline, 1-(3-pyridylazo) 2-naphthol and 1-(3-thiazolylazo)-2-naphthol) did not enhance copper toxicity. Addition of substituents to a ligand which increased its water solubility substantially reduced the toxicity of its copper(II) complex.

Copper(II)(oxinate)$_2$ is a common fungicide. Both oxine and its copper complex severely inhibited both photosynthesis and growth in *Nitzschia*. Copper oxinate, which was completely adsorbed by *Nitzschia* cells within a few hours, dissociated within the algal cell. Copper was retained in the cell and the oxine was expelled unchanged (**Paper 4**).

Ethylxanthate, which is used in industrial flotation processes for ore concentration, forms a toxic hydrophobic copper complex that stimulated respiration, mitochondrial electron transport, ATP formation and photosynthesis in *Nitzschia* under conditions of strongly inhibited cell division (**Paper 6**).

Phenanthroline derivatives form some of the most toxic copper complexes. The toxicity of one copper complex, copper-2,9-dimethyl-1,10-phenanthroline, to algae is described (**Paper 2**). Concentrations as low as 5 nM significantly decreased the growth of *Nitzschia closterium* Bioaccumulation of this highly toxic lipid-soluble copper complex depended on the reduction of copper(II) to (I), since only the copper(I) complex was lipophilic. It was proposed (**Paper 2**) that hydrogen peroxide, produced by the alga during photosynthesis was the reductant which rapidly reduces the copper(II)-2,9-DMP A reaction mechanism was proposed which leads to the destruction of the copper(I) complex when excess hydrogen peroxide is present and liberation of singlet oxygen, copper ions, oxygen free radicals and bipyridyl compounds, all of which contribute to the extreme toxicity of this copper complex.

2.3.3 Amelioration of copper toxicity

The toxicity of some lipid-soluble complexes can be ameliorated by humic and fulvic acids (**Paper 24**). Using a combination of algal bioassays, visible spectroscopy and anodic stripping voltammetry, it was shown that humic acid sequestered copper(II)

from the hydrophobic complex of copper oxine, releasing the ligand molecule. Toxicity was reduced providing that the ligand itself was not toxic. Humic acid, which forms more stable complexes with copper, was more effective in reducing toxicity than fulvic acid.

The toxicity of ionic copper, but not lipid-soluble complexes, can be ameliorated by trivalent metal ions such as iron, manganese, cobalt, aluminium and chromium **(Papers 1, 3 and 6)**. Measurements of intracellular and extracellular iron and copper, and algal growth rate bioassays were used to show that iron, carried over from culture media, forms a layer of iron (III) hydroxide around the algal cell. Copper is adsorbed to this colloidal iron layer at the cell surface and is therefore unable to penetrate the cell to cause a toxic effect **(Paper 1)**. This work was extended to investigate amelioration of copper toxicity by manganese **(Paper 3)** Manganese, like iron, was shown to form a layer of manganese(III) oxide around the cell which prevented copper penetration into the algal cell This research was particularly important because it disproved Sunda and Huntsman's theory (1983) that copper toxicity was caused by competition between copper and manganese for cellular uptake of manganese. It was shown that although there is competitive binding of copper and manganese at the algal cell surface, copper has no effect on intracellular manganese Thus the primary toxic effect of copper is not that it induces cellular manganese deficiency but rather that it oxidises thiol groups in the cell, disrupting cell division Manganese was particularly effective in reducing copper toxicity because it could also catalytically scavenge superoxide radicals, produced by the reduction of molecular oxygen in the chloroplast.

Further work showed that other metals including Co(III), Al(III) and Cr(III) could also protect against copper toxicity by the same mechanism, providing that they formed metal(III) hydroxides in seawater **(Paper 6)**. The degree of insolubility of the metal(III) hydroxide and its stability indicated its ability to protect against copper toxicity. Cobalt and manganese were the most effective, followed by iron, chromium and aluminium. As little as 2.5 μg Co/L, 5 μg Mn/L, 50 μg Fe/L and 100 μg Al/L reduced the toxicity of copper to *Nitzschia* by a factor of ten. Cobalt(III) was even more effective that manganese because it could also scavenge hydrogen peroxide. The trivalent metals did not afford any protection against copper to the freshwater green alga *Chlorella protothecoides*, presumably because the pH of the growth medium (7 0) was too low to promote sufficient hydrolysis of the metals.

2.4 Mechanism of Toxicity of Zinc to Algae

Few studies have investigated the mechanism of toxicity of zinc to phytoplankton. Fisher and Jones (1981) found that zinc can depress algal cell division, leading to an uncoupling of cell division and photosynthesis. Zinc can also inhibit fucoxanthin synthesis in algae (Gillan *et al.*, 1983). Some workers have suggested that zinc is bound intracellularly to protein or polyphosphate (Davies, 1978; Bates *et al.*, 1985).

I investigated the effect of zinc on cell division, photosynthesis, ultrastructure, respiration, ATP levels, mitochondrial electron transport (ETC), total thiols and glutathione in *Nitzschia closterium* (**Paper 21**). Although 65 µg Zn/L halved the cell division rate, photosynthesis and respiration were unaffected by zinc concentrations up to 500 µg/L. Most of the zinc was bound to the algal cell surface, with only 3-4% of the extracellular zinc penetrating the cell membrane. Once inside the cell, zinc exerted its toxicity at a number of sites. Zinc enhanced ATP production, ETC, cellular thiols and total glutathione in this alga, indicative of the possible production of phytochelatins to bind and detoxify zinc. Increased ATP production may provide the energy required to synthesise these metal-binding proteins, at the expense of other energy-requiring processes such as cell division. Some zinc-treated cells also contained more thylakoids in the chloroplast than control cells, although photosynthetic rates on a per cell basis were similar.

Both copper and zinc induce the production of phytochelatins in this alga. Zinc binds the phytochelatins as Zn-SH, effectively detoxifying much of the zinc, whereas copper binds and oxidises the -SH to Cu-S. This oxidation of thiols by copper alters the ratio of oxidised to reduced thiols in the cells, causing toxic effects on cell division. Thus much lower concentrations of copper are required to cause toxic effects in this species, compared to zinc.

2.5 Conclusions

My research has shown that the mechanisms of toxicity of copper and zinc to algae differs between species and is largely dependant on the chemical form of the metal With a basic understanding of modes of action of these metals, microalgae can increasingly been used as tools to assess metal bioavailability in polluted waters. By taking into account the chemical speciation of the metal, which in turn controls its bioavailability, we are now in a better position to be able to predict the toxicity of copper and zinc and to identify those factors which ameliorate toxicity in natural

waters.

Chapter 3

Development and Application of Bioassays with Unicellular Algae

3.1 Introduction

Until recently in Australia, the assessment and control of potentially toxic effluents has been based on chemical analyses. These are not only expensive, but often fail to detect all of the chemical species which may elicit a toxic response. Toxicity tests or bioassays use living organisms as indicators of the potential toxicity of a sample It has been traditional to use acute toxicity tests based on fish or invertebrate mortality, however, ethical constraints on using fish are restricting the continued use of such bioassays. Moreover, regulatory agencies worldwide now recognise the inadequate environmental protection offered by criteria based on acute lethality tests. There is now a major research thrust towards the development of bioassays based on sub-lethal or chronic responses, rather than mortality

Chronic toxicity tests with unicellular algae were originally developed by the USEPA to assess the eutrophication potential in freshwaters receiving effluents (USEPA, 1971). However, because of their sensitivity, reproducibility and ecological relevance, algal bioassays have been more widely used to assess biostimulatory and inhibitory effects of natural waters, wastewaters, specific chemicals and sediment leachates (Van Coillie *et al.*, 1983; Soniassy *et al.*, 1977; Florence *et al.*, 1994).

Species commonly used in North America include the freshwater green alga *Selenastrum capricornutum* and the marine diatom *Minutocellus polymorphus* which are not widely distributed in Australian waters. There was a need to develop sensitive tests with local species suitable for monitoring impacts in our warmer waters. This chapter describes the development of algal growth and enzyme bioassays and illustrates their use in batteries of toxicity tests for monitoring discharges for the mining and pulp/paper industries.

3.2 Development of Growth Inhibition Bioassays

Most algal bioassays are growth inhibition tests which measure the decrease in the growth rate (cell division rate) or final cell biomass after a few days in the presence of

toxicant, compared to controls in dilution water. Other effect parameters, apart from growth inhibition, have also been used, including carbon assimilation in photosynthesis (Van Coillie *et al.*, 1983; Kusk and Nyholm, 1991), respiration (**Paper 6**), ATP (Kwan, 1989; Hickey *et al.*, 1991) and enzyme activity (**Paper 21**).

Cell density measurements in growth inhibition bioassays are usually determined by counting cells in a haemocytometer under a phase contrast microscope This method is both imprecise and time consuming, so automation of the counting procedure was desirable. The counting technique was successfully automated with a Coulter Multisizer II particle analyser (**Paper 35**). This had a number of advantages over the manual techniques as both cell numbers and cell size distribution in the presence of toxicant could be determined precisely and quickly.

Development of algal growth inhibition tests with local species of marine and freshwater algae is reviewed in the context of protocols used overseas (**Paper 35**). These bioassays differed from standard USEPA protocols in the type of media used, inoculum pretreatment, incubation conditions and test duration, all factors which affect toxicity.

The algal bioassays were unique in that the tests were carried out in minimal media, without the addition of nutrients such as EDTA, silica and iron, which would complex or adsorb metals and reduce toxicity. We showed that the concentration of copper giving a 50% reduction in growth rate (EC50) of the freshwater green alga *Chlorella pyrenoidosa* increased from 16 µg/L in synthetic softwater to greater than 200 µg/L in high nutrient MBL medium (**Paper 19**). Similarly, marine medium f reduced copper and zinc toxicity to *Nitzschia closterium* by 10-20 fold compared to unsupplemented seawater For this reason, our protocols for toxicity testing with marine and freshwater algae recommended using natural filtered seawater or synthetic water enriched only with nitrate and phosphate to maximise the sensitivity of the test. Protocols in use overseas (USEPA, Environment Canada, OECD) use nutrient medium in the bioassay which grossly underestimates toxicity. Most of the toxicity data currently available for algae are therefore of limited use. With increasing use of algal bioasssays to generate toxicity data for risk assessments, such underestimation of toxicity could have dire consequences.

Another factor not usually considered in bioassay test protocols is inoculum pretreatment. For example, the toxicity of copper to *Nitzschia* depended on the concentration of iron in the culture medium used to grow the cells prior to their inoculation into the assay flasks **(Paper 1)** Cells cultured under the iron levels in normal f medium (790 µg/L) were more tolerant to copper than cells cultured in iron deficient medium Similarly, manganese, cobalt, aluminium and chromium (III) protected against copper toxicity by forming metal(III) hydroxides around the cells which adsorbed copper and reduced copper penetration into the cells **(Papers 3 and 6)**. This led to the recommendation to standardise the trace metal concentrations in culture media used to grow cells for test inocula and to wash the cells prior to use in the algal bioassays.

USEPA protocols originally used 14-day tests, which were reduced to 96-h test durations to prevent loss of toxicants by degradation or evaporation. Test duration has been further reduced to 72 h to avoid changes in pH which may alter algal metabolism and chemical behaviour of the toxicant **(Paper 35)**

The sensitivity of our algal bioassays to a range of metals and chlorophenolic compounds was compared with standard North American test species **(Paper 35)**. Our bioassays were generally more sensitive than overseas test species and also compared favourably with higher organism tests.

3.3 Development of Enzyme Inhibition Bioassays

Although growth inhibition tests are sensitive and use an ecologically relevant test endpoint, their duration of 72 h is long when rapid results are required. Serious environmental damage could be averted if the hazard of an effluent could be determined and managed in hours rather than days. There has therefore been an increasing demand for tests that are both rapid and inexpensive. Bioassays that detect sub-lethal endpoints such as inhibition of enzyme activity (Gilbert *et al.*, 1992; Jung *et al.*, 1995; Mariscal *et al.*, 1995) show promise in meeting these requirements.

The enzyme ß-D-galactosidase has recently been used in rapid toxicity tests with bacteria such as *Escherichia coli* (Dutton *et al.*, 1988; 1990) and the invertebrate *Daphnia magna* (Janssen and Persoone, 1993). ß-D-galactosidase is a catabolic enzyme that hydrolyses 1,4-ß-glucosidic linkages in carbohydrates. Specifically, this enzyme breaks down the sugar lactose into galactose and glucose.

A ß-D-galactosidase assay has been developed in our laboratory to detect coliforms and sewage pollution in seawater and drinking waters (Apte and Batley, 1994). With the recent discovery of this enzyme in some algae, enabling these algae to grow heterotrophically as well photosynthetically (Davies *et al* , 1994), it was decided to use this enzyme as the basis for developing a rapid algal toxicity test applicable to marine and freshwaters. A wide range of algae were screened for ß-D-galactosidase activity. High activity was found in two marine and three freshwater species of green algae. Of these, the marine alga *Dunaliella tertiolecta* and the freshwater alga *Chlamydomonas reinhardii*, were chosen as test species because they were available in axenic culture (bacteria-free), were amenable to automatic cell counting and were ecologically relevant to Australian waters (Report 71).

A rapid 2-h test based on enzyme inhibition in the marine alga *Dunaliella tertiolecta* was developed (**Paper 42**). The bioassay uses a fluorescent-labelled substrate 4-methylumbelliferyl ß-D-galactoside, which is cleaved by the algal enzyme and then the fluorescent moiety released into solution is detected by fluorimetry. Toxicants decrease enzyme activity and consequently less fluorescence is detected in solution. The new bioassay was particularly sensitive to metals, with 2-h EC50 values as low as 0.035 mg/L for copper. The bioassay was rapid, sensitive and reproducible, with coefficients of variation of 3-22% between experiments. Results correlated well with other ecologically relevant endpoints such as algal growth and proved superior to other enzyme assays with bacteria and invertebrates in terms of sensitivity and reproducibility The major advantage of the new test is that toxicity information can be obtained in 2 h, rather than 72 h in a typical algal growth test, making the algal enzyme bioassay a cost effective way to monitor the toxicity of a range of samples.

An improved bioassay protocol, together with its application to monitoring complex effluents and natural waters is given in Stauber *et. al* (1995). By optimising the substrate concentration, the sensitivity of the enzyme bioassay was further improved, with the EC50 for copper being reduced to 9µg Cu/L In addition, no pre-incubation with toxicant is required, further reducing the time of the bioassay to just 1 h. A similar bioassay with the freshwater green alga *Chlamydomonas reinhardii* was also developed, for application in freshwater aquatic systems.

It is proposed to further develop the rapid enzyme bioassay into a commercially-available toxicity test kit for use in field testing. Such a toxicity test will have

immediate applicability to assessing potential impacts of urban and industrial pollution. Instrumentation currently being developed by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the Australian Defence Industries (ADI) for a faecal coliform test, based on fluorescence measurement, will be adapted to the rapid algal assay. For such a test kit to be useful, techniques for preserving algae indefinitely, without loss of enzyme activity, will need to be developed.

3.4 Application of Algal Toxicity Tests

3.4.1 Introduction

The growth inhibition bioassays with the marine alga *Nitzschia closterium* have been extensively applied to monitoring the toxicity of pollutants in aquatic systems. Because different organisms from different trophic levels have different sensitivities to pollutants, algal tests have been used as part of a suite of tests for hazard assessment. **Papers 30 and 35** describe specific applications of the *Nitzschia* bioassay to assessing the toxicity of effluents, natural waters receiving industrial discharges, and ore elutriates.

3.4.2 Impacts of mining

Specific applications of the algal bioassays to the mining industry include a risk assessment of the movement of nickel ores, a study of the bioavailability of copper in the Fly River system, Papua New Guinea (PNG) and a toxicity assessment of the mine-impacted Macquarie Harbour in South-western Tasmania. Although these studies were performed under contract and were often documented in restricted reports, aspects of each study have been published in journal papers. These studies demonstrated the broad applicability of algal bioassays for assessing mine-derived contaminants in natural systems.

Toxicity of nickel ores, North Queensland

A battery of chemical and biological tests were used in a risk assessment for Queensland Nickel, who proposed to import nickel ores from New Caledonia and Indonesia through Halifax Bay in North Queensland This study investigated the potential for impacts of spilt ore on the ecological health of the Bay (**Paper 30**).

Chemical leaching studies showed that nickel and chromium (VI) were the only metals released in sufficient quantities to be of concern. The toxicity of the ore leachates,

nickel and chromium was determined using bioassays with temperate and tropical clones of the alga *Nitzschia closterium.* The algal bioassays showed that toxicity of the ore leachates was due entirely to chromium(VI) leached from the ores and that the tropical strain was less sensitive than the temperate algal strain. Similar toxicity was observed using the juvenile banana prawn *Penaeus merguiensis* and the amphipod *Allorchestes compressa* in a series of experiments carried out in collaboration with the Australian Nuclear Science and Technology Organisation. From all the toxicity data, a conservative maximum safe concentration of nickel in seawater was calculated. The nickel ore was not highly toxic and if spilt in the quantities estimated, it was predicted that it would not have a significant impact on the ecological health of the Bay.

Toxicity of copper tailings, Papua New Guinea

Ok Tedi Mining Ltd (OTML) commenced operation of a large copper mine at Mt Fubilan, Western Province, Papua New Guinea in 1984. Due to the high rainfall and geological instability of the region, it was not possible to build a tailings dam to retain waste rock and tailings from the copper mine. Consequently, 80,000 tonnes of tailings/day and 55,000 tonnes of waste rock overburden/day have been discharged directly into the Ok Tedi River. OTML is currently investigating the aquatic behaviour and fate of mine-derived sediments in the Ok Tedi/Fly River systems, with the ultimate aim of determining the bioavailability of copper released from the tailings.

Earlier studies had shown that particulate copper in this system was not toxic to invertebrates and fish (Smith, Ahsanullah and Batley, 1990). My interest was in the bioavailability of the dissolved copper, which can reach concentrations of 20 µg/L in the Fly River, hundreds of kilometres from the source of the tailings. Using bioassays with local species of copper-sensitive freshwater algae, it was possible to detect copper toxicity at dissolved copper concentrations as low as 3 µg/L if the copper in the riverwater was bioavailable. It was shown that none of the Fly River waters were toxic, with growth rates in the river waters being the same as the controls (**Paper 35**). This suggested that less than 3 µg Cu/L was bioavailable, otherwise significant decreases in algal growth would have been observed. Even waters containing up to 15 µg Cu/L were not toxic, suggesting that copper in the Fly River was complexed or bound to dissolved organic matter and unavailable for uptake into algal cells This

was a particularly significant finding for OTML because it confirmed earlier chemical studies that showed that the complexing capacity of the river waters was greater than the riverine dissolved copper concentrations. It was concluded that copper was unlikely to be available to cause an impact on aquatic organisms in this system.

Impact of copper tailings, Tasmania

The 100-year operation of the Mount Lyell Mining and Railway Co. Ltd's copper mine in Queenstown, Tasmania, has resulted in the deposition of over 100 million cubic metres of mine tailings, smelter slag and topsoil into the King River and Macquarie Harbour. Following cessation of tailings discharge into the rivers in December 1994, the Commonwealth and Tasmanian governments undertook the Mount Lyell Remediation, Research and Demonstration Program to assess the environmental impact of metal release from the mine and smelter, as part of the development of a remediation strategy. In particular, the potential biological impact of elevated copper levels in Macquarie Harbour waters was required. A suite of toxicity tests, including our *Nitzschia closterium* growth inhibition test and *Dunaliella tertiolecta* enzyme inhibition test, was used to determine the concentration of copper which could be tolerated in Macquarie Harbour waters without causing detriment to aquatic life (Report 77)

Total dissolved copper in Macquarie Harbour waters collected for use in the bioassays, ranged from 10-42 µg Cu/L, with the highest concentrations immediately below the outflow of the King River. Dissolved copper concentrations exceeded the complexing capacity of the dissolved organic matter in all samples. Electrochemical methods showed that significant amounts of dissolved copper in the Macquarie Harbour waters were present in labile forms (6-24 µg Cu/L) and potentially bioavailable.

Toxicity testing of mid-salinity Macquarie Harbour waters revealed that there were no significant effects on algal growth, amphipod and juvenile flounder survival, or osmoregulation and copper accumulation in flounder, although chemical measurements of copper in the Macquarie Harbour waters, together with the preliminary risk assessment, suggested that copper was potentially bioavailable. Of these biological endpoints, only algal growth inhibition and copper accumulation in flounder were sufficiently sensitive to detect potential effects at copper concentrations found in Macquarie Harbour

The growth inhibition bioassay with the marine alga *Nitzschia closterium* was particularly sensitive to copper, with ionic copper concentrations as low as 5 µg/L causing a significant reduction in algal cell division rate over 72 h. However, in general, no toxicity of either filtered or unfiltered Macquarie Harbour waters was observed, suggesting that copper in these waters was not present in bioavailable forms. There was, however, sufficient bioavailable copper to cause inhibition of enzyme activity (ß-D-galactosidase) in the marine alga *Dunaliella tertiolecta* and this bioavailable copper was weakly correlated with labile copper in the Macquarie Harbour waters measured using anodic stripping voltammetry. It is possible that colloidal iron, manganese and aluminium oxyhydroxides bind copper at the algal cell surface, preventing its uptake into the cell Enzyme inhibition may occur at the cell membrane, whereas growth is not affected because copper adsorbed to metal hydroxides cannot penetrate the cell membrane.

Taking into account these results, and using the results of the risk analysis of literature values of copper toxicity in marine and estuarine waters, we estimated that the maximum acceptable concentration of copper in Macquarie Harbour mid-depth waters lies between 10 and 20 µg/L. This study indicated the useful information that a combination of bioassays can provide and highlighted the value of such data in risk prediction.

3.5 Conclusions

Both the marine and freshwater algal bioassays developed in our laboratory are an important component of batteries of tests with organisms from all trophic levels for use in environmental risk assessments. Their sensitivity and reproducibility makes them a convenient and cost effective tool for monitoring the potential toxicity of chemicals, complex effluents and polluted natural waters. With increasing restrictions by animal ethics legislation on the use of fish lethality tests, such short-term chronic microbial tests will play an important role in the protection of the aquatic environment in future.

Chapter 4

Toxicity of Metals to Humans

4.1 Introduction

While much of my research focused on the development of techniques for understanding the mechanisms of toxicity of metals to simple organisms such as algae, this work was extended to developing novel monitoring methods for metals in humans. With the increase, throughout the world, of blood-related communicable diseases, non-invasive techniques for biological monitoring were required. In particular, there was a need for methods for analysing metals in non-invasive samples such as urine, faeces, hair, saliva and sweat, for comparison to blood analyses to determine body burdens of metals.

This chapter describes the development of methods for analysing metals in urine, sweat and hair, with particular emphasis on lead and manganese. These techniques have been used to study skin absorption of lead in occupationally-exposed workers and in the study of manganese toxicity in aborigines from Groote Eylandt, Northern Territory, Australia.

4.2 Lead

The major sources of lead exposure for humans are inhalation and ingestion. Skin absorption has been largely ignored, although considerable quantities of lead from sources such as soil, water and dust, and from workplace exposure, can be in continuous contact with the skin.

Lead can affect the nervous system, bone formation, haem synthesis, kidney function and reproduction. It accumulates in red blood cells (3%), soft tissue (up to 5%) and bones and teeth (90-95%), with lead excretion via the kidneys, renal tubules, sweat, hair, nails and through the gut (Coyle *et al.*, 1994). Recent studies have suggested that even small intakes of lead can reduce intelligence in children (McMichael *et al.*, 1988) A 10 µg/dL increase in blood lead concentration has been associated with a decrease between 2-8 IQ points in young children.

4.2.1 Development of biological monitoring techniques

Electrochemical techniques such as anodic stripping voltammetry (ASV) have been widely used for measuring toxic metals in body fluids, including blood, sweat and urine However, direct determination of metals in urine posed considerable problems in electroanalysis due to its high organic content which leads to adsorption at the electrode and suppression of ASV waves. More usually, a pre-treatment step such as acid digestion to remove organic interferences is required.

A novel method was developed for the direct determination of lead in urine (**Paper 22**). Fumed silica, added to urine directly in the ASV cell, completely removed sorption interferences by urinary organic constituents This simple, rapid method was successfully used to measure lead in urine at the µg/L level in unexposed individuals.

While chloride levels in sweat have been used extensively for the detection of cystic fibrosis in newborn babies, metals in sweat have only recently been used to indicate nutritional status and toxicity in humans There had been little previous interest in the determination of trace metals in sweat due to the problems of collecting uncontaminated samples of sufficient size for analysis. A technique was developed for collecting sweat onto membrane filters using pilocarpine iontophoresis (**Paper 7**). After leaching the membrane filter, zinc, copper, cadmium and lead in sweat could be determined using ASV. Choice of the membrane filter type was critical to prevent adsorption of the metals and to avoid interferences by wetting agents. This method was used to compare sweat metal levels in males and females It was hoped to use metals in sweat as a convenient non-invasive technique for measuring occupational exposure to toxic heavy metals and to identify abnormal copper/zinc ratios in various disease states in humans.

4.2.2 Application to monitoring toxic metals such as lead in humans

A new method for determining metals in sweat after collection by pilocarpine iontophoresis was used to provide background data on the levels of copper, cadmium, zinc and lead in 63 volunteers (**Paper 12**). Copper, cadmium, zinc and lead in human sweat were compared with ceruloplasmin, alkaline phosphatase, and total and mobile metal levels in blood sera in males and females. Although concentrations of lead and cadmium in serum and sweat were similar, zinc and copper in sweat were much lower than in serum. This lack of correlation for zinc and copper limited the usefulness of

sweat as as surrogate for blood measurements Sweat was identified as an important route of excretion for heavy metals, as high metal concentrations were detected in the sweat of exposed individuals when blood concentrations were in the normal range. This suggested that sweat may be a useful tool for screening occupationally-exposed workers.

While pursuing the usefulness of sweat as a monitoring tool, it was discovered that some lead workers had extremely high levels of lead in their sweat. To avoid surface contamination of the skin during sweat collection, a number of skin washing procedures were tested. During these control experiments, it was noticed that if lead was applied to the skin surface on one arm, high levels of lead were detected in sweat collected from the opposite arm, suggesting that inorganic lead was absorbed through the skin and circulated throughout the body in the blood and lymph systems (**Papers 13 and 14**).

This work represented a major breakthrough in understanding the routes of exposure for inorganic lead. Skin absorption of lead was not usually considered to be a significant mode of uptake unless the lead was present as lipid-soluble organic complexes, such as tetraethyl lead (Rastogi and Clausen, 1976). However, a variety of inorganic lead compounds (lead chloride, lead nitrate, lead acetate, lead oxide and finely powdered lead metal) were shown to be rapidly absorbed through human skin *in vivo* (**Paper 13**) When solutions of the lead compound were placed on a small area of forearm skin for 1-24 h, increased concentrations of lead were subsequently found in sweat and saliva within one hour of skin application However, no increase in lead in blood or urine was detected using anodic stripping voltammetry.

The stable lead isotope ^{204}Pb was used with more sensitive analytical techniques (thermal ionisation mass spectrometry and inductively coupled plasma mass spectrometry), to show that skin-absorbed lead was detectable in blood and urine within 6 h of skin application (**Papers 26 and 29**). Increases in ^{204}Pb concentration and abundance in blood and urine were found, however, no increase in *total* lead in blood was detected. It was proposed that trans-dermal transport of lead occurred by two distinct mechanisms: rapid diffusion through the sweat glands and hair follicles, and slower diffusion through the stratum corneum, the skin's upper layer

Although skin-absorbed lead appeared in high concentrations in sweat and saliva, very little was detectable in blood. Similarly, Moore *et al* (1980) found only minute quantities of lead in blood (0-0.3% of the applied dose) in human volunteers using hair dyes containing lead acetate. It appears that lead absorbed through the skin was in a different physico-chemical form (presumably a protein complex) which has a low affinity for red blood cells, but partitions strongly into extracellular fluid. This was quite different to the behaviour of inorganic lead salts injected directly into the blood stream, where 50% of the lead is associated with red blood cells after 1 h (Chamberlain, 1985, Campbell *et al.*, 1984).

Although these human experiments established that inorganic lead compounds can be absorbed through the skin, the *fate* of skin-absorbed lead was unknown. If all the absorbed lead is exported from the body via sweat and saliva, it is unlikely that skin-absorbed lead would be a health risk to occupationally-exposed workers. However, if a significant fraction is retained in organs, tissues or bones, deleterious health effects may result. In order to study the fate of skin-absorbed lead, animal studies were required. With the help of an ANZECC grant, the fate of skin-absorbed lead was compared with intravenous (i.v.) -injected lead in mice. Skin-absorbed lead concentrated in extracellular fluid, skin and muscle remote from the site of application and was not detectable in blood, confirming our previous work with humans. In contrast, i.v.-injected lead concentrated in blood and other organs **(Paper 44)**.

The ability of lead salts to enter the body through the skin without significantly raising blood lead levels has important implications for occupational health control because measurement of lead in blood is the main criterion used to assess lead exposure. The inadequacy of blood lead measurements to detect lead which has been absorbed through the skin highlights the dangers of only using this monitoring technique to determine lead body burden and lead exposure Consequently, other biological monitoring techniques which can detect skin-absorbed lead need to be developed.

4.3 Manganese

Until recently, manganese salts were generally regarded as being of low toxicity to humans. Manganese has recently been implicated in a number of neurological diseases in occupationally-exposed workers, including manganese miners in Chile who suffered similar symptoms to Parkinson's disease. Manganese is absorbed through the lungs and gastrointestinal tract and deposited in tissues, particularly liver, kidney,

and brain. Manganese is excreted rapidly in the faeces via bile, with very little excreted in urine. Because of its short biological half-life (4 days for the fast fraction and 40 days for the slow fraction) monitoring manganese at the low concentrations found in blood and urine, can only indicate relatively recent exposure.

The most convenient record of manganese exposure over a few months is the manganese concentration in scalp hair. However, problems with using scalp hair include pre-treatment of the hair before manganese analysis, and the need to distinguish between endogenous (manganese that enters the hair via the blood supply to the hair follicle) and exogenous sources of manganese, including dust and sweat.

A washing procedure was developed for the pre-treatment of hair before analysis of metals, including manganese, by ASV and neutron activation analysis (**Paper 8**). This washing effectively removed all surface dust and gave reproducible results for multiple hair samples from the same person Manganese in dust trapped in the hair before sampling may be solubilised in sweat, incorporated into the hair shaft and measured as endogenous manganese Up to 15 μg Mn/g hair could be incorporated into hair via this route. To obtain reliable estimates of manganese taken up from blood, manganese in hair from these exogenous sources must be eliminated The contribution of exogenous manganese to total manganese in hair was investigated and a number of methods proposed to overcome this problem (**Papers 8 and 17**). Selective leaching of hair with chemical chelators, thiols, acids and solvents was unsuccessful because these agents also removed endogenous manganese in hair. More useful was an extrapolation technique in which manganese along the length of the hair strands were analysed and extrapolated back to zero length to estimate the amount of manganese in the hair as it emerged from the scalp Using this technique, manganese was determined in the scalp hair of aborigines living on Groote Eylandt.

Aborigines from Angurugu, a small township on Groote Eylandt in the Gulf of Carpentaria, have a high incidence of neurological disturbances, referred to as the Groote Eylandt Syndromes (Cawte, 1984). One syndrome, probably a form of motor neurone disease, is characterised by weakness in the legs and onset occurs in early childhood. The second syndrome is characterised by a cerebellar syndrome and oculomotor disturbances, with onset in middle age. It has been suggested that this endemic disease may be caused by environmental manganese, as similar neurological symptoms have been associated with Chilean miners suffering from manganese

poisoning (Cotzias *et al.*, 1968). The Angurugu population is exposed to an environment naturally high in manganese, with soils containing up to 4% manganese. In addition, the Groote Eylandt Mining Company (GEMCO) has mined manganese since the early 1960s.

Manganese, as a potential causative agent in the Groote Eylandt syndromes, was investigated (**Papers 8, 11 and 17**). Manganese body burdens in both unaffected and affected aborigines, together with caucasians in the township, were estimated using analyses of blood, urine, sweat and hair. Aborigines had scalp hair manganese concentrations which were much higher than aborigines living in other parts of Northern Australia, however there was no significant difference in hair manganese between affected and unaffected individuals. Those with the disease, however, had elevated levels of manganese in blood.

It was concluded that all Angurugu aborigines had a high intake of manganese due to their "close to the earth living", relatively poor hygiene and their diet. Only some aborigines developed the disease however, and these individuals were closely related, suggesting that at least some genetic factor was also involved. It is possible that some susceptible individuals had genetic/metabolic defects which either enhanced their uptake of manganese or reduced their ability to clear manganese from the body. While manganese was strongly implicated in the disease, a causal-effect relationship was not clearly established

In order to understand the possible role of manganese in the Groote Eylandt syndromes, the mechanism of manganese toxicity was investigated (**Paper 18**). The neurotoxicity of manganese was believed to result from its catalysis of the oxidation of dopamine by oxygen, causing depletion of this neurotransmitter In the presence of manganese, oxidation of dopamine by oxygen proceeds via a manganese(II)/(III) redox couple, the superoxide radical and a semiquinone intermediate Neurotoxicity of manganese results from depletion of dopamine and from the production of neurotoxic oxidation products dopamine quinone and hydrogen peroxide.

The ability of a wide range of compounds to inhibit dopamine oxidation in vitro was examined (**Paper 15**). Some electrophilic compounds such as ascorbic acid (vitamin C) and thiamine (vitamin B1) effectively inhibited dopamine oxidation by stabilising the intermediate semiquinone free radical. Groote Eylandt Aborigines, who are likely

to be deficient in vitamin C and thiamine at some stages of the year due to poor diet and alcohol intake, may therefore be predisposed to manganese intoxication The significance of this work is reflected in its publication in the highly prestigious medical journal The Lancet (**Paper 15**).

4.4 Conclusions

This novel research on the uptake and mechanism of toxicity of lead and manganese has important implications in human health. It identified skin absorption as a new route for lead exposure in humans. The National Institute of Occupational Safety and Health in the USA has recognised this work and has now introduced safety precautions for occupationally-exposed workers to prevent lead uptake through the skin. Because lead which is skin-absorbed is not detectable in blood, new monitoring procedures to identify skin-absorbed lead in the body are urgently required Manganese is now recognised as a potential toxicant and precautions, such as minimising manganese dust exposure, are being implemented throughout mining communities.

Chapter 5

Extent of Originality and Participation in Collaborative Research

Many of the publications used as the basis for this thesis involved co-authorship In most cases, I was the senior and corresponding author. In some instances, the co-authors were staff undertaking research under my direction, while in others the collaboration involved scientists who contributed a new and complementary expertise to the study. In all cases, I made original and significant contributions to the research. In general, a listing as first author in the research papers indicates the person who wrote the majority of the paper.

Much of my initial research was undertaken jointly with Dr Mark Florence, who was my immediate supervisor until 1989. His expertise in analytical and environmental chemistry complemented my own background in biochemistry and microbiology. Together we made a number of significant advances in the understanding of mechanisms of metal toxicity in microalgae and humans.

My research interest in the development of new bioassay techniques has increasingly extended beyond the chemical focus of my joint work with Florence, and encompasses aspects of ecotoxicology including plant, invertebrate and fish bioassays I have co-ordinated a number of large multi-disciplinary research projects for the National Pulp Mills Research Program, the Australian pulp/paper industry, state government departments and mining companies, involving collaboration with researchers in universities and environmental protection agencies throughout Australia. I was the senior author in joint publications arising from these collaborations and was responsible for the research program, interpreting the data and writing the final papers and reports.

My contribution to each of these joint publications is summarised in the following table

Table 1. Contribution of the Candidate to Joint Publications Submitted as Part of this Thesis

Publication No.	Authors	Title	Contribution of the Candidate	Summary of Candidate's contribution
1	Stauber and Florence	The influence of iron on copper toxicity to the marine diatom *Nitzschia closterium*.	I planned the research, carried out the experimental work, interpreted the results and wrote the paper.	Major
2	Florence, Stauber and Mann	The reaction of copper-2,9-dimethyl-1,10-phenanthroline with hydrogen peroxide	I carried out part of the experimental work and interpreted the appropriate parts of the results.	Minor
3	Stauber and Florence	Interactions of copper and manganese: a mechanism by which manganese alleviates copper toxicity to the marine diatom *Nitzschia closterium*	I planned the research, carried out the experimental work, interpreted the results and wrote the paper	Major
4	Florence and Stauber	Toxicity of copper complexes to the marine diatom *Nitzschia closterium*	I carried out the experimental work, and interpreted and discussed the results.	Substantial
5	Stauber and Florence	Reversibility of copper-thiol binding in *Nitzschia closterium*	I planned the research, carried out the experimental work, interpreted the results and wrote the paper.	Major
6	Stauber and Florence	The mechanism of toxicity of ionic copper and copper complexes to algae.	I planned the research, carried out the experimental work, interpreted the results and wrote the paper.	Major

Publication No.	Authors	Title	Contribution of the Candidate	Summary of Candidate's contribution
7	Stauber and Florence	The determination of trace metals in sweat by anodic stripping voltammetry	I planned the research, carried out the experimental work, interpreted the results and wrote the paper.	Major
8	Stauber, Florence and Webster	The use of scalp hair to monitor manganese in aborigines from Groote Eylandt	I planned the research, carried out the experimental work, interpreted the results and wrote the paper.	Major
11	Florence, Stauber and Fardy	Ecological studies of manganese on Groote Eylandt	I carried out the experimental work, and interpreted the results.	Minor
12	Stauber and Florence	A comparative study of copper, lead, cadmium and zinc in human sweat and blood	I planned the research, carried out the experimental work, interpreted the results and wrote the paper.	Major
13	Lilley, Florence and Stauber	The use of sweat to monitor lead absorption through the skin	I initially planned the research, and helped supervise the experimental work. The research was funded by a Worksafe Grant.	Minor
14	Florence, Lilley and Stauber	Skin absorption of lead	I initially planned the research, and helped supervise the experimental work.	Minor
15	Florence and Stauber	Neurotoxicity of manganese	I planned the research, carried out the experimental work and interpreted the results.	Substantial

Publication No.	Authors	Title	Contribution of the Candidate	Summary of Candidate's Contribution
17	Stauber and Florence	Manganese in scalp hair: problems of exogenous manganese and implications for manganese monitoring in Groote Eylandt Aborigines	I planned the research, carried out the experimental work, interpreted the results and wrote the paper	Major
18	Florence and Stauber	Manganese catalysis of dopamine oxidation	I carried out the experimental work and interpreted the results.	Substantial
19	Stauber and Florence	The effect of culture medium on metal toxicity to the marine diatom *Nitzschia closterium*	I planned the research, carried out the experimental work, interpreted the results and wrote the paper.	Major
21	Stauber and Florence	Mechanism of toxicity of zinc to the marine diatom *Nitzschia closterium*	I initiated and planned the research, carried out the experimental work, interpreted the results and wrote the paper.	Major
22	Stauber and Florence	Fumed silica for the direct determination of lead in urine by differential pulse ASV	I carried out the experimental work, interpreted the results and wrote the paper.	Major

Publication No.	Authors	Title	Contribution of the Candidate	Summary of Candidate's Contribution
24	Florence, Powell, Stauber, Town	Toxicity of lipid-soluble copper(II) complexes to the marine diatom *Nitzschia closterium:* amelioration by humic substances	This research was part of a PhD project by Town. I helped supervise the algal part of the work and helped in interpretation of these results.	Minor
25	Florence, Morrison and Stauber	Determination of trace element speciation and the role of speciation in aquatic toxicity	I carried out some of the experimental work used in this review and contributed to the ideas through discussions with the major authors.	Minor
26	Dale, Stauber, Farrell, Florence and Gulson	A comparative study of ICPMS and TIMS for measuring skin absorption of lead	I initiated and planned the research, supervised or carried out part of the experimental work, interpreted the results and helped write the paper.	Major
29	Stauber, Florence, Gulson and Dale	Percutaneous absorption of inorganic lead compounds	I initiated and planned the research, supervised or carried out the experimental work, interpreted the results and wrote the paper.	Major
30	Florence, Stauber and Ahsanullah	Toxicity of nickel ores to marine organisms	I planned and carried out the experimental work, helped interpret the results and wrote the paper.	Major

Publication No.	Authors	Title	Contribution of the Candidate	Summary of Candidate's Contribution
35	Stauber	Toxicity testing using marine and freshwater unicellular algae	This was an invited review for the first issue of the new Australasian Journal of Ecotoxicology	Sole author
42	Peterson and Stauber	A new algal enzyme bioassay for the rapid assessment of aquatic toxicity	This paper describes research undertaken with an Environmental Trust Grant from the NSWEPA. I obtained the grant, planned the experimental work, supervised the project, interpreted the results and co-wrote the paper.	Major
44	Florence, Stauber, Dale, Henderson, Izard and Belbin	Skin absorption of lead compounds	This research was funded by ANZECC. I obtained the funding, helped design the experimental program, supervised the work, interpreted the results and wrote the final report to ANZECC.	Major

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Appendix 1

List of Research Publications - J. L. Stauber

The following is a complete listing of the research publications of the candidate. Publications in bold type in Part A are those which relate specifically to the topic of this submission, and are included in full in Appendix 2. Part B is a listing of reports prepared as part of the CSIRO report series. These reports include those in the public domain, together with restricted reports (denoted by an R in the report number) prepared for companies under commercial-in-confidence arrangements

A. External Publications

1. **Stauber, J.L. and Florence, T.M. (1985) The influence of iron on copper toxicity to the marine diatom *Nitzschia closterium* (Ehrenberg) W. Smith. *Aquatic Toxicology* 6, 297-305.**
2. **Florence, T.M., Stauber, J.L. and Mann, K.J. (1985) The reaction of copper-2,9-dimethyl-1, 10-phenanthroline with hydrogen peroxide. *Journal of Inorganic Biochemistry* 24, 243-254.**
3. **Stauber, J.L. and Florence, T.M. (1985) Interactions of copper and manganese: A mechanism by which manganese alleviates copper toxicity to the marine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith. *Aquatic Toxicology* 7, 241-254.**
4. **Florence, T.M. and Stauber, J.L. (1986) Toxicity of copper complexes to the marine diatom *Nitzschia closterium*. *Aquatic Toxicology* 8, 11-26.**
5. **Stauber, J.L. and Florence, T.M. (1986) Reversibility of copper-thiol binding in *Nitzschia closterium* and *Chlorella pyrenoidosa*. *Aquatic Toxicology* 8, 223-229.**
6. **Stauber, J.L. and Florence, T.M. (1987) The mechanism of toxicity of ionic copper and copper complexes to algae. *Marine Biology* 94, 511-519.**
7. **Stauber, J.L. and Florence, T.M. (1987) The determination of trace metals in sweat by anodic stripping voltammetry. *The Science of the Total Environment* 60, 263-271.**
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17. Stauber, J.L. and Florence, T.M. (1989) Manganese in scalp hair: problems of exogenous manganese and implications for manganese monitoring in Groote Eylandt Aborigines. *The Science of the Total Environment* 83, 85-98.

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65. Vaughan, G.T. and Stauber, J.L. (1994) Technical review of the potential application of ecotoxicity tests in the risk assessment of multiple-contaminated sites CSIRO Investigaton Report CET/IR289, 117 pages .

66. Abel, K., Nelson, P., Johannes, B , Stauber, J., Vaughan, G., Bond, W., Kookana, R. and Rogers, S (1994) Bagasse pulp mill effluent. literature review CSIRO Report, May 1994, 108 pages

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72. Ahsanullah, M , Florence, T M. and Stauber, J.L. (1995) Ecotoxicology of copper to marine and brackish water organisms. Report CET/IR402, 69 pages.

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Appendix 2

Principal Publications In Full

Paper 1

Aquatic Toxicology, 6 (1985) 297–305
Elsevier

AQT 00158

THE INFLUENCE OF IRON ON COPPER TOXICITY TO THE MARINE DIATOM, *NITZSCHIA CLOSTERIUM* (EHRENBERG) W. SMITH

J.L. STAUBER and T.M. FLORENCE

CSIRO Division of Energy Chemistry, Private Mail Bag 7, Sutherland, N.S W., 2232, Australia

(Received 19 December 1984; accepted 25 March 1985)

The effect of iron on the toxicity of copper (0–60 μg Cu l^{-1}) to the marine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith was studied using unsupplemented sea water for growth rate experiments. Intracellular and extracellular iron and copper concentrations were determined by inductively coupled plasma emission spectrometry and anodic stripping voltammetry.

Cells cultured under normal f medium iron levels (790 μg Fe $\cdot l^{-1}$) were more tolerant to copper than cells cultured in iron deficient medium (79 μg Fe l^{-1} and 7.9 μg Fe $\cdot l^{-1}$). Lower extracellular iron and higher intracellular copper concentrations were found in cells grown previously in iron deficient medium compared to cells grown in normal iron medium. Iron, however, had no effect on the toxicity of the lipid soluble copper complex, copper–oxine

It is proposed that copper toxicity is reduced by colloidal ferric hydroxide, carried over in the assay inoculum, which binds to the diatom cell membrane, absorbs copper and prevents copper penetration into the cell.

Key words: *Nitzschia closterium*; iron; copper; toxicity

INTRODUCTION

Copper, at concentrations as low as 1 ppb, has been shown to be toxic to a number of marine unicellular algae (Mandelli, 1969; Erickson, 1972; Lumsden and Florence, 1983). Copper reacts at the cell membrane, affecting cell division and permeability. Within the cell, copper may inactivate the Hill reaction in photosynthesis, or it may inactivate essential enzymes by displacing the active metal or binding sulphydryl, amino or carboxylic groups (Huntsman and Sunda, 1980).

Copper toxicity to a species depends on growth conditions – cell density, composition of the medium, including salinity and pH, and culture conditions including light, light/dark cycles and temperature (Whitton, 1968; Mandelli, 1969). In particular, addition of certain micronutrients to the medium can partially overcome the inhibitory effects of copper and other trace metals, e.g. iron, and chelators such as citric acid and EDTA.

The toxicity of copper to estuarine algae may be reduced due to the presence of iron and organic chelators, e.g. humic and fulvic acids, from land runoff, compared to algae growing in the open ocean environment. Iron at a level of 1 mg $\cdot l^{-1}$ has

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been shown to reduce cadmium toxicity to *Chlorella pyrenoidosa* (Gipps and Coller, 1982).

Steeman Nielsen and Kamp-Nielsen (1970) suggested that the influence of iron on copper toxicity may be due to the adsorption of copper onto colloidal ferric hydroxide in the alkaline growth medium. Hydrous ferric oxide may also adhere to the surfaces of diatoms by intermolecular attraction (Davies, 1966). It has also been suggested that iron might compete with copper for binding sites on the cell membrane (Kanazawa and Kanazawa, 1969).

Increased toxicity of copper in cultures of *Nitzschia closterium* grown under low iron conditions has been noted elsewhere (Lumsden and Florence, 1983). It is possible that colloidal ferric hydroxide bound to the organic layer surrounding the diatom silica frustule, may prevent penetration of copper into the cell. This hypothesis was tested by growing the diatom *N. closterium* in culture media of varying iron concentrations, before exposure to copper concentrations of 0–60 $\mu g\ Cu \cdot l^{-1}$ in unsupplemented sea water, and measurement of intracellular and extracellular iron and copper.

MATERIALS AND METHODS

Sea water

Surface sea water was collected off Port Hacking, New South Wales, in carefully cleaned polyethylene bottles and passed immediately through a 0.45 μm filter. Filtered sea water was stored at 4°C until used in the culture medium and algal assays. Total copper was in the range 0.07–0.3 $\mu g \cdot l^{-1}$ and total iron was 1.2–2.0 $\mu g \cdot l^{-1}$.

Algae maintenance

The unicellular marine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith[a] (originally obtained from CSIRO Division of Fisheries Algal Culture Collection) was cultured in f medium (Guillard and Ryther, 1962), except that ferric citrate/citric acid (4.5 $mg \cdot l^{-1}$ ferric citrate $\cdot 7\ H_2O$ + 4.5 $mg \cdot l^{-1}$ citric acid) replaced iron–EDTA, and the trace metal concentration was halved. The algal cultures were maintained at 21°C, on a 12 h light/dark cycle at 6,400 lux, transfer being made under axenic conditions every 2 wk.

Cultures for the algal assays were maintained in f medium, minus the trace metals, at three different iron concentrations (iron added as ferric citrate/citric acid in a 1:1.6 mole ratio): 'normal' iron – 790 $\mu g\ Fe \cdot l^{-1}$; iron/10 – 79 $\mu g\ Fe \cdot l^{-1}$; iron/100 – 7.9 $\mu g\ Fe \cdot l^{-1}$. Cells in exponential phase growth were used for all experiments.

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[a] The name *Nitzschia closterium*, rather than *Cylindrotheca closterium*, was used in agreement with Hasle (1964).

The effect of different forms of iron on growth was also determined by adding iron (750 μg Fe $\cdot$ l^{-1}) as ferric citrate/citric acid, ferric citrate alone, ferric chloride, iron–EDTA complex and iron–desferrioxamine complex. Age and size of inoculum, together with medium age after autoclaving were kept constant (Levandowsky and Hutner, 1975).

Algal assays

These were carried out in siliconised 150-ml conical flasks, containing 50 ml of sea water with an initial cell density of 2.75 $\times$ 10^4 cells $\cdot$ ml^{-1}, according to the method of Lumsden and Florence (1983). The inoculum was washed three times in sea water by centrifugation before use. Cultures were incubated at 21°C at 16,000 lux. Cell density was measured daily for 3 days, by counting microscopically in a haemocytometer. A regression line was fitted to a plot of $\log_{10}$ (cell density/initial cell density) versus time (in hours) for each sample, and the growth rate determined.

Iron determinations

Iron on the cells (intracellular and extracellular), in the medium, and on the walls of the culture flask, was determined by inductively coupled plasma emission spectrometry (ICP). A 12 ml aliquot of the algae inoculum, to be used in the algal assays, was centrifuged for 10 min at medium speed. The supernatant (10 ml) was acidified with 0.1 ml of 10 M HCl, and used directly to determine the iron in the medium by ICP. The cell pellet was resuspended in 0.5 ml of sea water and transferred to a covered 50-ml beaker for acid digestion. Cells were digested with 1 ml of 15 M HNO_3 and 0.5 ml of 72% $HClO_4$ to strong fumes of $HClO_4$, then diluted to 10 ml for iron determination on the cells. Iron in the f medium, to which no iron had been added, was also determined. Reagent blanks were subtracted from all iron measurements.

Intracellular and extracellular iron was determined by centrifuging 2 $\times$ 12 ml aliquots of *N. closterium* suspension. One aliquot was resuspended in culture medium, and the other was resuspended in 10 ml 0.02 M EDTA in sea water. The EDTA wash removed iron attached to the outside of the cells, without causing cell lysis. This enabled intracellular or metabolic iron (Davies, 1970) to be determined. After standing for 10 min, the tubes were centrifuged and the cells resuspended in 0.5 ml sea water, then transferred to a covered 50-ml beaker for acid digestion and iron determination as above.

A significant fraction of the iron adhered to the glass walls of the culture flask (Hayward, 1968; Lewin and Chen, 1971, 1973). The culture flask was emptied, and, after rinsing with distilled water, 10 ml of hot 2 M HCl was added. After transfer to a 10-ml volumetric, iron was determined by ICP. The error in iron determination by ICP was 1–5% for iron on the cells, and 10–20% for iron in the supernatant. All glassware was acid washed before use.

Iron associated with the cells during the assay was also determined. Before the algal assay, cells were grown in f medium containing radioactively labelled iron - ^{59}Fe (634 $\mu g\ Fe \cdot l^{-1}$). Aliquots taken over the 3-day assay were counted in a gamma well counter for 30 min to determine iron on the cells, in the supernatant and on the glass.

Copper determinations

Labile and total copper on algae were determined by anodic stripping voltammetry (a.s.v.) (E.G. and G. Princeton Applied Research model 384 Polarographic Analyser, with a model 303 static mercury drop electrode assembly). All analytical procedures were carried out at 25 ± 0.5°C in a class 100 clean room.

At the end of the assay (day 3), copper on the algae was determined by passing 20 ml of algal suspension through an acid-washed, 25 mm diameter 0.45 μm membrane filter. The filter was digested in a 50-ml covered beaker in a clean air cupboard with 1 ml of 15 M HNO_3 and 0.5 ml 72% $HClO_4$ to strong fumes of perchloric acid, then diluted to 10 ml with water. Copper was determined directly on this solution. An acid-washed membrane filter was used as a blank.

Intracellular copper concentration was measured by centrifuging 11 ml of algal suspension, resuspending in 0.02 M EDTA in sea water to remove copper adhering to the outside of the cells, passing through an acid-washed 0.45 μm filter and digesting with acid as above. The extracellular copper concentration was determined by subtracting the intracellular copper concentration from the total copper bound to the cells.

Total copper in the filtrate was determined by a.s.v. after adding 0.2 ml 4 M HNO_3 and 0.05 ml 30% H_2O_2 to 10 ml of filtrate, and irradiating with a 550 W mercury lamp for 12 hours. Sodium acetate (0.4 ml of 4 M) and 0.4 ml of 2% $(NH_2OH)_2 \cdot H_2SO_4$ were added to the irradiation tubes, which were heated in a boiling water bath for 20 minutes and cooled before measurement by a.s.v. (Florence et al., 1983).

RESULTS

N. closterium cells cultured under low iron conditions before the assay, were more sensitive to copper than cells cultured previously in 'normal' (f) iron medium (Table I). Control cultures, not exposed to copper, had a specific growth rate of 0.84 divisions/day.

Iron content of the cells

The iron content of the cells increased with increasing iron concentration in the medium (Table II) in agreement with the work of Hayward (1968). In particular,

TABLE I

Toxicity of copper to *Nitzschia closterium*, grown at iron concentrations from 7.9 to 790 μg Fe l^{-1}

Culture	Iron concentration in medium ($\mu g \cdot l^{-1}$)	Cell density after 3 days (20 μg Cu $\cdot l^{-1}$ added) (cells ml^{-1})	Copper concentration causing 50% inhibition of growth rate ($\mu g\ l^{-1}$)
Normal iron (+ trace metals)	790	9.98×10^4	> 50
Normal iron (− trace metals)	790	9.00×10^4	> 50
Iron/10 (− trace metals)	79	5.02×10^4	20
Iron/100 (− trace metals)	7.9	2.75×10^4	6

extracellular iron increased from 2×10^{-5} ng/cell in low iron-grown cells (7.9 μg Fe $\cdot l^{-1}$) to 56×10^{-5} ng/cell in 'normal' iron cells (790 μg Fe $\cdot l^{-1}$). Intracellular iron concentrations were somewhat lower in low iron cultures than in the 'normal' iron culture.

Mass balance was obtained between iron added to the flask (from medium and inoculum) and iron recovered (iron on cells, iron in medium and iron on flask) at the three iron growth levels used.

Copper content of the cells

Total copper bound to the cells varied between 55 and 68% of total copper added, and was not significantly affected by the iron concentration in the culture medium or by added copper in the range 10–50 $\mu g \cdot l^{-1}$.

TABLE II

Intracellular and extracellular iron content of *Nitzschia closterium*.

Culture	Iron content ($\mu g \cdot l^{-1}$)										Iron content ($\times 10^{-5}$ ng/cell)	
	Iron added				Iron recovered				Intracellular iron	Extracellular iron	Intracellular iron	Extracellular iron
	Iron stock	Iron in medium	Iron on inoculum	Total	Iron on cells	Iron in medium	Iron on glass	Total				
Normal iron	790	53	2	845	763	6	40	809	173	590	16	56
Iron/10	79	51	1	131	115	12	8	135	29	86	4	11
Iron/100	7.9	51	1	60	39	11	20	70	31	8	7	2

Intracellular copper concentration was highest in cells previously cultured in the lowest iron medium (iron/100), and ranged from 12.0 down to 3.3 $\mu g\ Cu \cdot l^{-1}$ in 'normal' iron-grown cells. Extracellular copper was highest in 'normal' iron-grown cells (6.9 $\mu g\ Cu \cdot l^{-1}$) and lowest (0.5 $\mu g\ Cu \cdot l^{-}$) in iron/100 cells (Table III).

On a per cell basis, the same trend is observed, with greater intracellular copper concentration (30×10^{-5} ng/cell) in lowest iron-grown cells. Less copper penetrates the cell when grown previously in 'normal' iron medium (2.8×10^{-5} ng Cu/cell).

Although extracellular copper ($\mu g \cdot l^{-1}$) decreased as iron concentration decreased, extracellular copper on a per cell basis in the iron/10 culture was higher than expected. This may be due to the lower cell density in this culture (Table I); i.e., the same amount of copper is bound to fewer cells, leading to higher extracellular copper when expressed per cell. In the iron/100 culture, a similar amount of copper is bound, but more penetrates the cell (Table III), therefore extracellular copper is again low (1.3×10^{-5} ng/cell).

To determine whether copper interferes with iron uptake into the cell, iron-limited cells (grown in 7.9 $\mu g\ Fe \cdot l^{-1}$) were assayed in the presence of 790 $\mu g\ Fe \cdot l^{-1}$ and copper in the range 0–70 $\mu g \cdot l^{-1}$. Both intracellular and extracellular iron were similar at all copper concentrations, showing that copper does not inhibit cellular iron uptake in this species.

Effect of iron complexes on growth

When establishing *N. closterium* in low iron medium for copper toxicity studies, it was noted that there was a much longer lag phase, reduced final cell densities, and less pigment compared to 'normal' iron-grown cells. This suggests that 7.9 $\mu g\ Fe \cdot l^{-1}$ is limiting under culture conditions for *N. closterium*. Cell densities improved with continued subculture in iron deficient medium, in agreement with Hayward (1968) and Davies (1970). The effect of different forms of iron on growth was determined by adding iron (750 $\mu g\ Fe \cdot l^{-1}$) as ferric citrate/citric acid, ferric citrate

TABLE III

Intracellular and extracellular copper concentrations of *Nitzschia closterium*, grown in varying concentrations of iron[a]

Culture	Intracellular copper ($\mu g\ l^{-1}$)	Extracellular copper ($\mu g\ l^{-1}$)	Intracellular copper ($\times 10^{-5}$ ng/cell)	Extracellular copper ($\times 10^{-5}$ ng/cell)
Normal iron	3 3	6.9	2.8	6.0
Iron/10	4.3	6.6	9 6	14.6
Iron/100	12 0	0.5	29.9	1.3

[a]Total copper concentration in assay flasks was 20 $\mu g\ l^{-1}$

alone, ferric chloride, iron-EDTA complex and iron-desferrioxamine complex. Growth of *N. closterium* with and without preadaptation to different iron complexes, was identical in all types of iron medium, except iron-desferrioxamine. Cells grown in this medium appeared large and swollen, had a longer lag period (2–5 days) and lower maximum cell densities than with other iron complexes. Desferrioxamine, believed to be a complexing agent released by bacteria and blue-green algae (McKnight and Morel, 1980), enhances iron uptake by specifically complexing ferric iron for transport across the cell membrane (Anderson and Morel, 1982). Desferrioxamine, however, appears to make iron unavailable for the growth of *N. closterium*. This may be a significant factor enabling blue-green algae to dominate shallow environments, at the expense of other algal groups (Murphy et al., 1976).

DISCUSSION

Cells cultured before the assay under normal (f-medium) iron conditions, were more tolerant to copper than cells cultured in low iron medium (Table I). This may be due to colloidal ferric hydroxide which binds to the diatom cell surfaces (Davies, 1966), adsorbs the copper, and prevents copper penetration into the cell. In support of this hypothesis, very little extracellular iron was found around cells grown in low iron medium, compared to cells grown in high iron medium (Table II). Copper may therefore be more able to penetrate the cell, as is shown by the very high intracellular and low extracellular copper concentrations in cells grown in iron/100 medium (Table III).

The algal assay was carried out in raw, unenriched sea water, with no iron added. As the cells divide, the ferric hydroxide coating the cells, carried over from the inoculum, may be sufficient to prevent copper penetration into the cells or, alternatively, the colloidal ferric hydroxide may be shed from the cell surface and copper adsorbed to this, preventing copper binding the diatom cell membrane. To test these alternative theories, iron associated with the cells during the assay was determined (Table IV). Most of the ferric hydroxide coating the cells, carried over from the in-

TABLE IV

Iron associated with dividing cells of *Nitzschia closterium* over the 4 day assay (measured by ^{59}Fe γ counts).

Day	Cell count ($\times 10^4$ cells/ml)	Iron on cells (μg/100 ml)	Iron on cells ($\times 10^{-5}$ng/cell)	Iron in supernatant (μg/100 ml)	Iron on glass (μg/100 ml)	Total iron recovered (μg/100 ml)
0	4 48	1.60	35.71	–	–	1.6
1	6.25	1.71	27.33	<0 004	0 037	1.75
2	7.63	1.59	20 79	<0 004	0 050	1.64
3	9.25	1 37	14.75	0.061	0 037	1 47

oculum, remained associated with the cells as they divided. Only a small amount of iron was shed from the cell surface, appearing in the sea water and on the walls of the flask.

In the competitive uptake experiments, intracellular and extracellular iron were not affected by copper in the range 0–70 μg Cu $\cdot$ l^{-1}. For this species, it appears copper toxicity is not due to competitive cellular uptake of copper at the expense of iron. Competitive cellular uptake between nutrient and toxic metal ions has been proposed to explain the effect of copper ions on manganese-limited algal cultures (Sunda and Huntsman, 1983) and of cadmium ions on iron-limited cultures (Foster and Morel, 1982; Harrison and Morel, 1983). Their experiments, however, were carried out in synthetic media containing EDTA, with both toxic and nutrient metals present, whereas our assays were made in natural, unsupplemented sea water, using washed algal cells which had previously been equilibrated with iron. Under these conditions, competitive processes could not be involved. Manganese dioxide, like hydrated ferric oxide, may also form a protective layer around *N. closterium* since cells cultured in manganese-supplemented (50 μg Mn $\cdot$ l^{-1}) low iron medium were as resistant as those grown in 'normal' iron medium (790 μg Fe $\cdot$ l^{-1}) (Stauber and Florence, to be publ.).

Whereas iron partially overcame the inhibitory effect of ionic copper on algal growth, it had no effect on the toxicity of the lipid-soluble copper complex, copper–oxine (5×10^{-8} M oxine plus 2 μg Cu $\cdot$ l^{-1}) which completely depressed algal growth at all iron concentrations, in agreement with Florence et al. (1983). Apparently, the copper–oxine complex readily diffuses through the hydrated iron oxide coating around the cells.

Low iron-grown cells may have decreased levels of iron enzymes e.g. the haemoprotein catalase, and possibly superoxide dismutase, which catalyse the removal of damaging H_2O_2 and superoxide dismutase. In addition, copper can catalyse the decomposition of H_2O_2 to produce hydroxyl radicals, which accelerate oxidative deterioration of membrane lipids (Florence, 1983). Thus increased copper toxicity to low iron-grown cells may be due not only to greater copper penetration into the cell, but also to reduced cell defence mechanisms against oxygen free radicals.

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Paper 2

The Reaction of Copper-2,9-Dimethyl-1,10-Phenanthroline with Hydrogen Peroxide

T. M. Florence, J. L. Stauber and K. J. Mann

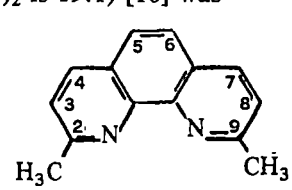
CSIRO Division of Energy Chemistry, Sutherland, Australia

ABSTRACT

The copper complex of 2,9-dimethyl-1,10-phenanthroline(2,9-dmp) is accumulated by a variety of organisms and is highly toxic. Bioaccumulation depends on reduction of copper(II) to (I), since only the copper(I)-2,9-dmp complex is lipophilic. In the case of the marine diatom, *Nitzschia closterium*, it is proposed that hydrogen peroxide, produced by the algae during photosynthesis, is the in vivo reductant. Hydrogen peroxide rapidly reduces copper(II)-2,9-dmp, but an excess of H_2O_2 leads to destruction of the yellow copper(I) complex. Rate constants for the formation and degradation of the yellow complex are reported. Oxygen, light, and a hydroxylating agent are released during the degradation reaction. A reaction mechanism is proposed for the destruction of copper-2,9-dmp by excess H_2O_2, involving attack on the 5, 6 positions of the phenanthroline ring by hydroxyl radical, then further oxidation by singlet oxygen and H_2O_2. These in vivo degradation reactions are believed to be the cause of the extreme toxicity of the complex.

INTRODUCTION

The copper complex of 2,9-dimethyl-1,10-phenanthroline (2,9-dmp)(I) has been shown to be highly toxic towards bacteria [1], fungi [2], mycoplasmas [3], tumor cells [4, 5], algae [6–8], and amphipods [9]. It was the most potent of all phenanthroline and bipyridyl compounds tested [1–3, 6–9]. Chelation with copper (log β_2 for Cu(I)(2,9-dmp)$_2$ is 19.1) [10] was



I. 2,9-dimethyl-1,10-phenanthroline

Address reprint requests to Dr. T. M. Florence, CSIRO Division of Energy Chemistry, Private Mail Bag 7, Sutherland, NSW, 2232, Australia.

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essential for the toxic effect. Smit et al. [1] concluded that the site of action of copper-2,9-dmp on *Paracoccus denitrificans* was the cytoplasmic membrane, resulting in the inhibition of respiratory electron transport.

Concentrations of copper-2,9-dmp as low as 5×10^{-9} *M* decreased the growth rate of the marine alga *Nitzschia closterium* [6–8]. The complex was strongly accumulated by the algal cells, and uptake was very rapid (95% in 3 hr), suggesting that copper-2,9-dmp has a high solubility in the cell membrane. Copper(II)-2,9-dmp is poorly soluble in *n*-octanol and other lipid-model solvents [7], and reduction to the yellow tetrahedral copper(I) complex is necessary for extraction into these solvents [7, 11]. It is likely, then, that reduction is also a prerequisite for strong bioaccumulation. Copper(II)-2,9-dmp underwent only very slow autoreduction (18% in 24 hr) in seawater [8], and the rapid uptake of the complex by *Nitzschia* indicates that the alga itself produced the reductant which yields the lipid-soluble copper(I) complex.

Hydrogen peroxide is an efficient reagent for the reduction of copper(II)-2,9-dmp [7, 12], and many algae, including *Nitzschia*, are known to produce H_2O_2 continuously during photosynthesis, and to release it into the growth medium [13]. It has been suggested [7] that H_2O_2 production by algae imposes a self-limiting control on their growth. Indeed, the addition of 0.02 mg ml^{-1} of catalase to a *Nitzschia* culture more than doubled the growth rate [7]. Hydrogen peroxide, therefore, is a likely candidate as the *in vivo* reductant of copper(II)-2,9-dmp.

Although stoichiometric concentrations of H_2O_2 produce the yellow copper(I)-2,9-dmp complex (reaction(1)) [12],

$$2\ \mathrm{Cu(II)(2,9\text{-}dmp)_2^{2+}} + \mathrm{H_2O_2} \rightarrow 2\ \underset{\text{yellow}}{\mathrm{Cu(I)(2,9\text{-}dmp)_2^{+}}} + \mathrm{O_2} + 2\mathrm{H^+} \qquad (1)$$

excess H_2O_2 causes the yellow color to fade, possibly as a result of the opening of the phenanthroline ring [14, 15]. The present study was carried out to determine the mechanism and products of the reaction between copper-2,9-dmp and excess H_2O_2, in an attempt to explain the extreme toxicity of this complex.

EXPERIMENTAL

Algal assays were carried out in filtered, unsupplemented seawater, as described previously [6, 16]. The total copper concentration naturally present in the seawater was 0.1–0.7 μg $liter^{-1}$, with labile copper, as measured by anodic stripping voltammetry [6], being in the range <0.1–0.3 μg $liter^{-1}$.

Spectrophotometric measurements were made on Cary model 16 manual, and model 118 recording, spectrophotometers. Dissolved oxygen was measured with an Orion model 97-08 oxygen electrode. A JEOL model JMS DX300 gas chromatograph mass spectrometer with a fast atom bombardment (FAB) probe was used to identify copper-2,9-dmp degradation products. Chemiluminescence measurements were made in scintillation vials (15 ml sample) using a Packard Tri-Carb model 3330 liquid scintillation counter with fully open windows and 90% gain. Hydrogen peroxide was determined by the titanium spectrophotometric method [17], and stock H_2O_2 solutions were standardized by titration with cerium(IV). Hydroxyl radical production was determined [18] by the hydroxylation of 4-nitrophenol to 4-nitrocatechol, which was measured spectrophotometri-

TABLE 1. Toxicity of Copper–Phenanthroline Complexes to the Marine Diatom *Nitzschia closterium*

Ligand[a]	*n*-Octanol extraction (%)[b]	Copper adsorbed by algae (%)[c]	Relative toxicity[d]
None ($CuSO_4$)	<10	42	1.0
1,10-phenanthroline	54	46	1.2
2,2′-bipyridyl	49	45	0.35
5,6-dmp	59	85	3.0
4,7-dmp	67	87	4.5
2,9-dmp	95[e]	>95	>20
Bathocuproine	93[e]	>95	7.3

[a] 1×10^{-7} *M* Cu^{2+} + 2×10^{-7} *M* ligand in unsupplemented seawater, pH 8.2. dmp = dimethyl-1,10-phenanthroline.
[b] In the presence of hydroxylamine hydrochloride as reducing agent.
[c] After 4-day bioassay.
[d] Ratio of growth rates (4-day) in absence and presence of ligand. Corrected for toxicity of excess ligand.
[e] Less than 10% extraction in absence of reducing agent.

cally. Standard copper solutions (EDTA titration) were prepared from reagent-grade $CuSO_4 \cdot 5H_2O$, and the phenanthrolines (Fluka) were analyzed by spectrophotometric titration with copper, and found to have a purity greater than 98%.

RESULTS

The toxicities of some copper-phenanthroline compounds to the marine diatom *Nitzschia closterium* are shown in Table 1. The copper-2,9-dmp complex was highly toxic and was strongly adsorbed by *Nitzschia*. In the absence of 2,9-dmp, only 42% of the copper partitioned to the algae [6]. Analysis of the filtrate from the algal suspension at the end of the 4-day growth experiments failed to detect any 2,9-dmp. The filtrate was treated with $CuSO_4$ and hydroxylamine hydrochloride, and the absorbance of copper(I)-2,9-dmp was measured at 460 nm. However, it was found that less than 3% of the added 2,9-dmp was in the filtrate. It appears, then, that the 2,9-dmp ligand is either retained by the algae cells, or discharged in an altered form.

The stoichiometry of the formation of copper(I)-2,9-dmp (reaction(1)) was studied over the pH range 6.5–8.5 using a Job's plot of the absorbance of the yellow complex at 460 nm (ϵ = 8413 liter mol^{-1} cm^{-1}), and by measurement of the oxygen evolved. Use of the chemical equilibrium program SIAS [19] showed that formation of copper(I)-2,9-dmp should be 99.5% complete under the above conditions. The stoichiometry of reaction (1), as reported by Davies et al. [12], was confirmed, but more than a twofold excess of H_2O_2 led to fading of the yellow complex (Fig. 1). With stoichiometric concentrations of the reactants in reaction (1), a second-order plot of $1/(A_m - A_t)$, where A_m is maximum absorbance and A_t is absorbance at time t, was linear for at least 90% of the reaction (r = 0.985). Second-order rate constants are shown in Table 2.

The kinetics of the destruction of the yellow complex were followed at 460 nm

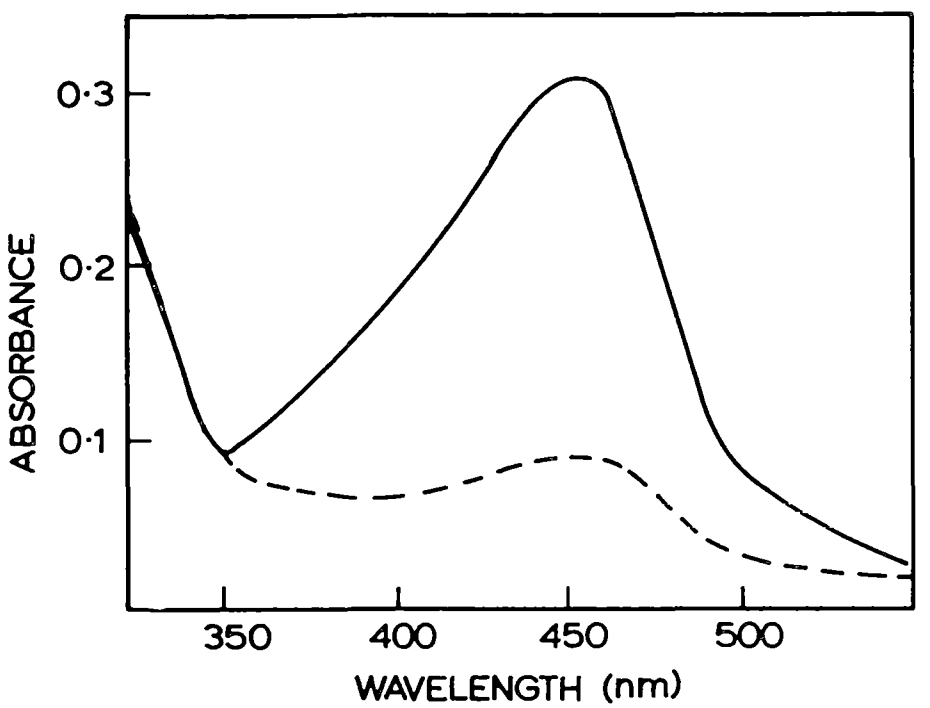


FIGURE 1. Visible spectrum of copper-2,9-dimethyl-1,10-phenanthroline (2,9-dmp). Phosphate buffer, 0.05 *M*, pH 7.70; Cu^{2+}, 1×10^{-4} *M*; 2,9-dmp, 2×10^{-4} *M*; Measured after 30 min. ——, 1×10^{-4} *M* H_2O_2; --- 4.8×10^{-3} *M* H_2O_2.

TABLE 2. Kinetics of Formation (k_f) and Degradation (k_d) of Copper-2,9-dimethyl-1,10-phenanthroline at 25°C

Addition	k_f (liter mol^{-1} $min^{-1}\times10^{-4}$)[a]	$k_d(min^{-1})$[b]
None	4.15	0.132[c]
HCO_3^-, 3×10^{-5} *M*	3.16	0.111
HCO_3^-, 3×10^{-4} *M*	2.40	0.096
Deaerate before adding H_2O_2	—	0.130
Dimethylfuran, 1×10^{-5} *M*	—	0.089
Mannitol, 0.2 *M*	—	0.136
Superoxide dismutase, 0.1 mg ml^{-1}	—	0.084
Sodium chloride, 0.5 *M*	—	0.288

[a] Phosphate buffer, 0.05 *M*, pH 7.70; Cu^{2+}, 1×10^{-4} *M*; 2,9-dmp, 2×10^{-4} *M*; H_2O_2, 5×10^{-5} *M*. Second-order rate constant.
[b] Pseudo-first-order rate constant. Phosphate buffer, 0.05 *M*, pH 7.70; Cu^{2+}, 1×10^{-4} *M*; 2,9-dmp, 2×10^{-4} *M*; H_2O_2, 4.8×10^{-3} *M*.
[c] Addition of 20% ethanol prevented degradation.

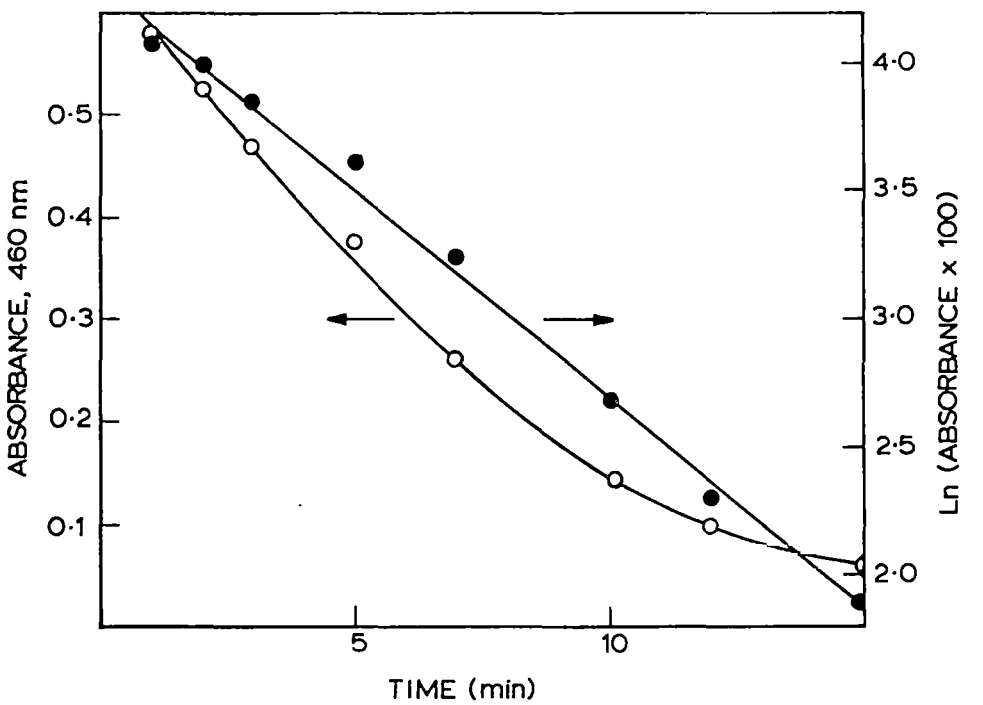


FIGURE 2. Kinetics of the degradation of the yellow copper-2,9-dimethyl-1,10-phenanthroline (2,9-dmp) complex by excess H_2O_2 at 25°C. Phosphate buffer, 0.05 *M*, pH 7.70, Cu^{2+}, 1×10^{-4} *M*; 2,9-dmp, 2×10^{-4} *M*; H_2O_2, 4.8×10^{-3} *M*.

(Fig. 2 and Table 2). With a large excess of H_2O_2, the reaction was pseudo-first-order ($r = 0.990$), and the rate constant (k_d) was directly proportional to H_2O_2 concentration. Using a 1:2 molar ratio of copper to 2,9-dmp, a value of $k_d = 27.0$ $[H_2O_2]min^{-1}$ at pH 7.7 and 25°C was calculated. In the pH range 6.5–8.5, a plot of log k_d versus log $[H^+]$ was linear with a slope of -0.22 (rate increased with increasing pH), yielding a rate constant of $k_d = 0.55\ [H_2O_2][H^+]^{-0.22}\ min^{-1}$ at 25°C.

The rate of fading decreased rapidly with increasing molar ratio of 2,9-dmp to copper (Table 3), showing that, as bound 2,9-dmp is oxidized, it is rapidly replaced by new, unaltered 2,9-dmp molecules to reform the yellow complex. Activation of the 2,9-dmp molecule by chelation with copper is necessary for H_2O_2 attack, since uncomplexed 2,9-dmp was not affected by the H_2O_2 concentrations used in these experiments.

Using 1×10^{-4} copper-2,9-dmp and 4×10^{-3} *M* H_2O_2, 1.0×10^{-4} *M* oxygen was liberated during the formation and degradation of the complex. Since 0.5 mole O_2/mole Cu is produced during formation of the complex (reaction (1)), an extra 0.5 mole of O_2 must be generated during degradation. No oxygen was produced when 4,7-dmp or 5,6-dmp were substituted for 2,9-dmp. Bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) appeared to yield about half as much oxygen as 2,9-dmp, but the concentration was difficult to quantify because of precipitation of the yellow copper(I) complex. Excess H_2O_2 caused little or no fading of the color.

TABLE 3. Effect of Concentration of 2,9-Dimethyl-1,10-Phenanthroline (2,9-dmp) on Rate of Degradation of Copper Complex at 25°C

2,9-dmp ($M \times 10^4$)[a]	k_d($min^{-1} \times 10^2$)[b]
1.00	27 2
2.00	13 2
3 00	1.12
4.00	0.84
5.00	0.72
10.0	0 38

[a] Phosphate buffer, 0.05 *M*, pH 7.70, Cu^{2+}, 1×10^{-4} *M*; H_2O_2, 4.8×10^{-3} *M*.
[b] Pseudo-first-order rate constant for conditions shown.

When copper-2,9-dmp was reduced with hydroxylamine hydrochloride or ascorbic acid, the yellow color was unaffected by the addition of H_2O_2, no fading occurring even after 24 hr. Reduction of the copper(II)-2,9-dmp complex by H_2O_2 is therefore a prerequisite for the destruction of the chromophore in 2,9-dmp by excess H_2O_2.

The liberation of hydroxyl radical during the reaction between some copper–phenanthroline complexes and H_2O_2 was studied (Table 4). Most hydroxylation of 4-nitrophenol occurred when 2,9-dmp was the ligand. However, the small effect of hydroxyl radical scavengers on hydroxylation suggests that *free* hydroxyl radical was not significantly involved in the reaction. It is more likely that some type of bound, or "crypto" hydroxyl radical [20, 21] is involved, where the OH· radical is associated with the copper atom in the 2,9-dmp complex and, in this way, is protected from OH· scavengers, but can react with 4-nitrophenol via a ternary complex [22]. No hydroxylation of 4-nitrophenol occurred when stoichiometric H_2O_2 was used for the reduction of copper-2,9-dmp (Table 4).

TABLE 4. Hydroxylation of Nitrophenol by Copper–Phenanthroline Complexes plus Hydrogen Peroxide

Ligand	Addition	4-Nitrocatechol formed ($M \times 10^6$)[a]
1,10-phenanthroline	none	0.55
5,6-dmp	none	1.11
4,7-dmp	none	0.46
2,9-dmp	none	2.20[b]
	no deaeration	0.49
	0.05 *M* mannitol	1.89
	0.013 *M* benzoate	1.31

[a] Deaerated phosphate buffer, 0.025 *M*, pH 7.70; 5×10^{-4} *M* Cu^{2+}; 1×10^{-3} *M* ligand (dmp = dimethyl-1,10-phenanthroline); 4.8×10^{-3} *M* H_2O_2; 1×10^{-3} *M* 4-nitrophenol; reaction time, 30 min.
[b] Stoichiometric H_2O_2 (2.5×10^{-4} *M*) gave less then 0.1×10^{-6} *M* 4-nitrocatechol.

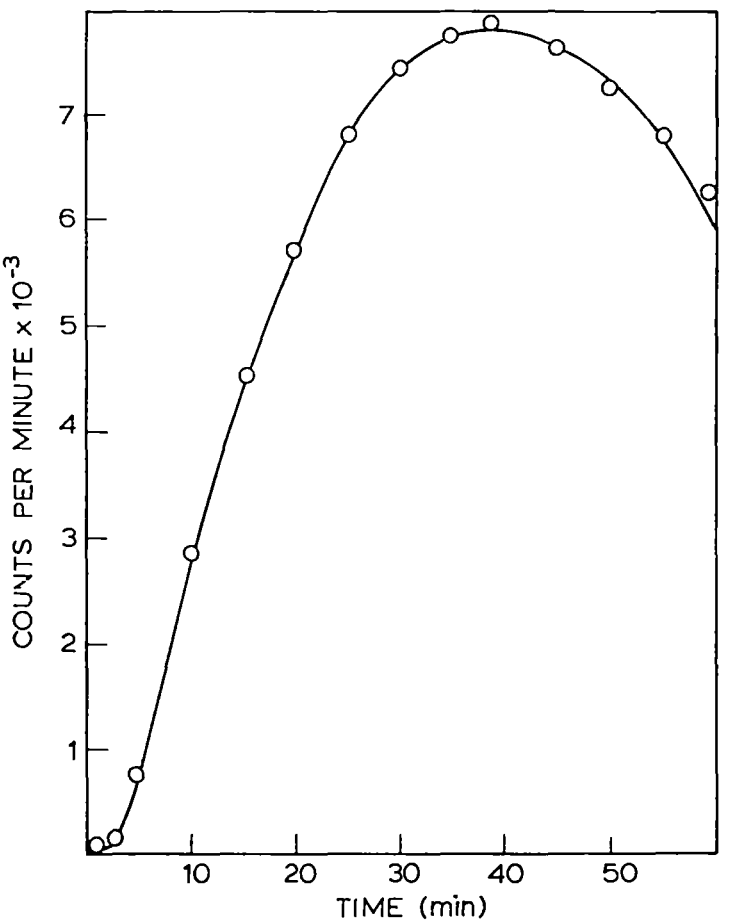


FIGURE 3. Chemiluminescence from the degradation of copper-2,9-dimethyl-1,10-phenanthroline (2,9-dmp) by hydrogen peroxide. Phosphate buffer, 0.05 *M*, pH 7.70; 1×10^{-4} *M* Cu^{2+}; 2×10^{-4} *M* 2,9-dmp; 4.8×10^{-3} *M* H_2O_2; 25°C.

The reaction of copper-2,9-dmp with excess H_2O_2 was accompanied by the emission of light (Fig. 3, Table 5). Count rate was proportional to H_2O_2 concentration, although a minimum of 1 mole H_2O_2/mole copper was needed to produce chemiluminescence (Fig. 4). With a constant concentration of H_2O_2, chemiluminescence increased with copper-2,9-dmp concentration, although the increase was less than proportional. Chemiluminescence was reduced by the hydroxyl radical scavengers ethanol, mannitol, and benzoate (Table 5). The singlet oxygen scavengers, dimethylfuran and β-carotene, also diminished chemiluminescence, as did sodium carbonate. A much higher light emission was measured when the solution was made 0.5 *M* in NaCl, and an increase in pH also increased the count rate. A plot of log (count rate) versus log $[H^+]$ was linear with a slope of -0.87. The time to reach maximum chemiluminescence (39 min, Fig. 3) was long compared to the half-time of the fading reaction (5 min, Table 2).

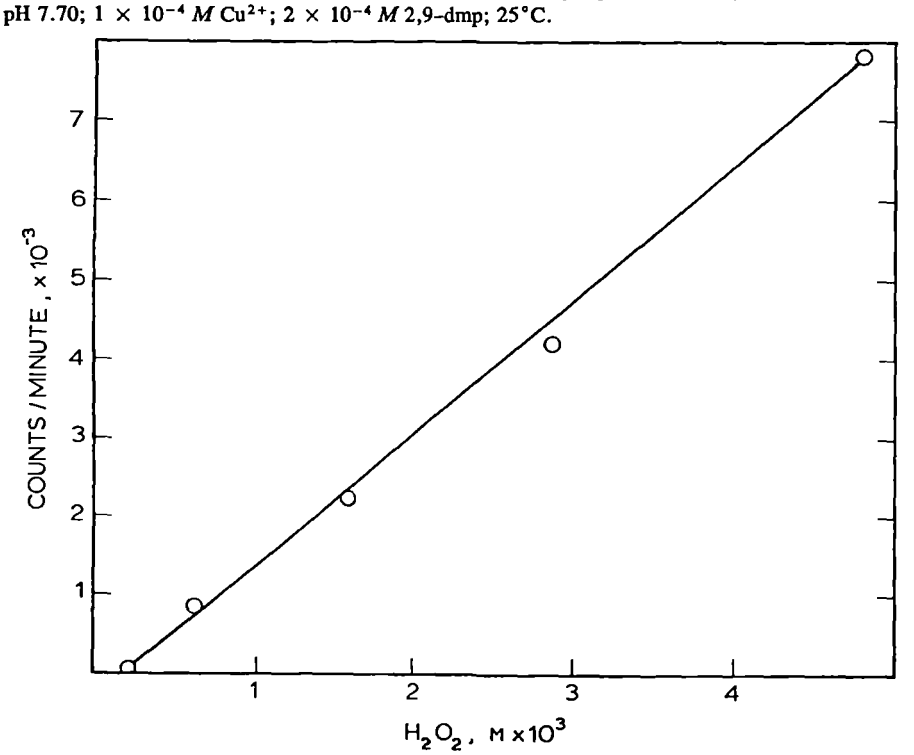
Much less chemiluminescence was produced when copper-2,9-dmp was first reduced with hydroxylamine hydrochloride, but deaeration of the solution before

TABLE 5. Chemiluminescence from Reaction Between Hydrogen Peroxide and Copper-2,9-Dimethyl-1,10-Phenanthroline (2,9-dmp)

Addition[a]	Maximum counts per minute	Time (min) to reach maximum counts
None (pH 7.70)	7,900	39
None (pH 6.91)	2,080	45
None (pH 7.82)	21,030	25
None (1×10^{-3} *M* 2,9-dmp)	< 100	—
Mannitol, 0.036 *M*	5,400	49
Benzoate, 0.03 *M*	720	> 100
Dimethylfuran, 0.02 *M*	3,100	20
β-Carotene, 0.001 *M*	3,500	30
Superoxide dismutase, 0.03 mg ml^{-1}	6,000	48
Sodium chloride, 0.5 *M*	67,600	18
Sodium carbonate		
3.3×10^{-4} *M*	2,200	59
1.7×10^{-3} *M*	1,300	60
Deaerated before H_2O_2	16,400[b]	22
Reduced with hydroxylamine	2,250[c]	5

[a] Phosphate buffer, 0.05 *M*, pH 7.70; 1×10^{-4} *M* Cu^{2+}; 2×10^{-4} *M* 2,9-dmp; 4.8×10^{-3} *M* H_2O_2; total volume, 15 ml; 25°C.
[b] Deaerated with nitrogen before adding H_2O_2.
[c] Reduced with 2×10^{-3} *M* hydroxylamine hydrochloride, then 6.8×10^{-3} *M* H_2O_2 added. Little fading of the yellow color occurred.

FIGURE 4. Effect of hydrogen peroxide concentration on chemiluminescence from the degradation of copper-2,9-dimethyl-1,10-phenanthroline (2,9-dmp) by hydrogen peroxide. Phosphate buffer, 0.05 *M*, pH 7.70; 1×10^{-4} *M* Cu^{2+}; 2×10^{-4} *M* 2,9-dmp; 25°C.



or after the addition of H_2O_2 significantly increased the count rate (Table 5). When copper was complexed by 1,10-phenanthroline, 4,7-dmp, 5,6-dmp, or bathocuproine instead of 2,9-dmp, and excess H_2O_2 added, the chemiluminescence was less than 5% of that produced using 2,9-dmp. When a large excess of 2,9-dmp was used, no chemiluminescence was produced (Table 5).

Degradation products of the reaction between stoichiometric copper-2,9-dmp and excess H_2O_2 were analyzed by FAB-mass spectrometry. After the yellow complex had completely faded, the solution was adjusted to pH 4.2, and extracted with diethyl ether. The ether extract was evaporated into a drop of glycerol on the FAB source, and the spectrum recorded. The only peaks found, other than the glycerol background peaks, were large peaks at mass numbers 385 and 399. When copper-2,9-dmp was reduced with hydroxylamine hydrochloride or stoichiometric H_2O_2 so that no fading occurred, peaks at 271 and 479 mass number were obtained, corresponding to the 1:1 and 1:2 copper-2,9-dmp complexes, respectively.

DISCUSSION

Although the free metal ion is usually considered the physicochemical form of a heavy metal most toxic to aquatic organisms, it was shown recently that some lipid-soluble metal complexes are even more potent [6]. Lipid-soluble complexes not only diffuse more rapidly through a biomembrane, but also carry the ligand molecule, which may exert its own toxicity, into the cytosol. Of a wide range of organocopper complexes tested for toxicity towards the marine diatom *Nitzschia closterium*, copper-2,9-dmp was the most toxic [6–8]. The lipophilicity of copper-2,9-dmp depends on copper being in the I valency state, since copper(II)-2,9-dmp, Cu^{2+}, and free 2,9-dmp are not lipid soluble.

Mohindru et al. [5], using tritium-labeled 2,9-dmp, found that its copper complex was taken up within 60 min by L1210 tumor cells, and that the radioactivity then gradually escaped from the cells. Our results showed that if absorbed 2,9-dmp is also ejected by *Nitzschia*, as is likely, the ligand must be metabolized to a new species which does not form a colored complex with copper.

Copper-2,9-dmp was much more toxic to *Nitzschia* than the complexes of the isomeric phenanthrolines substituted in the 4,7 and 5,6 positions (Table 1), and even copper bathocuproine, which is as lipophilic as the 2,9-dmp complex, had a lower toxicity. Redox potentials indicate that the copper(II) complexes of 4,7- and 5,6-dmp [23] would not oxidize H_2O_2 to oxygen, but that copper(II)-2,9-dmp, with $E_0 = +594$ mV versus NHE, would.

Excess H_2O_2 is required in the reaction with copper-2,9-dmp to produce chemiluminescence, a hydroxylating agent, and fading of the yellow complex. An additional 0.5 mole of oxygen per mole of copper is produced during the fading reaction. The most likely site of oxidative attack on copper-complexed 2,9-dmp is the 5,6-bridge, as was found by Fedorova and co-workers [14, 15] to be the case for 1,10-phenanthroline. The mass numbers of 385 and 399 found for the H_2O_2 degradation products of copper-2,9-dmp may be glycerol adducts; correction for glycerol leads to mass numbers of 292 and 306. These considerations suggest the following reactions for the degradation:

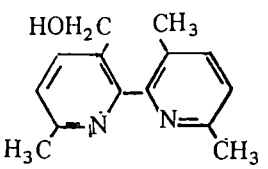
$$\underset{\text{5,6-bridge}}{-CH{=}CH-} + OH\cdot \rightarrow -\overset{OH}{\overset{|}{C}}H-\dot{C}H- \qquad (2)$$

$$\text{-CH(OH)-}\dot{\text{C}}\text{H(OH)-} + H_2O_2 \rightarrow \text{-CH(OH)-} + \text{-}\dot{\text{C}}\text{H(OH)-} + O_2 \quad (3)$$

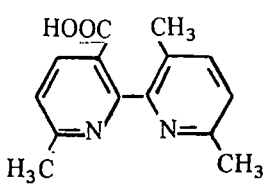
$$\text{-CH(OH)-} + O_2 \rightarrow \text{-COOH} + H_2O \quad (4)$$

$$\text{-}\dot{\text{C}}\text{H(OH)-} + H_2O_2 \rightarrow \text{-CH(OH)-} + HO_2^{\cdot} \quad (5)$$

The two products formed from the opening of the 5,6-bridge, according to this scheme, would be the 1:1 copper complexes of 3-hydroxymethyl-6,3′,6′-trimethyl-2,2′-bipyridyl(II, M.W. 292), and 3-carboxy-6,3′,6′-trimethyl-2,2′-bipyridyl(III, M.W. 306).



II. 3-hydroxymethyl-6,3′,6′-trimethyl-2,2′-bipyridyl



III. 3-carboxy-6,3′,6′-trimethyl-2,2′bipyridyl

The hydroxyl radical required in reaction (2) can be provided by

$$Cu(2,9\text{-dmp})_2^{2+} + H_2O_2 \rightarrow Cu(2,9\text{-dmp})_2^{+} + \tfrac{1}{2}O_2 + H^+ + OH\cdot \quad (6)$$

Alternatively, initial oxidative attack on the 5,6 bridge (reaction(2)) could be by singlet oxygen, liberated in reaction (1).

Some of the oxygen produced in reaction (3) may be in the excited, singlet state, leading to chemiluminescence from minor side reactions, (reactions 7 and 8) involving formation and dissociation of a 1,2-dioxetane. This unusual process is believed to be the main cause of light emission from reactions between singlet oxygen and olefins [22, 25].

$$\underset{\text{5,6-bridge}}{\text{-CH=CH-}} + {}^1O_2 \rightarrow \text{-CH(O-O)-CH-} \quad (7)$$

$$\begin{array}{l} O-O \\ | \quad | \\ -CH-CH- \rightarrow 2\text{-}C + h\nu \\ \qquad\quad H \end{array} \tag{8}$$

Maximum chemiluminescence is attained about 40 min from the addition of excess H_2O_2 (Fig. 3, Table 5), whereas the half-time of the degradation reaction, calculated from the rate constant (Table 2), is 5.3 min. This discrepancy supports the suggestion that chemiluminescence is produced by a side reaction, and not by the main degradation reactions.

Low concentrations of sodium carbonate markedly depressed the rate of formation of copper(I)-2,9-dmp, its degradation by H_2O_2, and the amount of chemiluminescence (Tables 2 and 5). Although the bicarbonate ion is a hydroxyl radical scavenger, its reaction with OH· is relatively slow [24]. It is possible, however, that the small size of the bicarbonate ion may facilitate its reaction with OH· bound in the copper-2,9-dmp molecule. The increase in chemiluminescence when the solution was deaerated (Table 5) was probably the result of removal of carbon dioxide.

Chloride ion also caused a large increase in chemiluminescence, and in the rate of the degradation reaction (Tables 2 and 5). This increase is probably due to the formation of hypochlorite, leading to enhanced production of singlet oxygen [25]:

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^- \tag{9}$$

$$HOCl + H_2O_2 \rightarrow HCl + H_2O + {}^1O_2 \tag{10}$$

Certainly, addition of NaOCl to a solution containing copper-2,9-dmp and H_2O_2 caused rapid fading of the yellow color. Direct chemiluminescence from singlet oxygen is unlikely to have contributed to the results in Table 5, since a mixture of NaOCl and H_2O_2, known to produce $^1O_2$ (reaction(10)) [25], gave no chemiluminescence.

The inability of excess H_2O_2 to cause degradation of the yellow complex formed between copper(I) and bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) can be attributed to steric hindrance by the bulky 4,7 phenyl groups protecting the 5,6 bridge from oxidative attack. The stability of copper(I)-bathocuproine could explain why it is much less toxic than the 2,9-dmp complex, despite the two complexes having similar lipid solubilities (Table 1). The degradation of copper(I)-2,9-dmp by H_2O_2 inside a cell, liberating singlet oxygen, copper ions, oxygen free radicals, and bipyridyl compounds, may be the cause of its extreme toxicity towards a variety of organisms.

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Paper 3

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INTERACTIONS OF COPPER AND MANGANESE: A MECHANISM BY WHICH MANGANESE ALLEVIATES COPPER TOXICITY TO THE MARINE DIATOM, *NITZSCHIA CLOSTERIUM* (EHRENBERG) W. SMITH

J.L. STAUBER and T.M. FLORENCE

CSIRO Division of Energy Chemistry, Private Mail Bag 7, Sutherland, NSW 2232, Australia

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The mechanism by which manganese (4.2 and 42 μg $Mn \cdot l^{-1}$) ameliorates the toxicity of copper (0–60 μg $Cu \cdot l^{-1}$) to the marine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith, was investigated using unsupplemented sea water for growth rate experiments. Speciation of manganese associated with the cellls, intracellular and extracellular manganese and copper concentrations, and competitive binding between copper and manganese were studied using ultrafiltration, anodic stripping voltammetry and radiochemical techniques.

Cells cultured before the sea-water assay and in the absence of manganese accumulated high intracellular copper, and their growth rate was more sensitive to copper than cells cultured in the presence of manganese. Manganese associated with the cells was present as manganese (II) and/or (III) hydroxides, rather than as the fully oxidized MnO_2. It is proposed that manganese (III) hydroxide, like iron (III) hydroxide and cobalt (III) hydroxide, adsorbs copper very effectively on the membrane surface and prevents its penetration into the cell. Competitive interactions between manganese and copper occurred at the cell surface, but copper had no effect on intracellular manganese. Only 4 μg $Mn \cdot l^{-1}$ was required to alleviate copper toxicity, compared to 790 μg $Fe \cdot l^{-1}$. In the presence of algae, copper ions had a greater affinity for manganese in sea water than iron at similar concentrations, which may partially account for the relative effectiveness of manganese as a protective agent. In addition, manganese can scavenge the toxic superoxide radical (O_2^-), catalyzing its dismutation to H_2O_2 and O_2.

Manganese was unable to reverse copper toxicity, nor did it inhibit the toxicity of lipid-soluble copper complexes, such as copper oxinate, to *N. closterium*.

Key words: *Nitzschia closterium*; copper; manganese

INTRODUCTION

Hydrous iron and manganese oxides are efficient scavengers of heavy metals in natural aquatic systems due to their low solubility and large surface area (Singh and Subramanian, 1984). Ferric hydroxide and manganese dioxide readily incorporate metals by adsorption, ion exchange and coprecipitation.

Alleviation of copper toxicity to unicellular algae by iron and manganese has been noted previously (Steeman Nielsen and Kamp-Nielsen, 1970; Stauber and Florence, 1985). Using measurements of intracellular and extracellular copper and iron,

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Stauber and Florence showed that the toxicity of copper to the marine diatom, *Nitzschia closterium*, was greatly reduced by ferric hydroxide bound to the algal cell wall, which adsorbed copper and prevented copper penetration into the cell. They noted that manganese, at much lower concentrations (50 μg $Mn \cdot l^{-1}$) than iron, also reduced copper toxicity.

Antagonistic interactions between copper and manganese have been reported for both algal growth (Sunda et al., 1981) and photosynthesis (Habermann, 1969). Sunda and Huntsman (1983) proposed competition between manganese and copper for a critical intracellular site, i.e., copper leads to manganese deficiency in the cell, which can be reversed by the addition of manganese.

Manganese is also an effective scavenger of superoxide radical (O_2^-) produced in the chloroplast (Kono et al., 1976). Manganese catalyzes the dismutation of O_2^- to H_2O_2 and O_2, its action being similar to that of superoxide dismutase which has been shown (Florence et al., 1983) to stimulate algal growth. This function of manganese, together with its physico-chemical speciation, may be important in determining copper toxicity to algae.

Whether manganese protects against copper toxicity by competing with binding sites within the cell, by acting as a redox catalyst, by adsorbing copper and preventing copper penetration into the cell, or by a combination of these effects, was unclear. This paper investigates the mechanism which enables manganese to ameliorate the toxicity of copper to the marine diatom *N. closterium* and, in particular, why it is more effective than iron.

MATERIALS AND METHODS

Sea water

Surface sea water was collected off Port Hacking, New South Wales, in clean polyethylene bottles and passed immediately through a 0.45 μm filter. Filtered sea water was stored at 4°C until used in the culture medium and algal assays. Total copper was in the range 0.07–0.3 $\mu g \cdot l^{-1}$.

Algae maintenance

The unicellular marine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith* (originally obtained from CSIRO Division of Fisheries Algal Culture Collection) was cultured in f medium (Guillard and Ryther, 1962), at one hundredth the normal iron concentration (i.e. 7.9 $\mu g\ Fe \cdot l^{-1}$ as ferric citrate) and without trace metals. Manganese in the culture medium was $< 1\ \mu g \cdot l^{-1}$. Cultures for the algal assays were grown in three media with the following added manganese concentrations (manganese added as radioactively labelled $^{54}MnCl_2$ – Amersham): 0 $\mu g\ Mn \cdot l^{-1}$,

* The name *Nitzschia closterium*, rather than *Cylindrotheca closterium*, was used in agreement with Hasle (1964)

4.2 μg $Mn \cdot l^{-1}$ and 42.3 μg $Mn \cdot l^{-1}$. Cells in log phase growth were used for all experiments.

Algal assays

These were carried out in siliconized 150-ml conical flasks, containing 50 ml of sea water with an initial cell density of $2.75–3.75 \times 10^4$ cells $\cdot ml^{-1}$, according to the method of Lumsden and Florence (1983). The inoculum was washed three times in sea water by centrifugation before use. Cultures were incubated at 21°C on a 12:12 h light/dark cycle at 16000 lux (Philips TL 40 W white). Cell density was measured daily for three days by counting microscopically in a haemocytometer. A regression line was fitted to a plot of $\log_{10}$ (cell density/initial cell density) versus time (in hours) for each sample, and the growth rate determined.

Total manganese determinations

Manganese on the cells (intracellular and extracellular), in the medium, and on the walls of the culture flask was determined at the beginning (day 0) and the end (day 3) of the algal assay in sea water by counting in a gamma well counter.

Intracellular and extracellular manganese concentrations were determined by centrifuging 2×10 ml aliquots of cell suspension at medium speed. To determine manganese in the medium, 5 ml of supernatant was retained. The algae concentrate from one aliquot was resuspended in 10 ml sea water for total Mn determination, and the other in 10 ml of 0.02 M ascorbic acid in sea water for determination of intracellular Mn. Preliminary experiments showed that MnO_2 (prepared by slowly adding 150 ml of 0.1 M $MnSO_4.H_2O$ to 100 ml of hot 0.1 M $KMnO_4$) was reduced to Mn^{2+} by 0.02 M ascorbic acid in sea water, and that ascorbic acid removed extracellular $Mn(OH)_2$, $Mn(OH)_3$ and MnO_2 without causing cell lysis. This enabled intracellular Mn to be determined on the washed cells. After standing for 5 min the tubes were centrifuged and the cells resuspended in 5 ml sea water for counting in a gamma well counter. Extracellular Mn was then determined by subtracting intracellular from total Mn.

A significant fraction of the manganese adhered to the glass walls of the culture flask. The flask was rinsed with 10 ml of 0.02 M ascorbic acid and 5 ml was counted to determine manganese on the glass.

Determination of Mn^{2+} by anodic stripping voltammetry

Free Mn^{2+} in the medium was determined by anodic stripping voltammetry (ASV) using an E.G. and G. Princeton Applied Research model 384 Polarographic Analyser with a model 303 static mercury drop electrode assembly. All analytical procedures were carried out at 25 $\pm$ 0.5°C in a class 100 clean room.

A 15 ml aliquot of algal suspension was centrifuged at medium speed. To 7 ml of supernatant, 0.25 ml of 1 M citrate (pH 9) and 2.5 ml of 0.1 M borate buffer (pH 9.5) were added and made up to 10 ml vol in sea water (Colombini and Fuoco,

1983). Mn^{2+} was determined by ASV, with 30 min deposition at -1.75 V, scanning to -0.9 V. Correction was made for a 10 ml sea-water blank.

Determination of Mn^{2+} by ultrafiltration

Mn^{2+} in the medium was also determined by membrane ultrafiltration. 10 ml of algae, grown in radioactively labelled manganese medium (42.3 μg $Mn \cdot l^{-1}$), was centrifuged for 8 min. Next, 5 ml of the supernatant was centrifuged for 25 min in an Amicon Centriflo membrane ultrafilter cone (CF-25), which retains molecules greater than 25,000 MW. The volume of the filtrate was measured and counted for 10 min in a gamma well counter. To ensure that all colloidal MnO_2 (and no Mn^{2+}) was retained on the cone, radioactively labelled $MnCl_2$ and MnO_2 solutions were run as standards. All colloidal MnO_2 was retained on the cone, while all the Mn^{2+} passed through the cone. This method was used to determine Mn^{2+} and MnO_2 in aged sea water and in medium, with and without algae.

Copper determinations

Copper on the algae and in the filtrate was determined by ASV (Florence et al., 1983; Stauber and Florence, 1985). At the end of the assay (day 3), copper on the algae was determined by passing 20 ml of algal suspension through an acid-washed, 25 mm diameter, 0.45 μm membrane filter. The filter was digested in a 50 ml covered beaker in a clean air cupboard with 1 ml of 15 M HNO_3 and 0.5 ml 72% $HClO_4$ to strong fumes of perchloric acid, then diluted to 10 ml with water. Copper was determined directly on this solution. An acid-washed membrane filter was used as a blank.

Intracellular copper concentration was measured by centrifuging 11 ml of algal suspension, resuspending in 0.02 M EDTA in sea water to remove copper adhering to the outside of the cells, passing through an acid-washed 0.45 μm membrane filter and digesting with acid as above. The extracellular copper concentration was determined by subtracting the intracellular copper concentration from the total copper bound to the cells.

Total copper in the filtrate was determined by ASV after adding 0.2 ml 4 M HNO_3 and 0.05 ml 30% H_2O_2 to 10 ml of filtrate, and irradiating with a 550 W mercury lamp for 12 h. Sodium acetate (0.4 ml of 4 M) and 0.4 ml of 2% $(NH_2OH)_2 \cdot H_2SO_4$ were added to the irradiation tubes, which were heated in a boiling water bath for 20 min and cooled before measurement by ASV (Florence et al., 1983).

Determination of hydrogen peroxide and oxygen

Superoxide radical (O_2^-) was generated from 1.67×10^{-4} M dihydroxyfumaric acid in sea water. Oxygen consumed in the reaction was measured using an Orion Research oxygen electrode in a sealed BOD bottle. Hydrogen peroxide was determined spectrophotometrically by the method of Sellers (1980).

TABLE I

Toxicity of copper to *Nitzschia closterium*, grown at manganese concentrations from 0 to 42.3 μg Mn l^{-1}.

Manganese concentration in medium (μg l^{-1})	Cell density after 3 days (20 μg Cu·l^{-1} added) (cells·ml^{-1})	Copper concentration[a] causing 50% inhibition of growth rate (μg·l^{-1})
0	2.75×10^4	6
4.2	14.75×10^4	65
42.3	7.25×10^4	37

[a] Relative standard deviation was ± 10%.

RESULTS

N. closterium cells grown before the assay in the presence of manganese (4.2 and 42.3 μg Mn·l^{-1}) were less sensitive to copper than cells cultured previously in medium without manganese (Table I). However, cells cultured in low manganese (4.2 μg Mn·l^{-1}) were more tolerant to copper than cells grown at high manganese (42.3 μg Mn·l^{-1}). Growth rates of control cells assayed without copper were similar at all manganese concentrations.

Manganese content of the cells

Cellular manganese (day 0) increased with increasing concentration of manganese in the culture medium (Table II). Both intracellular and extracellular manganese were higher in medium containing 42.3 μg Mn·l^{-1} compared to low manganese (4.2 μg Mn·l^{-1}).

Most of the manganese added to the medium was associated with the cells,

TABLE II

Intracellular and extracellular manganese content of *Nitzschia closterium* before assay in sea water (day 0).

Culture	Manganese added (μg l^{-1})	Manganese content (μg·l^{-1})				Intracellular manganese[a] (μg·l^{-1})	Extracellular manganese (μg·l^{-1})	Intracellular manganese ($\times 10^{-5}$ ng/cell)	Extracellular manganese ($\times 10^{-5}$ ng/cell)
		Manganese on cells	Manganese in supernatant	Manganese on glass	Total Manganese recovered				
Low Mn	4.2[b]	4.2	0.01	0.26	4.50	1.10	3.13	0.14	0.40
High Mn	42.3[b]	37.0	1.6	7.9	46.5	6.1	30.9	0.97	4.9

[a] Relative standard deviation in cellular manganese determinations was ± 10%

[b] Blank Mn in the culture medium was less than 1 μg·l^{-1}.

TABLE III
Intracellular and extracellular manganese and copper concentrations of *Nitzschia closterium* on day 3 of the assay. Total copper concentration in the assay flask was 20 $\mu g\ Cu \cdot l^{-1}$.

Manganese concentration in culture medium ($\mu g \cdot l^{-1}$)	Intracellular manganese ($\mu g \cdot l^{-1}$)	Extracellular manganese ($\mu g \cdot l^{-1}$)	Intracellular manganese ($\times 10^{-5}$ ng/cell)	Extracellular manganese ($\times 10^{-5}$ ng/cell)	Intracellular copper[a] ($\mu g \cdot l^{-1}$)	Extracellular copper ($\mu g \cdot l^{-1}$)	Intracellular copper[a] ($\times 10^{-5}$ ng/cell)	Extracellular copper ($\times 10^{-5}$ ng/cell)
0	–	–	–	–	12.0	0.53	29.9	1.33
4.2	0.007	0.162	0.011	0.260	3.40	6.68	3.09	6.08
42.3	0.048	1.19	0.067	1.77	4.68	4.74	6.44	6.52

[a] Relative standard deviation in cellular copper determinations was ± 11%.

although some manganese remained on the glass, similar to that found for iron (Stauber and Florence, 1985). In the high manganese medium, 80% of the manganese was associated with the cells, while 17% was retained on the glass and 3% in the medium. In the low manganese cells, 94% of the manganese was on the cells and only 6% on the glass. Mass balance was obtained between manganese added and manganese recovered.

As expected, both intracellular and extracellular manganese were lower, on a per cell basis, by the end of the assay in sea water (day 3; Table III). Both intracellular and extracellular manganese in cells cultured before the assay in high manganese remained higher than that cultured in low manganese.

Cellular copper

Intracellular copper was highest in cells cultured previously without manganese (29.9×10^{-5} ng Cu/cell), correlating well with the extreme toxicity of copper to cells grown in the absence of manganese (Table III). However, intracellular copper in low-manganese grown cells (4.23 $\mu g\ Mn \cdot l^{-1}$) was slightly lower than cells cultured previously in 42.3 $\mu g\ Mn \cdot l^{-1}$ (3.09×10^{-5} ng Cu/cell and 6.44×10^{-5} ng Cu/cell respectively). Extracellular copper increased with increasing concentration of manganese in the culture medium.

While manganese ameliorates the toxicity of ionic copper, it does not prevent the lipid-soluble copper complex, copper oxinate (5×10^{-8} M oxine + 10 $\mu g\ Cu \cdot l^{-1}$) penetrating the cell. Copper oxinate, which completely depressed algal growth after 2 days, readily penetrated the cell as shown by the high intracellular copper in both high and low-manganese grown cells (25.0×10^{-5} ng/cell and 25.2×10^{-5} ng/cell, respectively) (Table IV). Manganese, like iron, does not alleviate the toxicity of lipid soluble copper complexes to *N. closterium*.

Reversal of copper toxicity by manganese

To determine whether manganese could reverse copper toxicity, cells grown in low iron (7.9 $\mu g\ Fe \cdot l^{-1}$) were assayed in sea water in the presence of copper (0–60 μg

TABLE IV
Intracellular and extracellular copper concentrations of *Nitzschia closterium* (grown at high and low manganese) after 2 days of exposure to 5×10^{-8} M oxine + 10 μg Cu l^{-1}).

Manganese concentration in culture medium (μg·l^{-1})	Final cell density after 2 days (cells ml^{-1})	Intracellular copper[a] (μg l^{-1})	Extracellular copper (μg·l^{-1})	Intracellular copper ($\times 10^{-5}$ ng/cell)	Extracellular copper ($\times 10^{-5}$ ng/cell)
Low manganese (4.2 μg·l^{-1})	0.75×10^4	1.89	3.64	25.2	48.5
High manganese (42.3 μg·l^{-1})	1.75×10^4	4.37	5.53	25.0	31.6

[a] Relative standard deviation in cellular copper determinations was ± 11%.

Cu·l^{-1}). On day 2, when growth was significantly reduced by copper, or no growth at all had occurred (compared to the blank without copper), manganese (4.7 μg·l^{-1} and 47 μg·l^{-1}) was added, and the effect on growth determined.

Manganese (4.7 μg·l^{-1} and 47 μg·l^{-1}) did not reverse copper toxicity to *N. closterium* at any copper concentration. Manganese (47 μg Mn·l^{-1}) did slightly stimulate growth of *N. closterium* in sea water whether or not copper was present ($\mu = 0.0071$) compared to the blank ($\mu = 0.0050$).

Competitive binding between copper and manganese

Cells cultured in the absence of manganese were assayed in sea water in duplicate in the presence of 0–40 μg Cu·l^{-1} and 21 μg Mn·l^{-1} (added as $^{54}MnCl_2$). On day 3 of the assay, intracellular and extracellular manganese were determined, as previously, to establish whether copper interfered with manganese binding or uptake (Table V). On a per cell basis, extracellular manganese, in the presence of copper ($2.5–2.9 \times 10^{-6}$ ng/cell), was significantly lower than the blank without copper

TABLE V
Intracellular and extracellular manganese concentrations in *Nitzschia closterium*, assayed in the presence of 0–60 μg Cu l^{-1} and 21.2 μg Mn·l^{-1}. Results on day 3 of the assay expressed ± σ

Copper concentration (μg l^{-1})	Intracellular manganese (μg l^{-1})	Extracellular manganese (μg l^{-1})	Intracellular manganese ($\times 10^{-6}$ ng/cell)	Extracellular manganese ($\times 10^{-6}$ ng/cell)
0	0.062 ± 0.002	1.22 ± 0.012	0.4 ± 0.10	8.3 ± 1.7
10	0.042 ± 0.001	0.418 ± 0.007	0.3 ± 0.05	2.9 ± 0.4
20	0.047 ± 0.001	0.349 ± 0.007	0.3 ± 0.01	2.5 ± 0.1
40	0.038 ± 0.001	0.248 ± 0.006	0.4 ± 0.10	2.9 ± 0.8

(8.3×10^{-6} ng/cell). However, intracellular manganese was similar at all copper concentrations in the range 0–40 μg $Cu \cdot l^{-1}$. Copper may interfere with extracellular manganese binding but it has no effect on manganese uptake into the cell (intracellular manganese).

Speciation of manganese

In all experiments, manganese was added to the medium as Mn(II). As the rate of oxidation of manganese in sea water is very slow, it is uncertain whether manganese remains as Mn(II) or is oxidized, perhaps at the cell surface, to Mn(III) or Mn(IV).

Sea water spiked with Mn(II) (42.3 μg $Mn \cdot l^{-1}$) was allowed to stand for 3 mth in the laboratory, and the Mn(II) remaining was determined using the Centriflo cone ultrafiltration method. Ninety percent of the manganese passed through the cone, while only 10% was retained. Thus, after 3 mth, most of the manganese was still present as Mn(II), with only 10% oxidized to colloidal MnO_2 or other insoluble species.

Medium to which 42.3 μg $Mn \cdot l^{-1}$ was added, was allowed to stand on the light platform for 4 wk, and the Mn(II) remaining determined as above. Only 8% of the manganese passed through the Centriflo cone as Mn(II), with the remaining 92% retained. The fraction retained may have included some colloidal MnO_2; however, most was probably Mn(II) adsorbed on silica and iron particles in the culture medium.

This experiment was repeated with algae grown for 4 wk in culture medium on the light platform. Cells were centrifuged, and Mn(II) in the supernatant was determined by Centriflo cone ultrafiltration. Ninety-seven percent of the manganese in the supernatant was present as Mn(II), with only 3% retained on the cone.

Mn(II) in the supernatant was also determined by ASV. Total manganese in the supernatant of the high manganese-grown cells was 1.6 μg $Mn \cdot l^{-1}$, of which less than 10% was ASV-labile.

To determine whether manganese around the cells was present as Mn(II) and/or Mn(III), i.e. $Mn(OH)_2$ and $Mn(OH)_3$, or Mn(IV), i.e. MnO_2, cells which had been grown in low iron, high manganese medium (42.3 μg $Mn \cdot l^{-1}$) were washed with sea water acidified to pH 2 with 0.01 M HNO_3. Preliminary experiments showed that a 10-min wash in pH 2 sea water did not visibly lyse the cells. Manganese present as $Mn(OH)_2$ and $Mn(OH)_3$ is soluble in 0.01 M HNO_3, whereas MnO_2 is not, and can be removed only by using a reductant such as ascorbic acid. Manganese on the cells and in each wash was determined by γ-counting. The acidified sea-water wash removed 98% of the extracellular manganese, with only a further 2% (MnO_2) being removed by the ascorbic acid wash. To ensure that acidified sea water (pH 2) did not simply cause the desorption of MnO_2 from the cells at this pH, any MnO_2 in the acid wash was determined using the Centriflo cone ultrafiltration method. Only 0.9 μg $Mn \cdot l^{-1}$ was retained on the cone, while 21.4 μg $Mn \cdot l^{-1}$ passed through as

Mn(II). Therefore, most extracellular manganese is present as $Mn(OH)_2$ or $Mn(OH)_3$, and not as colloidal MnO_2.

Removal of copper from solution by iron and manganese

To determine how effectively iron and manganese remove copper from solution, iron alone (7.9 and 790 $\mu g\ Fe \cdot l^{-1}$), manganese alone, as Mn(II) (4.7 $\mu g\ Mn \cdot l^{-1}$) and Mn(IV), (8.7 $\mu g\ MnO_2 \cdot l^{-1}$), and iron and manganese together, were added to flasks of f medium (without trace metals) and sea water. Medium flasks were autoclaved and cooled, while sea water was used without autoclaving, and 20 μg $Cu \cdot l^{-1}$ was added to the flasks, which were shaken and allowed to settle for at least 2 h. Copper remaining in solution was determined by ASV (5-min deposition at -0.6 V) each day for 4 days. The experiments were repeated in sea water with the addition of *N. closterium* (6.75×10^4 cells/ml).

Medium alone, without the addition of trace metals, removed all (95%) copper from solution after 48 h, probably by adsorption onto silica in the medium (Lumsden and Florence, 1983). Addition of iron or manganese did not enhance copper removal. In sea water alone, 18% of copper was lost after 48 h. Addition of iron (790 $\mu g\ Fe \cdot l^{-1}$) caused the loss of 87% of copper, but low iron (7.9 $\mu g\ Fe \cdot l^{-1}$), MnO_2, and Mn(II) were ineffective. Manganese and iron together were similar to iron alone at both iron levels (7.9 and 790 $\mu g\ Fe \cdot l^{-1}$).

In the presence of algae, 71% of copper was removed from sea water after 48 h owing to adsorption of copper onto algal cells. Manganese(II) caused an additional loss of 12%. Iron alone (7.9 $\mu g \cdot l^{-1}$) did not remove any additional copper compared to the blank. In the presence of algae, manganese, as Mn(II), Mn(III) or Mn(IV) appears to be more effective than iron (at similar concentrations) in adsorbing copper.

Decomposition of H_2O_2 by manganese

Thorne and Roberts (1948) showed that MnO_2 catalytically decomposes H_2O_2. Under akaline conditions the following reactions may occur:

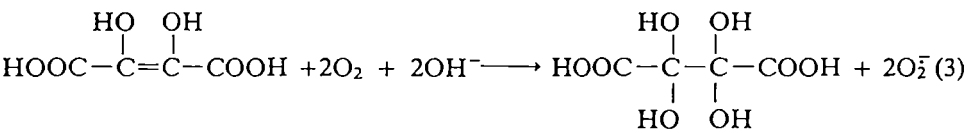
$$MnO_2 + H_2O_2 \longrightarrow Mn(OH)_2 + O_2 \quad (1)$$

$$Mn(OH)_2 + H_2O_2 \longrightarrow MnO_2 + 2H_2O \quad (2)$$

We examined these reactions in sea water by measuring the oxygen produced, using an Orion Research oxygen electrode. If MnO_2 was catalyzing these reactions, we would expect more than stoichiometric amounts of oxygen to be released. However, MnO_2 produced only stoichiometric (reaction 1) amounts of oxygen. As expected, no oxygen was produced in the presence of Mn(II). We conclude that reaction 2 does not proceed and that MnO_2 does not catalyze the decomposition of H_2O_2 in sea water, pH 8.2.

Dismutation of superoxide (O_2^-) by manganese

The ability of manganese(II) to scavenge O_2^- catalytically in sea water was tested, using the oxygen electrode and dihydroxyfumaric acid as a source of O_2^- (reaction 3):



$$HOOC-C(OH)=C(OH)-COOH + 2O_2 + 2OH^- \longrightarrow HOOC-C(OH)(OH)-C(OH)(OH)-COOH + 2O_2^- \quad (3)$$

Figure 1 shows that more oxygen was consumed (1.0×10^{-4} M) in reaction 3 in the presence of 2×10^{-5} M Mn(II), than in its absence (3.1×10^{-5} M O_2 consumed). The ability of Mn(II) to catalyze reaction 3 is probably due to its catalytic decomposition of O_2^- (reactions 4–6):

$$Mn^{2+} + O_2^- + 2H^+ \longrightarrow Mn^{3+} + H_2O_2 \quad (4)$$

$$Mn^{3+} + \tfrac{1}{2}H_2O_2 \longrightarrow Mn^{2+} + H^+ + \tfrac{1}{2}O_2 \quad (5)$$

$$\text{net: } O_2^- + H^+ \longrightarrow \tfrac{1}{2}H_2O_2 + \tfrac{1}{2}O_2 \quad (6)$$

Reactions 4–6 are believed to occur in illuminated chloroplasts (Kono et al., 1976), and copper can produce O_2^- from H_2O_2 (Florence, 1984).

To investigate reactions 4–6, we measured the H_2O_2 produced from O_2^- in the presence and absence of manganese (Table VI). H_2O_2 produced in the presence of manganese was nearly double that measured in its absence. Manganese (1×10^{-5} M), with excess O_2^-, produced 1.95×10^{-4} M H_2O_2 (20 times the stoichiometric amount). Thus manganese must be acting as a catalyst for reactions 4 and 5. The H_2O_2 produced decreased with high concentrations of manganese. In the presence of excess manganese, a complex may form with O_2^-,

$$Mn^{2+} + O_2^- \longrightarrow MnO_2^+ \quad (7)$$

The MnO_2^+ complex is 10 times more stable than O_2^- (Bielski and Chan, 1978), therefore less H_2O_2 will be measured.

Manganese in sea water is an effective scavenger of O_2^- by catalyzing its dismuta-

TABLE VI

Hydrogen peroxide production in sea water at varying manganese concentrations, in the presence of superoxide (O_2^-) generated from dihydroxyfumaric acid.

Manganese (M)	H_2O_2 produced (M)
0	1.06×10^{-4}
1×10^{-5}	1.95×10^{-4}
1×10^{-4}	1.93×10^{-4}
5×10^{-4}	1.87×10^{-4}
2×10^{-3}	1.23×10^{-4}

tion to H_2O_2 and O_2. The ability of other metals to catalyze this reaction was also tested (Fig. 1); Ni(II), Co(II) and Fe(II) led to the consumption of much less oxygen than Mn(II). It appears that, under the conditions used, these metals are unable to catalyze the production of O_2^- from dihydroxyfumaric acid or the dismutation of O_2^-.

DISCUSSION

Manganese(II) in sea water is oxidized very slowly to MnO_2. Manganese-oxidizing bacteria have been shown to catalyze the oxidation of Mn(II) to Mn(IV) in freshwater lakes (Chapnick et al., 1982). Our ultrafiltration results showed that most of the manganese added to sea water remained as Mn(II), with only 10% oxidized to MnO_2 after 3 mth. However, in the presence of algae, manganese (II) may be oxidized by O_2^- at the cell surface to manganese (III). Of the extracellular manganese on *Nitzschia*, 98% was present as manganese (II) and/or (III) hydroxides.

Cells cultured in the presence of manganese, before the sea-water assay, were much more tolerant to copper than cells cultured in the absence of manganese. Cells grown without manganese had high intracellular copper, correlating with the ex-

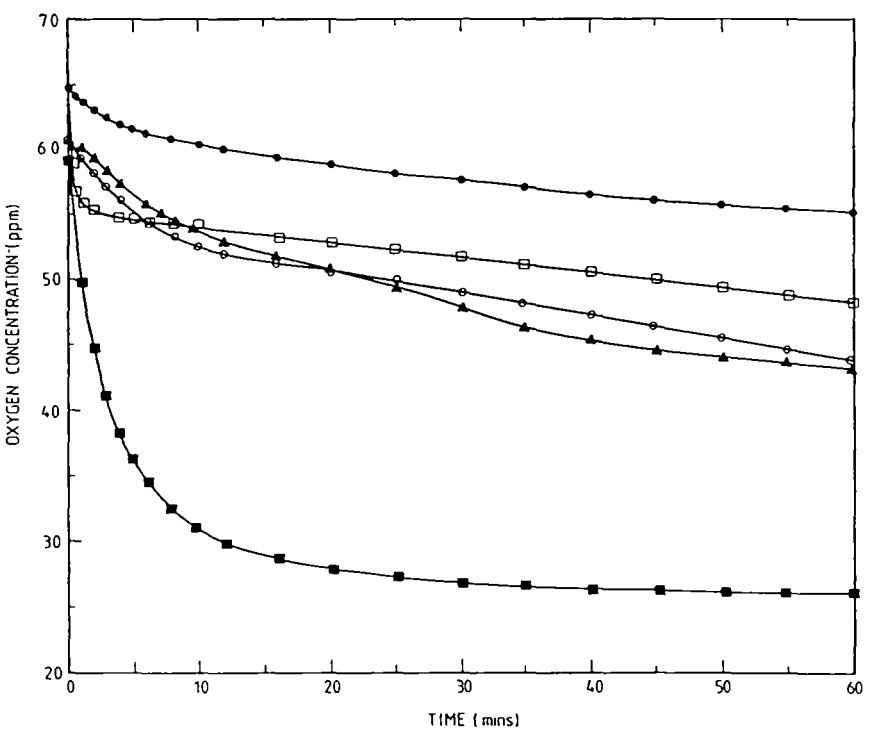


Fig. 1. Oxygen consumed in the reaction of oxygen and dihydroxyfumaric acid (1.67×10^{-4} M) in sea water, in the presence of different metals (2×10^{-5} M). (●—●) Blank; (□—□) iron(II); (○—○) nickel(II); (▲—▲) cobalt(II); (■—■) manganese(II).

treme toxicity of copper to these cells, compared to cells grown with manganese, which had low intracellular copper. Manganese hydroxides, like iron(III) hydroxide (Stauber and Florence, 1985), effectively adsorb copper and prevent its penetration into the cell.

Only very small amounts of manganese (4 $\mu g \cdot l^{-1}$) are needed to protect against copper toxicity. Extracellular binding sites must be saturated with manganese at this level, as increasing manganese (42 $\mu g \cdot l^{-1}$) gives no additional protection. In fact, intracellular copper was slightly higher in high-manganese grown cells, corresponding to greater copper toxicity to these cells, compared to cells grown in low manganese. We have no evidence to suggest that manganese (42 $\mu g \cdot l^{-1}$) is itself toxic, as cells grown in culture medium at different levels of manganese had similar growth rates.

Manganese is much more effective in protecting against copper toxicity than is iron (Stauber and Florence, 1985). Huntsman and Sunda (1980) noted that one thousand times more iron than manganese is needed to stimulate algal growth in deep sea water. Hydrous iron oxides are believed to have slightly greater affinity for cupric ions than manganese oxides in fresh-water (Vuceta and Morgan, 1978). Our results suggest that in the presence of algae, manganese (4–8 $\mu g \cdot l^{-1}$) is more effective than iron at similar concentrations in removing copper from sea water. Greater affinity for copper ions may account in part for the greater effectiveness of manganese than iron in alleviating copper toxicity. Also, manganese (II) is much more soluble in sea water than is iron (III); the solubility products, pK values, for $Fe(OH)_3$ and $Mn(OH)_2$ are 37.4 and 12.7, respectively (Meites, 1963). Because the oxidation of Mn(II) to either Mn(III) or Mn(IV) by dissolved oxygen in sea water was found to be very slow, and manganese was an effective adsorbent for copper ions only in the presence of *Nitzschia*, it is likely that there is a *de novo* formation of $Mn(OH)_3$ on the surface of the algal membrane. This oxidation of Mn(II) to (III) is probably caused by superoxide radical, O_2^- (Davies, 1969), and may involve a manganese–algal exudate complex. Our selective leaching experiments showed that the oxidation of Mn(II) by *Nitzschia* did not proceed to Mn(IV), i.e., to MnO_2.

Sunda et al. (1981) found that the addition of 10^{-7} M $MnCl_2$ totally reversed the toxicity of copper (3×10^{-8} M) to *Chaetoceros socialis* in deep sea water. They proposed a mechanism involving cupric ion competition for manganese-activated sites within the algal cell, i.e., copper induces manganese deficiency which can be overcome by increasing the manganese concentration. We were unable to demonstrate reversal of copper toxicity (10^{-7} M) by manganese (10^{-6} M) in *N. closterium*. Manganese did stimulate growth slightly, regardless of whether copper was present or not. Anderson and Morel (1978) also reported that manganese did not reverse the toxicity of copper to flagellar motility in *Gonyaulax tamarensis*. Huntsman and Sunda (1980) concluded therefore, that copper-manganese interactions occur within the cell at specific loci, rather than at the membrane surface.

Our experiments to test the competitive binding theory suggest that it is not valid

for *N. closterium*. We showed that copper does interfere with extracellular manganese binding, but has no effect on intracellular manganese over the range 0–40 μg Cu·l^{-1}. These results do not support Sunda and Huntsman's (1983) conclusion that copper competitively blocks cellular manganese uptake or binding of manganese within intracellular pools. In their experiments, cellular manganese concentrations were determined on ^{54}Mn labelled cells which had been filtered on a 3 μm filter, then rinsed with 5 ml sea water. They assumed that manganese on the filter was equal to manganese present in the cells (since manganese adsorbed to the filter in blank medium without algae was negligible). However, they did not take into account any manganese adsorbed to the outside of the cells, which we have shown to be much greater than intracellular manganese. To check whether a 5-ml sea-water wash removed any extracellular manganese, we repeated our competitive binding experiments, washing with sea water instead of 0.02 M ascorbic acid in sea water. The sea-water wash did not remove any extracellular manganese. Thus Sunda and Huntsman's cellular manganese values are actually total manganese associated with the cells – both intracellular and extracellular manganese. We have shown that, although there is competitive binding on the cell membrane between copper and manganese, copper does not affect intracellular manganese. Therefore the primary toxic effect of copper is not that it induces cellular manganese deficiency, but rather that it may oxidize thiol groups on the membrane and within the cell, disrupting protein structure and cell division (Florence and Stauber, in press).

Manganese, although effective in preventing toxicity from ionic copper, does not reduce the toxicity of lipid soluble copper complexes, such as copper oxinate, which rapidly penetrate the cell (Florence et al., 1983).

Superoxide radical (O_2^-) is produced by the reduction of molecular oxygen in illuminated chloroplasts (Kono et al., 1976) and its concentration may be increased by the presence of copper (Florence, 1984). Manganese (II) has been shown to scavenge O_2^- catalytically in the chloroplast (Kono et al., 1976) and in the bacterium, *Lactobacillus plantarum* (Archibald and Fridovich, 1981). From oxygen and hydrogen peroxide measurements we have shown that Mn(II) in sea water catalytically scavenges O_2^-. It appears that nickel(II), cobalt(II) and iron(II) are ineffective in catalyzing this reaction. Cobalt, however, significantly reduces copper toxicity in *N. closterium*, whereas nickel does not. We believe this is caused by the ability of cobalt to be oxidized by H_2O_2 or O_2^- in sea water to form cobalt (III) hydroxide. Co(III), like Mn(III), is a most effective adsorbent for copper.

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Paper 4

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TOXICITY OF COPPER COMPLEXES TO THE MARINE DIATOM *NITZSCHIA CLOSTERIUM*

T.M. FLORENCE and J.L. STAUBER

CSIRO Division of Energy Chemistry, Private Mail Bag 7, Sutherland, N.S.W., Australia 2232

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Copper ions initially bind to *Nitzschia* with a stability constant, β_1, in the range $\log\beta_1 < 13 > 10$, a value which precludes sulfur bonding and suggests involvement of a membrane protein. Although the algal growth rate in sea water was halved by 20 μg Cu·l^{-1}, photosynthesis was not affected until the copper concentration was above 100 μg Cu·l^{-1}. This decoupling of growth and photosynthesis did not occur to the same extent with other algae species tested, and it is proposed that it results from an intracellular reaction between Cu^{2+} and GSH, leading to a lowering of the GSH/GSSG ratio and suppression of mitosis. Water-soluble ligands generally decreased the toxicity of copper, whereas lipid-soluble copper complexes were highly toxic. Oxine, 2,9-dimethyl-1,10-phenanthroline, and pyridyl- and thiazolyl-hydroxyazo compounds formed exceptionally toxic complexes. The toxicity of many redox-active compounds and their copper complexes appears to be due, at least partly, to the intracellular generation of hydrogen peroxide during oxidation of these compounds, an effect exacerbated by the ability of copper to inhibit catalase. Hydrogen peroxide is highly toxic towards marine algae, and may be a natural growth-inhibiting factor. Hydroxyl radicals (OH·), generated intracellularly from a mixture of hydrogen peroxide and the lipid-soluble thiol, cysteine, were also extremely toxic. However, when OH or superoxide radicals ($O_2^{\cdot-}$) were generated extracellularly, no effect on growht rate was observed, even though singlet oxygen ($^1O_2$) did seriously reduce growth.

Key words: toxicity; copper complexes; *Nitzschia closterium*

INTRODUCTION

Various theories have been proposed to explain the toxicity of copper ions to organisms. These theories include: competitive inhibition by copper of manganese uptake in phytoplankton (Sunda and Huntsman, 1983), depression of ATP production (Viarengo et al., 1981), blockage of membrane-bound sulfhydryl groups (Hochstein et al., 1980), interference with silicon metabolism (Thomas et al., 1980), disruption of cell separation (Thomas et al., 1980), inhibition of enzymes (Metz and Sagone, 1972), increase in membrane permeability (Hwang et al., 1972; Lambert et al., 1984), catalysis of the oxidation of membrane lipids (Hochstein et al., 1980), and catalysis of the formation of hydrogen peroxide and oxygen free radicals from oxygen (Florence et al., 1984). However, most of the evidence for these toxicity mechanisms has been indirect. Shaw and Grushkin (1957) pointed out the correla-

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tion between toxicity to aquatic organisms and metal–sulfur binding strength for a range of metals. The toxicity of free metal ion towards phytoplankton was found by Canterford and Canterford (1980) to decrease in the order, $Hg^{2+} > Ag^{+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Tl^{+}$, which is approximately the order of decreasing solubility product of the metal sulfides, or the stability constants of the metals with thiol ligands like cysteine or glutathione. The ratio of reduced to oxidized glutathione (GSH/GSSG) in sea urchin eggs and other organisms was found to fluctuate in a regular fashion with the mitotic cycle, a high GSH/GSSG ratio favouring cell division (Kosower and Kosower, 1978). This led Lumsden and Florence (1983) to suggest that copper complexing of GSH (thus lowering the GSH/GSSG ratio) was the reason why copper inhibited the growth of the marine diatom *Nitzschia closterium*, but had no effect on its photosynthesis.

If copper binding to SH-enzymes or thiols is the origin of copper toxicity to phytoplankton, then it might be expected that in a water sample there would be a correlation between the fraction of total dissolved copper that could be removed by a thiol resin, and the toxic fraction of copper in the sample. However, in studies with *Nitzschia* this was found not to be the case, thiol resin-labile copper being much larger than the toxic fraction (Florence et al., 1983).

Although the free hydrated cupric ion is usually considered to be the most toxic physico-chemical form of copper, some lipophilic organocopper complexes are even more potent (Albert, 1965; Ahsanullah and Florence, 1984; Florence et al., 1984). Lipid-soluble copper complexes can diffuse directly into a biomembrane, whereas cupric ions are transported by the slower process of facilitated diffusion (Williams, 1981). In addition, lipid-soluble complexes carry into the membrane and cytosol both copper and the ligand, which may exert its own toxicity. Albert (1965) showed that many organic compounds are toxic to bacteria and other organisms only when they are combined with a heavy metal such as copper or iron to form neutral, lipid-soluble complexes.

Some ligands, e.g., 1,10-phenanthroline, form copper complexes that catalyze the production of excited oxygen species from molecular oxygen (Florence, 1984; Florence et al., 1984). These oxygen species, including hydroxyl ($OH\cdot$) and superoxide ($O_2^{\cdot-}$) radicals, singlet oxygen ($^1O_2$) and hydrogen peroxide, if produced in the cell or the cell membrane, can destroy the organism. Hydrogen peroxide is toxic to a wide range of cells, and it is likely that its effects are the result of the ability of H_2O_2 to react with various biological reducing agents (e.g., quinols and amines) to produce the highly destructive hydroxyl radical (Florence, 1984),

$$H_2O_2 + e^- + H^+ \rightarrow H_2O + OH\cdot \qquad (1)$$

Reaction (1) is normally quite slow, but is catalyzed by some copper and iron complexes. Phytoplankton produce considerable amounts of H_2O_2 during photosynthesis (Stevens et al., 1973), and the presence of this toxic substance in the cells may

impose a self-limiting control on their growth (Florence et al., 1984). Any substance that inhibits catalase will cause a rapid increase in cellular H_2O_2.

This present study investigated the toxic mechanisms of a range of copper complexes to *Nitzschia closterium*, the relationship between toxicity and lipophilicity, and the ability of the complexes to inhibit enzymes and to catalyze the formation of excited oxygen species.

EXPERIMENTAL PROCEDURES

Algal cultures

Nitzschia closterium, (Ehrenberg) W. Smith a unicellular marine diatom (originally obtained from CSIRO Division of Fisheries Algal Culture Collection) was cultured in f medium (Guillard and Ryther, 1962), but with the trace elements halved, and only 45 μg iron citrate$\cdot l^{-1}$ (iron/100). The cultures were illuminated on a light box (Philips TL 40 W, fluorescent white, 6,400 lux) at 21°C with a 12 h/12 h light/dark cycle. The cultures were maintained axenic by using autoclaved media, and carrying out transfers in a laminar-flow cupboard.

Growth rate experiments

Algal assays were conducted as described previously (Lumsden and Florence, 1983) in filtered (0.45 μm), unsupplemented sea water collected 2 km off the coast of Sydney. *Nitzschia* was separated from the culture medium by centrifugation and washing, and an aliquot (0.1–0.3 ml) of the washed algal suspension was added to 50 ml of sea water in a 150 ml conical flask to give an initial cell density of 2 to 4×10^4 cells per ml. The flasks were covered with a loose glass cap, and placed on the light box. Cell density was measured initially and on three subsequent days using a haemocytometer. A regression line was fitted to a plot of log (cell density/initial cell density) vs. time, and growth rate (μ) in cell divisions/day was calculated from, $\mu = 3.32 \log(N/N_0)/t$, where t is growth time (days) and N and N_0 are the number of cells at time t and zero, respectively. Occasionally, there was a lag period in growth, and growth rate was then arbitrarily calculated from the initial and final cell densities.

Measurement of photosynthesis

Inhibition of photosynthesis by copper ion and copper complexes was determined by uptake of [^{14}C]carbonate, followed by removal of excess $H^{14}CO_3^-$ in acid solution, and liquid scintillation counting in Instagel (Lumsden and Florence, 1983).

ANALYTICAL PROCEDURES

Copper analysis

Copper was determined in sea water by anodic stripping voltammetry (ASV) after

U.V. irradiation, and in filtered algae by ASV after HNO_3-$HClO_4$ oxidation (Florence et al., 1983).

Hydrogen peroxide

Hydrogen peroxide was determined spectrophotometrically by the titanium method (Sellers, 1980). Oxidized diphenols interfere slightly by their brown colour, and this was corrected for by using a blank solution produced by removing H_2O_2 with catalase.

Octanol extractability

Sea water (10 ml) containing the copper complex was extracted with 10 ml of reagent grade (Fluka) *n*-octanol by shaking for 5 min in a separating funnel. Copper was then determined in the aqueous phase by ASV after U.V. irradiation. Octanol extractability is a useful model for lipid solubility (Davies and Dobbs, 1984).

Inhibition of enzymes

Standard assays (Colowick and Kaplan, 1955) were used for measurement of the activity of catalase, superoxide dismutase (SOD) and glutathione reductase; catalase by its ability to decompose H_2O_2, SOD by its destruction of the superoxide radical, O_2^- (where O_2^- was generated from dihydroxyfumaric acid and measured spectrophotometrically with nitro blue tetrazolium), and glutathione reductase by its catalysis of the reduction of GSSG to GSH by NADPH, followed by spectrophotometric determination of GSH with Ellman's reagent.

TABLE I

Effect of copper ion and copper complexes on the photosynthesis of *Nitzschia closterium*[a]

Copper ($M \times 10^{-7}$)	Ligand[b]	Conc ($M \times 10^{-7}$)	Photosynthesis (μmol CO_2 fixed per hour per 10^6 cells)	Growth rate, μ (div /day)
none	none	-	0.30 ± 0.05[c]	0.54 ± 0.06[c]
27.8	none	-	0.35	0.31
none	oxine	1.00	0.19	<0 05
0.79	oxine	1.00	0.17	<0 05
none	PAN	1.00	0.29	<0.05
0.79	PAN	1.00	0.28	<0.05
0.79	2,9-dmp	1.00	0.31	<0.05
1.58	EXA	5.00	0.37	0 05
3.15	NTA	400	0.28	0.45

[a] Algal culture was normal (f) iron concentration

[b] Oxine, 8-hydroxyquinoline; PAN, 4-(2-pyridylazo) naphthol; dmp, dimethyl-1,10-phenanthroline; EXA, ethylxanthogenate; NTA, nitrilotriacetic acid.

[c] 2σ.

RESULTS AND DISCUSSION

Growth rate and photosynthesis

It was noted previously (Lumsden and Florence, 1983) that whereas 20 μg $Cu \cdot l^{-1}$ caused 50% reduction in the growth rate of *Nitzschia*, even 50 μg $Cu \cdot l^{-1}$ had no measurable effect on photosynthesis. This unusual effect was investigated further by testing both copper sulfate and some copper complexes for inhibition of photosynthesis (Table I). Copper ion concentrations as high as 100 $\mu g \cdot l^{-1}$, which completely inhibited growth, had to be used before a reduction in photosynthesis occurred. With the exception of oxine, none of the other ligands or their copper complexes, either water soluble or lipophilic, had any significant effect on photosynthesis. Free oxine depressed photosynthesis, and the addition of copper did not enhance this inhibition. It should be noted that cell density affected the inhibition of algal growth by copper (Table II).

This decoupling of growth from the photosynthetic apparatus did not occur with the green alga *Chlorella pyrenoidosa* Chick or the marine pennate diatom *Asterionella glacialis* Castracane when they were tested with the same conentrations of copper; with these algae, both growth and photosynthesis were strongly depressed.

Copper (3.47×10^{-7} M) in sea water (pH 8.22) is computed (Fardy and Sylva, 1978) to be distributed between $Cu(OH)_2$, 43.7%; $CuCO_3$, 41.7%; $Cu(OH)^+$, 8.3%; Cu^{2+}, 2.5%; $CuCl^+$, 2.2%. However, in near-shore surface sea water, more than half the copper is bound in unidentified, highly stable complexes (Florence, 1982; Mackey, 1983), while in algal cultures, copper is also complexed, although relatively weakly, by algal exudate (Lumsden and Florence, 1983). For some natural complexing agents (fulvic, humic, and tannic acids) ASV-labile (i.e. electrochemically active) copper correlated well with the toxic fraction of copper (Florence et al., 1983). Earlier studies (Florence, 1982) with a wide range of ligands showed that the fraction of copper that could be removed from sea water by thiol or iminodiacetate resins was much higher than the toxic fraction. Therefore, the in-

TABLE II

Effect of cell density on copper inhibition of growth rate of *Nitzschia closterium* in sea water[a]

Cell density (cells/ml $\times 10^4$)		Growth rate, μ (div./day)	Inhibition of growth by 50 $\mu g \cdot l^{-1}$ (%)[b]
Initial	After 72 h		
1.6	2.9	0.53	100
3.1	9.6	0.54	52
6.6	13 4	0.34	21
14.1	23.1	0.24	11

[a] Cells were cultured in f-medium iron concentration, and growth experiments were made in unsupplemented sea water.

[b] Using 5×10^{-8} M oxine and 2 $\mu g \cdot l^{-1}$, negative growth was observed at all cell densities.

itial binding of copper to the algal cell membrane must be weaker than copper–thiol or iminodiacetate complexing. Examination of the effect of various ligands on the binding of copper ions to *Nitzschia* (Florence et al., 1983) indicated that the copper–algae stability constant (β_1) has a value of $\log\beta_1 < 13 > 10$. Gavis (1983) had estimated that $\log\beta_1$ for the binding of cupric ions to marine phytoplankton was 9–11. These results rule out the possibility that thiols are involved in the initial binding of copper to *Nitzschia*, since β_1 values many orders of magnitude higher would be expected if this were the case. The membrane groups which initially bind copper ions to algae are most likely protein carboxylic acid and amino residues. However, once copper has penetrated the membrane it may then possibly react with thiol compounds such as glutathione. Use of the lipid-soluble thiol reagent dithiodipyridine showed (Stauber and Florence, to be publ.) that treatment of *Nitzschia* with copper ions significantly lowered the concentration of intracellular thiol groups. These studies also showed that much of the copper adsorbed by *Nitzschia* is present as copper(I), further indicating that copper(II) is reduced to (I) by -SH groups, then bound as Cu(I)-S-complexes.

High iron or manganese in the algal culture medium efficiently protected against copper toxicity to *Nitzschia* by forming a protective layer of hydrated metal (III) oxide around the cell and preventing the diffusion of copper into the membrane (Stauber and Florence, 1985). These metals did not, however, protect against the toxicity of lipid-soluble copper complexes.

Toxicity of copper complexes with phenanthroline-type ligands

Table III shows data for the toxicity, octanol extractability (lipophilicity), and calculated formation of the 1:1 and 1:2 complexes (ML and ML_2) for some copper–phenanthroline type complexes. Ionic equilibrium calculations were made using SIAS (Fardy and Sylva, 1978) and published stability constants (Martell and Smith, 1977).

The most toxic copper complex was that of 2,9-dimethyl-1,10-phenanthroline (2,9-dmp or neocuproine), which caused a reduction in growth rate at concentrations as low as 5×10^{-9} M. The toxicity of free 2,9-dmp was much less than its copper complex. Table III shows that only the reduced, copper(I) complexes are lipid soluble; prior reduction of the copper(II) species is therefore a requirement of bioaccumulation. Copper(II)-2,9-dmp underwent only very slow (18% in 24 h) autoreduction in sea water illuminated on the light box, whereas more than 95% of the complex partitioned to the algae in 3 h (Florence et al., 1985). This result strongly suggests that *Nitzschia* itself produced the reductant which yielded the lipid-soluble copper(I) complex. It has been proposed (Florence et al., 1985) that this reductant is hydrogen peroxide. The inracellular reaction between excess H_2O_2 and copper(I)–2,9-dmp then gives rise to singlet oxygen, oxygen free radicals and bipyridyl compounds, these products causing the extreme toxicity of the complex. Other copper complexes of similar structure (e.g., copper–bathocuproine) are less toxic (Table

TABLE III

Toxicity of some phenanthroline-type copper complexes to *Nitzschia closterium* in sea water[a]

Ligand[b]	Conc. ($M \times 10^{-6}$)	Copper ($M \times 10^{-7}$)	Octanol extractability (%)[c]	Calculated formation of Cu(II) complex (%)		Toxicity index of copper complex[d]
				CuL_1	CuL_2	
none	-	3.15	<10	-	-	1.0[f]
1,10-phenanthroline	2.00	3.15	19	40	(99)[e]	1.2
4,7-dmp	2.00	3.15	58	7.0	92	4.7
5,6-dmp	2.00	3.15	61	12	87	3.0
2,9-dmp	0.05	0.32	92	26	(72)[e]	>25
4,7-dpp	2.00	3.15	52	-	-	5.0
4,7-dpp sulfonate	2.00	3.15	<10	-	-	0.40
bathocuproine	0.05	0.32	90	-	-	2.1
bathocuproine disulfonate	2.00	3.15	<10	-	-	< 0.1
2,2'-bipyridyl	2.00	3.15	11	5.8	(22)[e]	0.70
tripyridyl-*S*-triazine	2.00	3.15	20	-	-	1.5
2,2'-biquinoline	0.10	0.79	38	-	(35)[e]	3.9

[a] f/10 iron in culture medium.

[b] dmp, dimethyl-1,10-phenanthroline; dpp, diphenyl-1,10-phenanthroline; bathocuproine, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline.

[c] In presence of hydroxylamine hydrochloride as reducing agent. Little extraction in absence of reducing agent.

[d] Toxicity index is defined as the ratio of the apparent copper concentration (as determined from a growth rate–copper concentration curve) to the added copper concentration. A toxicity index greater than 1.0 indicates that the complex is more toxic than inorganic copper(II), and vice versa Results were corrected for the toxicity of excess ligand.

[e] Calculated formation of copper(I) complex ($Cu(I)L_2$)

[f] 47% depression of growth rate.

III) because they do not enter into this reaction with H_2O_2. The sulfonated derivatives actually decrease the toxicity of copper ion, because they bind copper in stable, hydrophilic complexes which do not diffuse into the cell.

Toxicity of other copper complexes

Table IV shows the toxicity, octanol extractability and degree of formation of some copper complexes formed from various ligands. In general, ligands (e.g., PAR and TAR) which produced water-soluble complexes with the copper ion reduced its toxicity, whereas ligands which strongly chelated copper in sea water to yield lipid-soluble complexes (e.g., PAN and TAN) greatly increased toxicity. Hemoglobin and bovine serum albumin (10 $\mu g \cdot ml^{-1}$) completely eliminated the toxicity of 20 $\mu g\ Cu \cdot l^{-1}$.

Oxine, PAN and TAN form especially toxic complexes with copper (Table IV). Other metal oxinates (Fe(III), Al, Sn(II), Ni, V(IV), Mo(VI)) were non-toxic when

TABLE IV

Toxicity of copper complexes to *Nitzschia closterium* in sea water

Ligand	Conc. ($M \times 10^{-6}$)	Mole ratio ligand/Cu	Calculated formation of complex (%)	Octanol extractable (%)	Toxicity index[a]
Nitrilotriacetic acid	2.0	6.0	91	<10	0 6
8-hydroxyquinoline	0.05	2.0	84	78	18
4-hydroxyquinoline	0.05	2.0	-	<10	1.0
8-aminoquinoline	2.0	6.0	2.3	45	2.5
3-aminoquinoline	2.0	6.0	-	<10	0.8
8-hydroxyquinoline-5-sulfonate	2.0	6.0	97	<10	0 35
8-hydroxyquinaldine	0.05	2.0	-	75	18
1-(2-pyridylazo)-2-naphthol	0.05	2.0	100	84	>25
1-(3-pyridylazo)-2-naphthol	0.05	2.0	-	<10	1.7
1-(2-pyridylazo)-4-naphthol	0.05	2.0	-	<10	< 0.5
1-(2-pyridylazo)-resorcinol	2.0	6.0	100	<10	< 0.1
1-(2-thiazolylazo)-2-naphthol	0.05	2.0	-	90	>25
1-(3-thiazolylazo)-2-naphthol	0.05	2.0	-	<10	1 6
4-(2-thiazolylazo)-resorcinol	2.0	6.0	-	<10	< 0.1
1-hydroxypyridine-2-thione	2.0	6.0	-	75	2 1
cupferron	2.0	6.0	-	82	0 7
penicillamine	2.0	6.0	100[b]	<10	< 0.1
cysteine	2.0	6.0	100[b]	<10	0.6
glutathione	2.0	6.0	100[b]	<10	< 0.1
lysine	2.0	6.0	< 1	<10	0 9
uracil	2.0	6.0	-	<10	0.5
sodium ethylxanthate	0.10	4.0	-	86	3.5
sodium-*i*-propylxanthate	0.10	4.0	-	-	1.9
sodium amylxanthate	0.10	4.0	-	-	1.5
salicyclic acid	2.0	6.0	1.3	15	1 0
sulfosalicyclic acid	2.0	6.0	3.6	<10	0.9
kojic acid	2.0	6.0	-	<10	1.1
catechol	2.0	6.0	82	-	1.0

[a] For definition see footnote to Table III.

[b] Copper(I) complex.

tested at the same molarity as copper. Equilibrium calculations (SIAS) showed that for Al, Ni and Fe(III), little oxine complex formation would occur under the conditions used. Albert and co-workers (1965), Block (1956) and Sijpesteijn et al. (1957) found that the toxicity of copper oxinate to various organisms varied with the ratio of the 1:1 and 1:2 copper–oxine complexes in the test solution. We did not find this effect with *Nitzschia*. By changing the oxine concentration with a fixed total copper concentration of 4.76×10^{-8} M (3 μg Cu·l^{-1}), the ratio of the 1:1 to 1:2 copper–oxine complexes in the test solution was varied from 0.17 to 4.2, as calculated by the ionic equilibrium program SIAS. However, toxicity to *Nitzschia* was found to be

related to the total percentage of copper complexed by oxine, and not to the ratio of the 1:1 and 1:2 complexes. The earlier workers used much higher copper concentrations than were used here, and their results may have been an experimental artefact caused by precipitation of the water-insoluble 1:2 copper–oxine complex.

Copper oxinate was almost completely (>95%) absorbed by *Nitzschia* cells within a few hours. However, at the end of the 4-day bioassay, copper remained in the cells but all the oxine had been ejected. The filtered, 4-day-old algal suspension contained no copper, but after the addition of excess copper sulfate, it gave a U.V.-visible absorbance spectrum identical to that of copper oxinate, with an oxine recovery of greater than 95%. Copper oxinate apparently dissociates within the algal cell, copper being retained and the oxine expelled. However, since the excreted oxine still retains its chelating ability towards copper, the molecule is either unchanged, or metabolized to a very similar new species which still reacts with copper, such as a hydroxy derivative (Williams, 1959). This type of behaviour, where the metal is retained by the organism, but the ligand expelled, has been observed previously for the copper-kethoxal bis (thiosemicarbazone) complex (Booth and Sartorelli, 1967) and copper – 2,9-dmp (Mohindru et al., 1983) in tumour cells, and copper-2,9-dmp in *Nitzschia* (Florence et al., 1985). *Nitzschia*, however, metabolizes 2,9-dmp to bipyridyl compounds before expelling them.

The importance of the chelate effect in algal toxicity is demonstrated (Table IV) by the decrease in toxicity when the heterocyclic nitrogen in the pyridylazo and thiazolylazo compounds is shifted from the chelate-forming 2-position to the relatively non-chelating 3-position, and when the hydroxy group is moved from the ortho-position. A similar effect is observed with the hydroxy- and aminoquinolines.

Effect of metabolic inhibitors and other compounds on growth of Nitzschia

The metabolic inhibitors tested which were most toxic to *Nitzschia* are acetylphenylhydrazine and 2,4-dinitrophenol (Table V). Acetylphenylhydrazine causes rapid and extensive oxidation of cellular glutathione, while 2,4-dinitrophenol is a potent uncoupler of oxidative phosphorylation, leading to inhibition of ATP synthesis.

The herbicides methyl viologen (Paraquat) (Hassan and Fridovich, 1978) and 3-amino-1,2,4-triazole (Cohen and Hochstein, 1965) unexpectedly had relatively low toxicities, but this may be due to the lack of organic matter (electron source) in the unsupplemented sea water used for the algal assays (Hassan and Fridovich, 1978).

Table V shows that catalase and superoxide dismutase (SOD) greatly enhance the growth of *Nitzschia*, and that this enhancement was due to the catalytic activity of the enzymes, since they were much less effective when deactivated by boiling. These results suggest that intracellular H_2O_2 and superoxide radical (O_2^-) control the growth of *Nitzschia*, and that their intracellular concentrations can be lowered by extracellular catalase or SOD by a mass action effect. Both H_2O_2 and O_2^- can diffuse

TABLE V

Toxicity of some metabolic inhibitors and other compounds to *Nitzschia closterium* in sea water

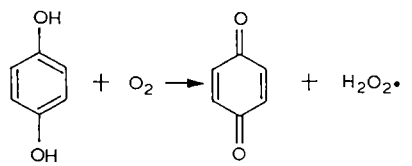
Compound	Conc. ($M \times 10^{-5}$)	Algal growth rate (% of control)
Metabolic inhibitors		
dichloroindophenol	10	45
alloxan	1.0	95
2,4-dinitrophenol	0.50	35
acetylphenylhydrazine	0.20	33
p-chloromercuribenzene sulfonate	1.00	76
3-amino-1,2,4-triazole	1.00	78
sodium azide	1.00	89
indomethacin	0.50	41
methyl viologen	1.00	78
potassium cyanide	1.00	50
Other compounds		
dihydroxyfumaric acid	1.00	92
catalase	0.02 $mg \cdot l^{-1}$	185
catalase (boiled)	0.02 $mg \cdot l^{-1}$	109
superoxide dismutase	0.04 $mg \cdot ml^{-1}$	172
superoxide dismutase (boiled)	0.04 $mg \cdot ml^{-1}$	118
urease	0.04 $mg \cdot ml^{-1}$	zero
hexokinase	0.04 $mg \cdot ml^{-1}$	85
trypsinogen	0.04 $mg \cdot ml^{-1}$	83
pepsin	0.04 $mg \cdot ml^{-1}$	66
Rose Bengal	0.50	28
xanthine oxidase	0.04 mg ml^{-1}	81
riboflavin	1.00	100
ascorbic acid	1.00	96
hydroquinone	0.02	65
1,4-benzoquinone	0.20	98
catechol	0.20	84
resorcinol	1.00	89

readily through membrane lipid material (Lynch and Fridovich, 1978; Voetman and Roos, 1980) although O_2^- generated extracellularly by xanthine oxidase, dihydroxyfumaric acid or riboflavin had no toxic effect (Table V). On the other hand, Rose Bengal, which on illumination generates singlet oxygen ($^1O_2$) was highly toxic, probably due to the oxidation of membrane lipids by $^1O_2$ (Foote, 1976).

Urease was by far the most toxic of several proteolytic and other enzymes tested (Table V). This may be the result of the ability of urease to catalyze the formation of ammonia, which severely inhibits the growth of algae.

The lipid solubility of dihydroxybenzene decreases in the order hydroquinone > catechol > resorcinol (Banerjee et al., 1980), which is the same order as

their toxicity towards *Nitzschia* (Table V). In addition, hydroquinone reacts with oxygen to produce a high concentration of H_2O_2 via the reaction,



(2)

It is likely that this H_2O_2 is the actual toxic agent (Florence and Mann, in prep.). Resorcinol (1,3-dihydroxybenzene), which cannot form a quinone structure, had a low toxicity and gave a small yield of H_2O_2.

Inhibition of enzymes

The effect of copper and some copper complexes on the catalytic efficiency of SOD and glutathione reductase (Table VI) and catalase (Table VII) was studied. Free 2,9-dmp seriously inhibited the activity of SOD in sea water, although the copper–2,9-dmp complex had no effect. Inactivation is probably the result of 2,9-dmp extracting copper from this copper, zinc enzyme. Copper ions and the copper-1,10-phenanthroline complex actually supplemented the effect of SOD by their own dismutase activity, as has been reported previously (Sorenson et al., 1984). Oxine and copper oxinate were the only compounds tested which seriously lowered the activity of glutathione reductase in phosphate buffer, pH 7.0 (Table VI).

The activity of catalase in sea water was significantly reduced by copper ions and by 1,10-phenanthroline and 2,9-dmp, but not by their copper complexes (Table VII). This inhibition of catalase may be due to removal of iron from the active centre of the enzyme by the phenanthroline-type ligands, with which iron forms strong complexes, and by replacement of iron by copper.

TABLE VI

Effect of copper ion, ligands, and copper complexes on the activity of superoxide dismutase (SOD) and glutathione reductase (GR)

Compound[a]	Conc. ($M \times 10^{-5}$)	Change in SOD activity (%)[b]	Change in GR activity (%)[c]
Cu^{2+}	4.0	+191	−27
1,10-phenanthroline	8.0	− 11	+29
Cu-1,10-phenanthroline (1·2)	4.0	+ 90	−
2,9-dmp	8 0	− 60	−
Cu-2,9-dmp	4.0	0	−24
oxine	8.0	+ 22	−78
Cu-oxine (1:2)	4 0	− 4	−69

[a] dmp, dimethyl-1,10-phenanthroline Mole ratios of copper to ligand are shown for complexes.

[b] Measured in sea water.

[c] Measured in phosphate buffer, pH 7.0.

TABLE VII

Inhibition of catalase by copper ion, ligands, and copper complexes in sea water

Compound[a]	Conc. ($M \times 10^{-5}$)	Residual H_2O_2 after 5 min reaction (%)[b]
none	-	< 0.5
Cu^{2+}	3.8	21
2,9-dmp	7.6	14
Cu-2,9-dmp (1.2)	3 8	< 0.5
1,10-phenanthroline	7.6	27
Cu-1,10-phenanthroline (1:2)	3.8	< 0.5
oxine	7.6	6
Cu-oxine (1:2)	3 8	< 0.5

[a] dmp, dimethyl-1,10-phenanthroline. Mole ratios of copper to ligand are shown for complexes.
[b] Initial solution contained 8×10^{-4} M H_2O_2 and 2 μg ml^{-1} catalase in sea water.

Toxicity of hydrogen peroxide

The effect of H_2O_2 on the growth of *Nitzschia* is shown in Table VIII. The blank H_2O_2 in a 7-day-old-culture of *Nitzschia* was about 1×10^{-8} M. During the growth experiments there was a substantial decrease in the concentration of H_2O_2 (Table VIII).

Low concentrations of catalase dramatically increased algal growth rate (Table V), which suggests that intracellular H_2O_2 can be drawn from the cell by a mass action effect and dissociated extracellularly by catalase. The lowered cell concentration of toxic H_2O_2 then leads to increased growth. Many species of algae are known to liberate H_2O_2 during photosynthesis (Stevens et al., 1973), and it has been suggested (Florence et al., 1984), that H_2O_2 production leads to self-regulation of algal growth. Table VIII shows that H_2O_2 in the growth medium (unsupplemented sea water) is highly toxic to *Nitzschia*, even though the concentration of H_2O_2 decreases rapidly with time. An initial concentration of 2.5×10^{-5} M H_2O_2 causes a 50%

TABLE VIII

Toxicity of hydrogen peroxide to *Nitzschia closterium* in sea water

H_2O_2 ($M \times 10^{-5}$)[a]				Algal growth rate (72 h) (% of control)
Initial	24 h	48 h	72 h	
8.0	3.45	0.56	<0.1	negative
6 0	2.72	0.45	<0.1	negative
5 0	1.85	0.33	<0 1	negative
4.0	1.53	<0.1	<0 1	21
3.0	0.93	<0.1	<0.1	40
2.0	0.57	<0.1	<0.1	69

[a] Concentration of H_2O_2 measured in sea water during the 72 h growth experiments.

decrease in growth rate. Hydrogen peroxide can be produced extracellularly by the oxidation of many organic compounds (e.g. phenols, amines, aminophenols) by dissolved oxygen in the growth medium (Florence, 1984), and intracellularly by the mixed function oxidase (cytochrome P450) system, or by a variety of redox compounds which divert electron flow during respiration (Hassan and Fridovich, 1979). Any agent (e.g., Cu^{2+} or phenanthroline-type ligands, Table VII) which inactivates catalase will also increase the cellular concentration of H_2O_2.

Although H_2O_2 has a low in vitro reactivity with many biological compounds, its high mobility in the cell may allow it to diffuse to a vulnerable region and react with a reducing agent (e.g., thiol, NADPH, flavin, diphenol) to produce the highly damaging hydroxyl free radical, OH· (Reaction (1)). Hydrogen peroxide is also formed during the photochemical oxidation of humic matter in natural water systems (Cooper and Zika, 1983); this process may exert some control on algal density.

Toxicity of hydroxyl radical generating systems

Hydroxyl radical, OH·, is produced during the oxidation of thiols by H_2O_2, e.g.,

$$GSH + H_2O_2 \rightarrow GSSG + H_2O + OH\cdot \quad (3)$$

TABLE IX

Toxicity of hydroxyl radical generating systems to *Nitzschia closterium* in sea water

Thiol[a]	Catalyst[b]	H_2O_2 added before or after algae[c]	Algal growth rate (% of control)
GSH	nil	nil	105
GSH	nil	before	88
GSH	nil	after	69
cysteine	nil	nil	95
cysteine	nil	before	85
cysteine	nil	after	37
nil	Fe-EDTA	nil	89
nil	Cu-phen	nil	81
nil	nil	after	40
cysteine	Fe-EDTA	nil	92
cysteine	Fe-EDTA	before	69
cysteine	Fe-EDTA	after	negative
GSH	Fe-EDTA	before	78
GSH	Fe-EDTA	after	72
cysteine	Cu-phen	before	65
cysteine	Cu-phen	after	negative
GSH	Cu-phen	after	70

[a] 3×10^{-5} M thiol.

[b] Catalysts were 1×10^{-5} M Fe(II) + 2×10^{-5} M EDTA or 1×10^{-7} M Cu^{2+} + 5×10^{-7} M 1,10-phenanthroline.

[c] All other reagents added to flask, then 3×10^{-5} M H_2O_2 added either 5 min before algae or 30 min after.

Reaction (3) is fairly slow, but is catalyzed by some metal complexes, including Fe(II)-EDTA and Cu-1,10-phenanthroline (Florence, 1984). Table IX shows that, even in the presence of a catalyst, GSH + H_2O_2 is not especially toxic to *Nitzschia*; neither is cysteine + H_2O_2 if the H_2O_2 and cysteine are mixed before adding the algae. However, if cysteine is equilibrated with the algae for 30 min before adding H_2O_2, a large decrease in growth rate results, and this decrease is accentuated by catalysts (Table IX). A similar effect does not occur with GSH.

Cysteine has a much higher lipid solubility than GSH (Berglin et al., 1982; Leo et al., 1971), and it is likely that the toxicity of the cysteine + H_2O_2 system is due to the production of OH· radicals in the cell membrane (Berglin et al., 1982). If H_2O_2 is mixed with the thiol before addition of the algae, the thiol is oxidized extracellularly, and less membrane damage occurs. Copper-1,10-phenanthroline and Fe(II)-EDTA have some lipid solubility (Borg et al., 1984), and these catalysts increase the rate of OH· production in the membrane.

CONCLUSIONS

While most ligands that form water-soluble complexes with copper ameliorate the toxicity of this metal towards algae, lipid-soluble copper complexes are usually more toxic than free copper ion. In addition to promoting the rapid diffusion of copper into the cell, these lipid-soluble complexes also transport the ligand into the membrane and cytostol, where it may exert its own toxic effect. For example, 2,9-dmp inhibits superoxide dismutase, oxine inhibits glutathione reductase, and 1,10-phenanthroline inhibits catalase. The toxicity of redox-active compounds such as diphenols and aminophenols is due, at least partly, to the intracellular formation of H_2O_2, which is highly damaging to algae. Copper ions strongly deactivate catalase, the loss of which would weaken cellular defences against H_2O_2, an effect exacerbated by the superoxide dismutase activity of Cu^{2+}, leading to the formation of additional H_2O_2,

$$2O_2^- + 2H^+ \xrightarrow{Cu^{2+}} H_2O_2 + O_2. \qquad (4)$$

The presence of lipid-soluble reducing agents (e.g. cysteine) plus H_2O_2 in the algal growth medium leads to a large decrease in growth rate as a result of the intracellular formation of hydroxyl radicals.

No unambiguous explanation can be offered for the unusual ability of copper ions to inhibit the growth, but not the photosynthesis, of *Nitzschia*, although the lowering of the cellular GSH/GSSG ratio by the reaction of Cu^{2+} with GSH may be involved in the inhibition of mitosis.

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REVERSIBILITY OF COPPER-THIOL BINDING IN *NITZSCHIA CLOSTERIUM* AND *CHLORELLA PYRENOIDOSA*

J.L. STAUBER and T.M. FLORENCE

CSIRO Division of Energy Chemistry, Private Mail Bag 7, Sutherland, NSW 2232, Australia

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The effect of ionic copper and copper complexes on intracellular thiols in the marine diatom *Nitzschia closterium* (Ehrenberg) W. Smith, and the green alga *Chlorella pyrenoidosa* Chick was studied using the lipid-soluble thiol reagent, dithiodipyridine. Treatment of cells with copper ions significantly reduced the concentration of intracellular thiol groups, whereas copper complexes and H_2O_2 had no effect

To test the reversibility of intracellular copper-thiol binding in *N. closterium* and *C. pyrenoidosa*, cells exposed to copper (60–200 μg Cu l^{-1}) were treated with various sulfhydryl compounds and chelating agents, with and without removal of copper from solution. Cysteine, glutathione (GSH), EDTA and bathocuproine disulfonate were most effective in reversing copper toxicity, whereas oxidized glutathione, cystine and glycine had no effect. It is proposed that copper (II) adsorbed to the algae is reduced to copper (I) by -SH groups, and bound as a Cu(I)-S-complex. Sulfhydryl compounds can reverse toxicity through thiol-disulfide interchange reactions, allowing algal growth to recommence, and powerful copper-chelating agents produce the same effect by withdrawing copper from the cell.

Key words: copper toxicity; sulfhydryl compounds; thiol-disulfide interchange

INTRODUCTION

Although ionic copper and lipid-soluble copper complexes are known to be very toxic to phytoplankton (Mandelli, 1969; Erickson, 1972; Lumsden and Florence, 1983), the mechanism of the toxicity is still uncertain. It has been proposed that initial copper binding on the membrane may be to protein carboxylic and amino residues, rather than to thiol groups, because the copper-algae stability constant is orders of magnitude lower than the thiol-copper binding constant (Gavis, 1983; Florence et al., 1983). Following copper penetration into the cell, copper may react with -SH enzyme groups and thiols, disrupting enzyme active sites, and leading to a lowering of the GSH/GSSG ratio and suppression of mitosis (Florence and Stauber, to be published). Thus, 20 μg Cu $\cdot l^{-1}$ inhibits growth of *Nitzschia closterium* but not photosynthesis.

During copper toxicity studies with *N. closterium*, we found that the toxicity of the thiol-oxidizing agent, *N*-ethylmaleimide (5×10^{-7} M), could be reversed by the addition of 5×10^{-5} M thiols, such as cysteine and reduced glutathione (GSH).

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Disulfide bonds may be broken by thiol-disulfide interchange reactions; these are oxidation-reductions which allow interconversions between stable compounds (Kosower and Kosower, 1978), e.g.

$$RS^- + GSSG \rightleftharpoons RSSG + GS^-.$$

If copper also exerts its toxicity by reversibly binding and oxidizing thiol groups, we could expect that copper toxicity would also be reversed by the addition of thiols. It has been shown that cysteine -SH groups, oxidized to disulfides by the copper-1,10-phenanthroline complex in air, were reduced by the addition of sulfhydryl compounds (Kobashi, 1968). Perhaps a similar mechanism could operate in the cell.

In this study, we examined thiol-copper binding in *N. closterium* by measuring intracellular thiol groups before and after treatment with copper ion and copper complexes, and the reversal of copper toxicity in *N. closterium* and *Chlorella pyrenoidosa* by sulfhydryl compounds.

METHODS

Algal cultures

The unicellular marine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith was cultured in f medium (Guillard and Ryther, 1962) with trace elements and the iron concentration halved. The freshwater green alga, *Chlorella pyrenoidosa* Chick, was maintained in MBL medium (Stein, 1973). Both species were originally obtained from the CSIRO Division of Fisheries Algal Culture Collection. Cultures were maintained axenically on a 12:12 h light/dark cycle (6400 lux) at 21°C.

Growth rate experiments

Toxicity of copper, and its reversal by sulfhydryl compounds, was studied using algal assays described previously (Lumsden and Florence, 1983). Algal assays using *N. closterium* were done in filtered (0.45 μm) unsupplemented seawater collected 2 km off Port Hacking, N.S.W. *C. pyrenoidosa* assays used 50 ml standard softwater ($NaHCO_3$, 48 $mg \cdot l^{-1}$; $CaSO_4 \cdot 2H_2O$, 30 $mg \cdot l^{-1}$; $MgSO_4$, 30 $mg \cdot l^{-1}$; KCl, 2 $mg \cdot l$) with the addition of 1 ml of 0.5 M HEPES buffer (pH 7.0), 0.5 ml $NaNO_3$ (2.1 $g \cdot l^{-1}$) and 0.5 ml K_2HPO_4 (0.22 $g \cdot l^{-1}$). Cell density was measured initially and on 4 subsequent days by counting microscopically in a haemocytometer. After exposure for 24 or 48 h to sublethal copper concentrations (60–200 μg $Cu \cdot l^{-1}$), which inhibited cell division, test compounds were added to the assay flasks, with and without the removal of copper by centrifugation and washing. Growth rates after addition of test compounds were compared to growth rates in the presence of copper only. Blank cultures, not exposed to copper, were used as controls, with and without each test compound.

Determination of intracellular thiol groups

Two methods were used to measure intracellular thiol groups after exposure for 24 h to copper and copper complexes; the method of Roberts and Rouser (1958) and Alexander (1958), using *N*-ethylmaleimide, and a modified version of the method of Grassetti and Murray (1967), using the lipid-soluble thiol reagent, dithiodipyridine. As the latter method was found to be more sensitive, only its results are reported here.

Algae were centrifuged, washed and resuspended in 3 ml of 1 M phosphate buffer (pH 7.2). Three millilitres of fresh 2,2′-dithiodipyridine (2×10^{-3} M) were added and the cells vortexed vigorously to ensure complete cell breakage. After 30 min, cell debris was removed by centrifugation and the absorbance of the supernatant measured at 342 and 233 nm on a Cary u.v. spectrophotometer. 2,2′-Dithiodipyridine reacts with thiols to form 2-thiopyridone with a change in the u.v. absorption spectrum. Absorbance at 342 nm for each flask was corrected for algae blanks, without dithiodipyridine treatment, and for a dithiodipyridine blank in phosphate buffer. The absorbance of this blank at 233 nm was used to correct for unused dithiodipyridine in each sample. A freshly prepared cysteine standard (6×10^{-5} M) was used for calibration.

RESULTS AND DISCUSSION

Treatment of *N. closterium* with copper (175 μg Cu·l^{-1}) for 24 h significantly lowered the concentration of intracellular thiol groups (Table I). Only 1.68 nmol SH/10^6 cells were detected in copper-treated cells, compared to 3.68 nmol SH/10^6 cells in cells without copper exposure. Cedeno-Maldonado and Swader (1974), using the *N*-ethylmaleimide method, also showed that cupric ions decreased the -SH content of *Chlorella sorokiniana* Shihira and Krauss, from 0.051 μmol SH/μg chlorophyll to 0.028 μmol SH/μg chlorophyll. To compare these values with ours, we expressed our results on a per chlorophyll basis, using a previous chlorophyll determination (0.75 μg chlorophyll $(a + c)/10^6$ cells) for the same *N. closterium* isolate grown under similar light and temperature conditions (Stauber, 1984). Our thiol results (0.005 μmol SH/μg chlorophyll) for untreated cells are an order of magnitude lower than those of Cedeno-Maldonado and Swader (1974), but within the range reported for spinach chloroplasts (Hirose et al., 1971).

Only ionic copper significantly reduced intracellular thiol groups; the lipid-soluble copper complexes Cu-1,10-phenanthroline, Cu-oxine, Cu-5,6-dimethyl-1,10-phenanthroline, Cu-2,9-dimethyl-1,10-phenanthroline and Cu-ethylxanthogenate, and the water-soluble complex Cu-nitrilotriacetic acid had no effect, or slightly increased -SH concentration (Table I). These stable complexes, although able to penetrate the cell rapidly, apparently did not liberate free copper ion and therefore did not reduce intracellular thiols over this period. Hydrogen peroxide (4×10^{-5} M), which is highly mobile in cell membranes and is known to react with

TABLE I

Cellular thiol groups (mol SH/10^6 cells) in *N closterium* after exposure to copper (175 $\mu g \cdot l^{-1}$) and copper complexes for 24 h

Treatment	nmol SH/10^6 cells
Blank	3.7 ± 0 9*
175 μg Cu·l^{-1}	1 7
175 μg Cu·l^{-1}, then 5 × 10^{-5} M cysteine	3 3
175 μg Cu l^{-1}, then 5 × 10^{-5} M GSH	2 9
4 × 10^{-5} M H_2O_2	3.3
5 μg Cu l^{-1} + 5 × 10^{-8} M 1,10-phenanthroline	3.8
5 μg Cu l^{-1} + 5 × 10^{-8} M oxine	4.2
5 μg Cu l^{-1} + 5 × 10^{-8} M 5,6-dmp[b]	4 2
5 μg Cu l^{-1} + 5 × 10^{-8} M 2,9-dmp	4 9
10 μg Cu·l^{-1} + 5 × 10^{-7} M EXA	4 5
20 μg Cu·l^{-1} + 2 × 10^{-5} M NTA	4 2

Oxine = 8-hydroxyquinoline; dmp = dimethyl-1,10-phenanthroline, EXA = ethylxanthogenate, NTA = nitrilotriacetic acid, * one standard deviation.

thiols and to inactivate sulfhydryl enzymes (Florence, 1984), had little effect on intracellular thiol concentration in *N. closterium*. Perhaps the oxidation of GSH by oxidants such as H_2O_2 is reversible, via enzymes such as glutathione reductase which cannot function efficiently in the presence of copper.

Cells treated with copper ions for 24 h, followed by sulfhydryl compounds, e.g. cysteine, for 24 h, had only slightly lower intracellular thiol groups compared to unexposed cells (Table I). Coper may reversibly oxidize -SH groups within the cell, some of which can be again reduced by cysteine and GSH through thiol-disulfide interchange reactions. However, washing and centrifugation appeared to remove some thiol from the cells, so the accuracy in thiol determination is poor in these two cases.

Cedeno-Maldonado and Swader (1974) concluded that copper-thiol binding in *Chlorella* was irreversible. To test the reversibility of thiol oxidation by copper, we treated *N. closterium* cells exposed to copper (60, 70 and 80 μg Cu·l^{-1}) for 48 h, with the thiols GSH, cysteine and thionicotinamide (Table II). Growth recommenced in the presence of 5 × 10^{-5} M cysteine and GSH, with growth rates equal to or greater than the blank without copper treatment. Similar results were obtained when excess copper was removed by centrifugation and washing, before addition of the thiol. Thionicotinamide had no effect, perhaps because of low lipid solubility, and oxidized glutathione (GSSG) and oxidized cysteine (cystine) did not help recovery of copper-treated cells. This suggests that copper may exert its toxicity by oxidizing intracellular thiols, and that this process is reversible through thiol-exchange reactions.

Copper toxicity to *C. pyrenoidosa* may also be reversed by the addition of

5×10^{-5} M cysteine and GSH, with growth rates much greater than copper-treated cells, but lower than the blank (Table III).

Cysteine reversed copper toxicity more effectively than GSH in *N. closterium*, whereas in *C. pyrenoidosa*, GSH was slightly more effective. Lipid solubility indicates how well a compound may penetrate biological membranes, and *n*-octanol is a reasonable model for membrane lipids. We measured the distribution ratio, D (total concentration in *n*-octanol/total concentration in seawater) of cysteine, GSH and thionicotinamide in *n*-octanol/seawater, and found cysteine ($D = 0.43$) to be more lipid-soluble than GSH or thionicotinamide ($D < 0.05$), in agreement with Yoshimura et al. (1982) who found that cysteine was more able to penetrate biological membranes than GSH.

TABLE II

Reversal of copper toxicity to *N. closterium* by test compounds Cells were exposed to 70 μg Cu l^{-1} for 48 h before the addition of the test compound.

Copper ($\mu g \cdot l^{-1}$)	Test compound	Growth factor
0	–	1 7
70	–	1 2
70	5×10^{-5} M cysteine	2 4
70	10^{-5} M bathocuproine disulfonate	2 2
70	10^{-4} M EDTA	2.0
70	5×10^{-5} M GSH	1 7
70	5×10^{-5} M cystine	1.4
70	5×10^{-5} M GSSG	1.3
70	10^{-5} M glycine	1.0
70	5×10^{-5} M thionicotinamide	0.8

Growth factor is the ratio of the final cell density after 96 h to the cell density at 48 h when the test compound was added. GSH = reduced glutathione; GSSG = oxidized glutathione.

TABLE III

Reversal of copper toxicity to *C. pyrenoidosa* by thiols. Cells were exposed to copper for 24 h before the addition of the test compound.

Copper ($\mu g \cdot l^{-1}$)	Test compound	Growth factor
0	–	4.9
100	–	2 0
200	–	1.0
100	5×10^{-5} M GSH	4 0
100	5×10^{-5} M cysteine	3.6

Growth factor is the ratio of the final cell density after 48 h to the cell density at 24 h when the test compound was added.

Bathocuproine disulfonate (10^{-5} M), a powerful, hydrophilic copper (I) complexing agent (log β_1 = 19.1), was better able to reverse copper toxicity than 10^{-4} M EDTA, a strong water-soluble copper (II) complexing agent (log β_1 = 18.7) (Table II). There was a lag of 48 h before growth recommenced in the presence of EDTA, due perhaps to slow transport of copper from the cell. By strongly complexing copper (II), EDTA may shift the Cu(I)-Cu(II) equilibrium towards Cu(II), and reverse copper toxicity by preferentially complexing copper. Glycine, which has a higher binding constant for copper (II) (log β_1 = 8.6) than has bathocuproine disulfonate (log β_1 = 5.2), but does not complex copper (I) (Smith and Martell, 1975) was unable to reverse copper toxicity. This suggests that copper (II), adsorbed on *N. closterium*, is reduced to copper (I) by -SH groups, and bound as a Cu(I)-S-complex:

$$4\ GSH + 2\ Cu^{2+} \rightarrow 2\ Cu\text{-}SG + GSSG + 4H^{+}$$

Sulfhydryl compounds, such as cysteine and GSH, can replace the bound thiol, and reduce GSSG by thiol-disulfide exchange reactions. Powerful water-soluble copper-complexing agents, e.g., bathocuproine disulfonate, appear to be able to reverse toxicity by withdrawing copper from the cell, thus allowing growth to recommence.

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Paper 6

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Mechanism of toxicity of ionic copper and copper complexes to algae

J. L. Stauber and T. M. Florence

CSIRO, Division of Energy Chemistry, Lucas Heights Research Laboratories; Private Mail Bag 7, Sutherland, New South Wales 2232, Australia

Abstract

The mechanism of toxicity of ionic copper and copper complexes to growth, photosynthesis, respiration, ATP levels and mitochondrial electron-transport chain-activity in two marine diatoms, *Nitzschia closterium* (Ehrenberg) W Smith (Hasle, 1964) and *Asterionella glacialis* Castracane, and one freshwater green alga, *Chlorella pyrenoidosa* Chick was investigated. Copper ions depressed both cell division and photosynthesis in *A glacialis* and *C pyrenoidosa*, whereas ionic copper concentrations which were inhibitory to cell division in *N. closterium* had no effect on photosynthesis, respiration, ATP production, electron transport or membrane ultrastructure This suggests that in *N. closterium*, copper does not act on the chloroplast, the mitochondrion, or the cell membrane, since if it did, the above parameters should be affected. Copper-ethylxanthogenate was exceptional amongst the copper complexes in that it stimulated respiration, mitochondrial electron-transport and ATP formation in *N closterium* under conditions of strongly inhibited cell division and slightly stimulated photosynthesis Ionic copper toxicity may result from an intracellular reaction between copper and reduced glutathione (GSH), leading to a lowering of the GSH:GSSG ratio and suppression of mitosis In addition, copper inhibits the enzyme catalase and reduces cell defence mechanisms against H_2O_2 and oxygen-free radicals. Lipid-soluble copper complexes are more toxic than ionic copper because both the metal and the ligand are introduced into the cell Toxicity of ionic copper is ameliorated by trivalent metal ions in the growth medium, including those of Mn, Co, Al, Fe and Cr which form a layer of metal (III) hydroxide around the algal cell, adsorb copper and reduce its penetration into the cell. The degree of insolubility of the metal (III) hydroxide is related to its ability to protect against copper toxicity. In addition, manganese and cobalt catalytically scavenge damaging H_2O_2 and superoxide radicals, respectively, produced by the cell

Introduction

Until recently, most studies of copper toxicity to aquatic organisms have assumed that free hydrated Cu^{2+} is the most toxic physico-chemical form of copper (Magnuson *et al*, 1979). Water-soluble copper complexes including Cu-nitrilotriacetic acid were shown to be less toxic to the marine diatom *Nitzschia closterium* and the marine amphipod *Allorchestes compressa* than was ionic copper. whereas lipid-soluble organo-copper complexes, e.g. of the fungicide 8-hydroxyquinoline, were much more toxic (Florence *et al.*, 1983; Ahsanullah and Florence, 1984) Lipid-soluble complexes can diffuse directly through the membrane into the cell, where both copper and the ligand may exert separate toxicity Highly stable organic copper complexes may form a significant fraction of the total copper in natural waters (Florence, 1982).

The mechanism of toxicity of copper complexes to the marine diatom *Nitzschia closterium* has been reported previously (Florence *et al.*, 1984; Florence and Stauber. 1986) In particular, toxicity of the Cu(I) complex of 2,9-dimethyl-1,10-phenanthroline has been shown to be due to reaction with H_2O_2 within the cell and production of highly destructive oxygen-free radicals (Florence *et al*. 1985) Other phenanthroline complexes may also catalyse the production of excited oxygen species (Florence, 1984)

Evidence for the mechanism of toxicity of ionic copper has, however, been indirect. It was proposed that initial copper-binding to the cell may be to membrane protein carboxylic and amino residues, rather than to thiol groups because the Cu-algae stability constant is orders of magnitude lower than the thiol-Cu binding constant (Florence *et al*, 1983; Gavis, 1983) At the cell membrane, copper may interfere with cell permeability or the binding of essential metals (Sunda and Huntsman, 1983). Following copper transport into the cytosol, Cu may react with -SH enzyme groups and free thiols (e.g glutathione). disrupting enzyme-active sites and cell division (Stauber and Florence. 1985b, Florence and Stauber, 1986) Copper may also

exert its toxicity in subcellular organelles, interfering with mitochondrial electron transport, respiration (Cedeno-Maldonado and Swader, 1974), ATP production (Viarengo *et al*, 1981) and photosynthesis in the chloroplast (Overnell, 1975)

The toxicity of copper has been shown to be alleviated by other metals, including manganese and iron (Steemann Nielsen and Kamp-Nielsen, 1970; Stauber and Florence, 1985a, b) Using measurements of intracellular and extracellular manganese, iron, and copper, Stauber and Florence (1985a, b) showed that copper toxicity to *Nitzschia closterium* is reduced by adsorption of Cu on Mn (III) and Fe (III) hydroxide coatings around the cell. Manganese was more effective than iron, possibly due to its ability to scavenge the toxic superoxide free-radical (O_2^-) Manganese and iron, however, were unable to protect against the toxicity of lipid-soluble copper complexes

For the present paper, we studied the mechanisms of toxicity of ionic copper and copper complexes to growth, photosynthesis, respiration, ATP production and mitochondrial electron-transport chain (ETC)-activity in two marine diatoms and one freshwater green alga The ability of Co, Al, Cr, Ni and Zn to alleviate copper toxicity to *Nitzschia closterium* was also investigated.

Materials and methods

Algae cultures

The unicellular marine diatoms *Nitzschia closterium* (Ehrenberg) W Smith (Hasle, 1964) and *Asterionella glacialis* Castracane were cultured in Medium *f* (Guillard and Ryther, 1962) with the trace elements and iron concentration halved. The *N. closterium* culture was axenic, whereas *A glacialis* was not. Experiments to determine the protective effect of trivalent metal ions on copper toxicity used *N closterium* cultured in Medium *f* at 1/100 the normal iron concentration (i.e, 7.9 μg Fe l^{-1} added as ferric citrate) and without trace metals *Chlorella pyrenoidosa* Chick was maintained in MBL medium (Stein, 1973) at normal and 1/100 the normal Fe (i.e, 6.5 μg Fe l^{-1}) and trace metal concentrations All cultures were maintained on a 12 h light: 12 h dark cycle (Philips TL 40 W fluorescent white, 6 400 lux) at 21 °C Cells in log-phase growth were used for all experiments.

Measurement of cell-division rate

Toxicity of Cu^{2+} and Cu complexes to *Nitzschia closterium* and *Asterionella glacialis* was studied using algal assays in filtered (0 45 μm), unsupplemented seawater as described previously (Lumsden and Florence, 1983) *Chlorella pyrenoidosa* assays used 50 ml standard softwater ($NaHCO_3$, 48 mg l^{-1}, $CaSO_4$ $2H_2O$, 30 mg l^{-1}, $MgSO_4$, 30 mg l^{-1}, KCl, 2 mg l^{-1}) with the addition of 1 ml of 0.5 *M* Hepes buffer (pH 7 0), 0 5 ml $NaNO_3$ (2 1 g l^{-1}), and 0 5 ml of K_2HPO_4 (0.22 g l^{-1}) Cell density was measured initially and on three subsequent days by counting in a haemocytometer A regression line was fitted to a plot of log (cell density/initial cell density) vs time, and cell division rate (μ) was determined from the slope or expressed as cell divisions day $^{-1}$ (3 32 μ)

Measurement of photosynthesis

Inhibition of photosynthesis after 24 and 48 h exposure to copper complexes was determined by uptake of carbon-14 carbonate, followed by removal of excess $H^{14}CO_3$ in acid solution, and liquid scintillation counting in Instagel (Lumsden and Florence, 1983) Results, expressed as μmol CO_2 fixed h^{-1} 10^{-6} cells, were corrected for dark fixation

Measurement of respiration

Algal dark respiration rates (μmol O_2 h^{-1} 10^{-6} cells) were determined in a sealed biological oxygen-demand bottle using an Orion Research Oxygen Electrode. Respiration was measured initially for 30 min and at 2, 4, 6, 24 and 48 h after exposure to copper and copper complexes

Determination of ATP

ATP was determined using a modified method of Holm-Hansen and Booth (1966). After a 24 h treatment with copper, ATP was extracted from the cells in boiling Tris buffer (0.025 *M*, pH 7 75). An aliquot of cell extract was added to 4 ml of Sigma firefly extract (reconstituted in buffer) to a final volume of 10 ml in Tris buffer, and light output was measured on a Packard liquid scintillation counter. ATP in the cells, with correction for a firefly extract blank, was determined from an ATP standard curve (area under the light decay curve vs ATP concentration).

Determination of electron transport chain (ETC) activity

Mitochondrial ETC activity in *Nitzschia closterium* and *Asterionella glacialis* was determined using an artificial electron acceptor, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), according to Kenner and Ahmed (1975) Cells exposed to copper complexes for 24 h were centrifuged and homogenised for 2 min in 6 ml cold buffer (0.05 *M* phosphate pH 8 0, 75 μM $MgSO_4$, 0 2% Triton X-100 and 0.15% polyvinylpyrrolidone) Extracts were centrifuged and assayed at 25 °C using 1 ml extract, 3 ml substrate buffer (0 05 *M* phosphate pH 8.0, 0 2% Triton X-100, 0.133 *M* disodium succinate, 8.35×10^{-4} *M* NADH and 2.4×10^{-4} *M* NADPH) and 1 ml of 2.5×10^{-3} *M* INT. After 15 min, the reaction was stopped with 1 ml of a 1·1 mixture of 1 *M* sodium formate (pH 3 5) and

formalin The absorption of INT-formazan was measured at 490 nm and corrected for a boiled homogenate blank which was assayed in the same way ETC activity was expressed as nmol formazan 10^{-6} cells

Preparation of cells for electron microscopy

Nitzschia closterium cells, exposed to copper and copper complexes for 48 and 72 h, were concentrated by gentle centrifugation ($1\,000\times g$ for 1 min) fixed in 3% glutaraldehyde in 0 1 *M* phosphate buffer (pH 7.2) and 0 6 *M* sucrose for 1 h at room temperature, washed in buffer and post-fixed in 1% OsO_4-0 1 *M* phosphate buffer for 1 h. Samples were dehydrated through a graded acetone series and embedded in Spurr's low-viscosity resin Thin sections were cut using a diamond knife on a Reichert-Jung Ultratome 3, sequentially stained in uranyl acetate and lead citrate, and examined in a Philips 400 electron microscope

To detect where copper was localized in the cell, sections were cut to give a purple interference colour and were collected on 300 mesh aluminium grids. The unstained sections were mounted in a Gatan 636 beryllium holder and examined in a Philips EM 430 operated at 250 kV. X-ray spectra were collected with an Edax detector The cytoplasm and chloroplasts from 12 treated and untreated cells were examined, together with the mitochondria from treated cells.

Trivalent metals and copper toxicity

The ability of metal ions to protect against copper toxicity to *Nitzschia closterium* was investigated using the method of Stauber and Florence (1985a). Cells were grown in 1/100 Fe culture medium with only the addition of the test trace metal being varied, before being washed and assayed in unsupplemented seawater for copper toxicity.

Results

Growth

Ionic copper concentrations greater than 7.9×10^{-7} *M* were required to give a 50% reduction in cell division rate in *Chlorella pyrenoidosa* (Table 1), similar to copper toxicity reported for *Nitzschia closterium* cultured prior to the assay in medium containing 790 μg Fe l^{-1} (Stauber and Florence, 1985a). Unlike *N. closterium*, increased concentrations of iron and trace metals in the growth medium gave no protection against copper toxicity to *C. pyrenoidosa* It is possible that Hepes buffer, added in the freshwater assay, complexes iron However, in the presence of Hepes, a ferric hydroxide precipitate was observed even when the pH was raised, whereas if significant Hepes complexing occurred, precipitation would be prevented Zinc ($1\,5\times10^{-7}$ *M*), Cd ($0\,9\times10^{-7}$ *M*) and Ni ($1\,7\times10^{-7}$ *M*) did not inhibit cell division in *C. pyrenoidosa*, Pb was toxic $0\,5\times10^{-7}$ *M* Pb giving a 50% reduction in cell division rate Increasing the pH from 7.0 to 8 0 did not decrease Pb toxicity. Lead at the same concentration had no effect on the growth of *N. closterium* in seawater pH 8 2

The most toxic copper complexes tested were those of the phenanthrolines, 2,9-dimethyl-1,10-phenanthroline (2,9-dmp), 5,6-dmp and 4 7-dmp, which completely inhibited cell division in *Chlorella pyrenoidosa* at concentrations as low as $0\,5\times10^{-7}$ *M* (Table 1). The Cu-2.9-dmp complex which is accumulated by, and highly toxic to, *Nitzschia closterium* (Florence *et al*, 1985), is reduced to the very lipid-soluble and highly toxic Cu(I)-2.9-dmp by H_2O_2 produced in the algal cell during photorespiration and photosynthesis The intracellular reaction between excess H_2O_2 and Cu(I)-2,9-dmp produces singlet oxygen, oxygen-free radicals and bipyridyl compounds, which cause the extreme toxicity of the complex (Florence *et al*, 1985).

Bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), which forms another stable, lipid-soluble Cu(I) complex, completely depressed cell division in *Chlorella pyrenoidosa* PAN [4-(2-pyridylazo)naphthol] and TAN [1-(2-thiazolylazo)-2-naphthol], which have been shown to strongly chelate copper in seawater to yield

Table 1. *Chlorella pyrenoidosa* Toxicity of copper complexes and other compounds Cells were grown in Fe/100 and trace metals/100 dmp dimethyl-1,10-phenanthroline, PAN 4-(2-pyridyl-azo)naphthol TAN 1-(2-thiazolylazo)-2-naphthol – no addition

Toxicant	Toxicant conc (10^{-7} *M*)	Cu conc (10^{-7} *M*)	Cell division rate as percentage blank[a]
–		7 9	59
2.9-dmp	0 5	0 31	0
5,6-dmp	2 5	0 79	0
4,7-dmp	2 5	0 79	0
1,10-phenanthroline	2 5	0.79	34
bathocuproine	2 5	0 79	0
2,2'-bipyridyl	2 5	0 79	62
2,2'-biquinoline	2 5	0 79	13
PAN	0 5	0 31	46
TAN	0 5	0 31	69
3-amino-1,2,4-triazole	200	–	82
p-aminophenol	10	–	93
m-aminophenol	10	–	105
ascorbic acid	1 000	–	69
resorcinol	100	–	103
catechol	100	–	95
H_2O_2	500	–	66
lead	0 5	–	51
zinc	1 5	–	107
cadmium	0 9	–	102
nickel	1 7	–	95
urease	20 mg l^{-1}	–	0
trypsin	20 mg l^{-1}	–	0
hexokinase	20 mg l^{-1}	–	111
pepsin	20 mg l^{-1}	–	100

[a] Blank cell division rate (μ) was 0 139±0 017, values are means of duplicates

Table 2. *Nitzschia closterium* and *Chlorella pyrenoidosa* Effect of copper, copper complexes and some other compounds on photosynthesis Cells were exposed to copper complexes for 24 h and to ionic copper for 48 h. Each experiment was made in duplicate, values are means and standard deviations of replicate (5–20) experiments using all individual results for the calculations Oxine 8-hydroxyquinoline, dmp dimethyl-1,10-phenanthroline, EXA ethylxanthogenate, PAN 4-(2-pyridylazo)naphthol, NTA nitrilotriacetic acid, APH acetylphenylhydrazine

Toxicant	Toxicant conc (10^{-7} *M*)	Cu conc (10^{-7} *M*)	Photosynthesis (μmol CO_2 fixed/ h/10^6 cells)	Cell-division rate (divisions/d)
Nitzschia closterium				
–	–	–	0 30±0 03	0.54±0 03
–	–	27 8	0 35	0.31
oxine	1 00	–	0 19	< 0 05
oxine	1 00	0 79	0 17	< 0 05
2,9-dmp	1.00	0.79	0 31	< 0 05
EXA	5.00	1.58	0 37	0.05
PAN	1 00	–	0 29	< 0 05
PAN	1 00	0 79	0 28	< 0 05
NTA	400	3 15	0 28	0 45
H_2O_2	400	–	0 34	< 0 05
APH	20	–	0 30	< 0 05
Chlorella pyrenoidosa				
–	–	–	0.16	0.37
–	–	10	0.01	< 0.05

lipid-soluble complexes highly toxic to *Nitzschia closterium*, were less toxic to *C. pyrenoidosa*. Hydrogen peroxide was less toxic to *C. pyrenoidosa* than to *Nitzschia closterium* (Tables 1 and 2).

Of the enzymes tested, urease and trypsin completely depressed cell division in *Chlorella pyrenoidosa* (Table 1) Urease has also been shown to be very toxic to *Nitzschia closterium* (Florence and Stauber, 1986).

Photosynthesis

It was observed previously (Lumsden and Florence, 1983) that, although the growth rate of *Nitzschia closterium* was halved by 20 μg Cu l^{-1}, photosynthesis was not affected until copper concentrations were above 100 μg Cu l^{-1}. It should be noted that our photosynthesis experiments used higher cell densities (2×10^5 cells ml^{-1}) than those used in cell division-rate experiments (2 to 4×10^4 cells ml^{-1}). Since copper inhibition of growth is dependent on cell density (Florence and Stauber, 1986), much higher copper concentrations were required to inhibit cell division in the photosynthesis experiments

With the exception of oxine and Cu-oxine, none of the other free ligands or their copper complexes, whether water-soluble or lipid-soluble, had any significant effect on photosynthesis even though they depressed algal cell division (Table 2) Hydrogen peroxide at concentrations inhibitory to cell division, had no effect on photosynthesis Acetylphenylhydrazine (20×10^{-7} *M*), which reacts with cellular glutathione and was extremely toxic to cell division in *Nitzschia closterium*, did not affect photosynthesis at this concentration Ionic copper at concentrations inhibitory to cell division (27.8×10^{-7} *M*) had no effect on photosynthesis in *N. closterium*. Inhibition of photosynthesis by ionic copper required concentrations exceeding 3.2×10^{-6} *M* applied for more than 6 h. After 24 and 48 h, when cells were no longer dividing, photosynthesis was depressed by 40%, perhaps due to negative feedback inhibition from accumulation of photosynthetic products, as suggested for *Chlorella pyrenoidosa* by Steemann Nielsen *et al.* (1969). This decoupling of growth and photosynthesis in *N closterium* was not observed in the two other algal species tested. For both *C. pyrenoidosa* and *Asterionella glacialis* at the same cell densities, growth and photosynthesis were strongly depressed by 10×10^{-7} *M* Cu (Table 2) and 1.6×10^{-6} *M* Cu (Table 3), respectively

Respiration, ATP and mitochondrial electron transport

Of the copper complexes tested (Table 4), only Cu-ethylxanthogenate had a significant effect, enhancing respiration in *Nitzschia closterium* (143% of the blank), ETC activity (182% of the blank) and ATP production (128% of the blank) after 24 h Copper-ethylxanthogenate may alter the activity of the dehydrogenases which funnel electrons into the ETC or alter components of the ETC itself ATP formation is coupled to the ETC, so any compound which enhances ETC activity, would also be expected to increase respiration and ATP formation. Hydrogen peroxide and catalase also significantly enhanced ETC activity in *N closterium*.

Ionic copper at concentrations inhibitory to cell division had no significant effect on respiration, ATP production or ETC activity in *Nitzschia closterium* Similarly, cop-

Table 3. *Asterionella glacialis* Effect of ionic copper on growth, photosynthesis, respiration and electron-transport chain (ETC) activity after 24 h exposure Values ± 1 SD

Compound	Cell division rate (divisions/d)	Photosynthesis (μmol CO_2 fixed/ h/10^6 cells)	Respiration (μmol O_2/h/ 10^6 cells)	ETC activity (nmol formazan/ 10^6 cells)
Blank	0.36	0 72 ±0 24	0 012±0.002	86.2± 12 9
Cu (1.6×10^{-6} *M*)	< 0 05	0.030±0 005	0 016±0.004	85 6± 11 9

Table 4. *Nitzschia closterium* Effect of copper and copper complexes on respiration, mitochondrial electron-transport chain-activity and ATP after 24 h exposure Values ± 1 SD EXA ethylxanthogenate, dmp dimethyl-1,10-phenanthroline, p phenanthroline, PAN· 4-(2-pyridylazo)naphthol

Treatment	Respiration (μmol O_2/h/10^6 cells)	ETC activity (nmol formazan/ 10^6 cells)	ATP (μg/10^6 cells)
Blank	0 0069±0 0006	38 2± 4 1	0.025±0.002
$3\ 15\times10^{-6}$ *M* Cu	0.0075±0.0006	45 7± 5.5	0.027±0.002
5×10^{-7} *M* EXA+$1\ 57\times10^{-7}$ *M* Cu	0 009±0 0009	69.5± 8.3	0 032±0.003
10^{-7} *M* 2,9-dmp+$0\ 79\times10^{-7}$ *M* Cu	ND	42 3± 5.1	ND
10^{-7} *M* oxine+$0\ 79\times10^{-7}$ *M* Cu	ND	49.1± 5.3	ND
10^{-6} *M* 1,10p+$1\ 57\times10^{-7}$ *M* Cu	ND	37.9± 5.4	ND
10^{-8} *M* PAN+$3\ 1\times10^{-8}$ *M* Cu	ND	32 4± 4 2	ND
6×10^{-5} *M* H_2O_2	ND	52.9±11.6	ND
Catalase 40 mg l^{-1}	ND	68 1±15 4	ND

per (1.6×10^{-6} *M*) had no significant effect on respiration and ETC activity in *Asterionella glacialis* (Table 3) It appears that in *N. closterium* and *A. glacialis*, the site of copper action within the cell is not the mitochondrion

Ultrastructure

No effect of ionic copper or copper complexes on the ultrastructure of the chloroplast, nucleus, mitochrondrion or cell membrane was observed (Fig. 1). Cells treated with Cu and Cu-2,9-dmp (Fig. 1 b, c) were swollen due to enlarged vacuoles Copper-oxine treated cells were the same size as untreated cells, as copper-oxine inhibited both cell division and photosynthesis (Fig. 1 a, d)

In untreated cells, no copper was detected by X-ray spectroscopy in chloroplasts or cytoplasm. Copper-treated cells showed copper only in the cytoplasm, not in chloroplasts or mitochondria

Role of trivalent metals in reducing copper toxicity

Protection against copper toxicity by Mn (III), Co (III), Al (III), Fe (III), Cr (III), Ni (II), and Zn (II), is shown in Table 5. Manganese, Co, Al and Fe were most effective in reducing Cu toxicity, 50 μg Cu l^{-1} being required to give a 50% reduction in cell division rate. compared with less than 10 μg Cu l^{-1} in the absence of trace metals Nickel and zinc were unable to protect against copper toxicity to *Nitzschia closterium* at the concentrations used

Manganese has been shown to be much more effective than iron in reducing copper toxicity due, in part. to its

Table 5. *Nitzschia closterium*. Role of metal ions in protection against copper toxicity Cells were grown at metal concentrations shown, prior to seawater assay for copper toxicity

Metal ion	Metal ion conc in growth medium (*M*)	Cu conc producing 50% reduction in cell-division rate (μg l^{-1})
None	–	< 10
Manganese(III)	8×10^{-8}	65
Cobalt(III)	4×10^{-8}	50
Aluminium(III)	4×10^{-6}	50
Iron(III)	1×10^{-5}	50
Chromium(III)	4×10^{-6}	20
Nickel(II)	4×10^{-8}	< 10
Zinc(II)	3×10^{-7}	< 10

ability to catalytically scavenge damaging superoxide radical (Stauber and Florence, 1985 b). Cobalt, which is even more effective than manganese in reducing copper toxicity, is unable to scavenge O_2^- but may decompose damaging H_2O_2. We followed Co^{2+} oxidation by H_2O_2 in seawater using an Orion Research oxygen electrode Excess H_2O_2 was determined by measuring oxygen evolved following addition of catalase With excess Co^{2+}, the reaction proceeded stoichiometrically, and a yellow/brown precipitate of Co (III) hydroxide formed (Reaction 1) This $Co(OH)_3$ precipitate catalytically decomposes H_2O_2 (Reactions 2–4):

$$Co(OH)_2 + \tfrac{1}{2}H_2O_2 \rightarrow Co(OH)_3 \quad (1)$$

$$Co(OH)_3 + H_2O_2 \rightarrow Co(OH)_2 + O_2^- + H^+ + H_2O \quad (2)$$

$$Co(OH)_2 + O_2^- + H^+ \rightarrow Co(OH)_3 + \tfrac{1}{2}O_2 \quad (3)$$

$$\text{net, (3)}-\text{(4)} \quad H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2 \quad (4)$$

Fig. 1. *Nitzschia closterium* cells treated with copper and copper complexes for 72 and 48 h, respectively (6 000×) (a) Untreated cells, (b) 200 μg Cu l^{-1}, (c) 5 μg Cu l^{-1} + 10^{-7} *M* 2,9-dimethyl-1,10-phenanthroline; (d) 5 μg Cu l^{-1} + 10^{-7} *M* oxine

Discussion

Although very low concentrations of copper inhibited cell division in *Nitzschia closterium*, photosynthesis was unaffected at copper concentrations up to 2.4×10^{-6} *M* Photosynthetic products accumulated, leading to enlarged cells which were unable to divide (Fig 1). In *Asterionella glacialis* and *Chlorella pyrenoidosa*, both growth and photosynthesis were strongly depressed by copper

Copper inhibition of photosynthesis has been observed previously in other diatoms (Erickson, 1972; Overnell, 1975) Inhibition of cell division without an effect on photosynthesis has been less frequently observed. Fisher and Jones (1981) showed that, in *Asterionella japonica*, copper-treated cells photosynthesised at normal rates, with cells continuing to enlarge when fixed carbon could not be excreted or utilised in cell division Davies (1976) also found that growth was uncoupled from cell division in mercury-treated *Dunaliella tertiolecta* and *Isochrysis galbana*. It appears that metals such as copper and mercury may, in some cases, inhibit the process of cell division independently of any effect on the production of new cell material.

Reported effects of copper on algal respiration vary from inhibition in *Chlorella sorokiniana* (Cedeno-Maldonado and Swader, 1974) and *Amphidinium carterae* (Samuel, 1976), to enhancement in *Nitzschia closterium* (Samuel, 1976) and *Chlorella vulgaris* (McBrien and Hassall, 1967), depending on time of exposure to copper. We found that ionic copper had no effect on dark respiration, ATP production or ETC activity in *Nitzschia closterium* and *Asterionella glacialis*. It appears that the site of copper action, at least in *N closterium*, is neither the chloroplast nor the mitochondrion, since photosynthesis is inhibited only at high copper concentrations after an effect on cell division. X-ray spectra of copper-treated cells confirmed that copper was in the cytoplasm, and not localized in chloroplasts or mitochondria.

Using a lipid-soluble thiol reagent, Stauber and Florence (1986) showed that treatment of *Nitzschia closterium* with copper ions significantly reduced the concentration of intracellular thiol groups from 7×10^{-3} to 3×10^{-3} *M* SH, whereas the copper complexes tested had no effect. It was proposed that Cu(II) adsorbed to the algae is reduced to Cu(I) by -SH groups and bound as a Cu(I)-S-complex A large proportion of intracellular -SH groups would be present as free glutathione (GSH), the concentration of which has been shown to be in the range 0 5 to 10×10^{-3} *M* in many types of cells (Kosower and Kosower, 1978) Copper toxicity may principally be due to copper binding to intracellular, rather than membrane, thiols, since protein thiol-Cu binding on the membrane would be expected to completely disrupt major cell functions (J.G Robertson, personal communication, 1985). In addition, sulphydryl compounds such as cysteine, which penetrate the cell, can reverse copper toxicity through thiol-disulphide interchange reactions, allowing algal growth to recommence (Stauber and Florence, 1986).

The primary toxic effect of copper may result from a cytosolic reaction between Cu and GSH, leading to a lowering of the reduced glutathione oxidised glutathione ratio and suppression of mitosis i e ,

$$4\,GSH + 2\,Cu^{2+} \rightarrow 2\,Cu^{1}\text{-}SG + GSSG + 4\,H^{+} \quad (5)$$

The ratio of GSH GSSG in sea urchin eggs was found to fluctuate regularly with the mitotic cycle, a high GSH GSSG ratio being associated with cell division (Kosower and Kosower, 1978) An increase in GSH immediately prior to nuclear division has also been reported for *Chlorella pyrenoidosa* (Hare and Schmidt, 1969) According to Reaction (5), a Cu GSH ratio of 0.5 is enough to oxidize all the GSH. Copper-treated *Nitzschia closterium* cells contain about 2×10^{-2} *M* Cu (Stauber and Florence 1985a), giving a Cu.SH ratio in the cell of 2.9 Therefore, the concentration of copper in *N closterium* cells is more than adequate to oxidise all the GSH to GSSG, and inhibit cell division Moreover, ionic copper was shown (Florence and Stauber, 1986) to inhibit glutathione reductase This enzyme, if present in algae, is responsible for the reduction of GSSG to GSH by NADPH Florence and Stauber showed that ionic copper, Cu-2,9-dmp and Cu-oxine reduce the activity of glutathione reductase in phosphate buffer (pH 7 0) by 27, 24 and 69% respectively. Thus, copper oxidation of GSH to GSSG in the cell may be irreversible, without the addition of sulphydryl compounds such as cysteine In further support for this mechanism of copper toxicity, we have found that compounds which oxidise glutathione, including acetyl-phenylhydrazine, 1-chloro-2,4-dinitrobenzene and H_2O_2, inhibit cell division, but not photosynthesis, in *N. closterium*.

Other mechanisms have been proposed to explain the effect of Cu-thiol binding on cell division in algae. Davies (1976) suggested that copper and mercury might prevent the production of methionine which appears necessary for cell division It has also been proposed that metals inhibit phytoplankton cell division by binding reactive thiols on the tubulin molecule, which is important in spindle formation during mitosis (Silio and Angulo, 1967, Onfelt, 1983).

Copper toxicity may also be partly due to its ability to reduce cell defence mechanisms against H_2O_2, which has been shown to be very toxic to algal growth (Florence and Stauber, 1986) Copper inhibits the H_2O_2-dissociating enzyme catalase, possibly by displacing iron from the enzyme active centre (Florence and Stauber, 1986) Copper also has superoxide dismutase activity, leading to the formation of more H_2O_2 from the superoxide radical ($O_2^{\cdot}$). Copper can also catalyse the decomposition of H_2O_2 to produce hydroxyl radical ($OH\cdot$), which may accelerate oxidative deterioration of membrane lipids (Florence, 1984)

Stauber and Florence (1985a, b) showed that the toxicity of ionic copper to *Nitzschia closterium* was reduced in the presence of Mn(III) and Fe(III) hydroxides, which coated the algal cells, effectively adsorbed copper, and reduced its penetration into the cell In the present paper,

Table 6 Calculated 1 1 conditional stability constants (β'_1) for metal(III)-hydroxide complexes, with correction for Cl^- complexing in seawater

Metal ion	log β'_1
Mn^{3+}	13 70
Co^{3+}	12 37
Fe^{3+}	10 52
Cr^{3+}	9.36
Al^{3+}	8 26

we have shown that other metals capable of forming insoluble metal(III) hydroxides in seawater, e g Co, Cr and Al, were also effective in reducing copper toxicity The degree of insolubility of the metal(III) hydroxide may indicate its ability to protect against copper toxicity Calculated conditional stability constants for the 1:1 metal(III) hydroxide complexes, with correction for Cl^- complexing in seawater (Ringbom, 1963), are shown in Table 6. It appears that the more stable the metal(III) hydroxide, the better the metal is able to reduce copper toxicity Manganese and cobalt are most effective, followed by Fe, Cr and Al. The protective role of aluminium, a non-essential element to the cell, provides further evidence that reduction in copper toxicity is dependent on the ability of the metal to form an insoluble metal(III) hydroxide; otherwise it could be argued that essential elements such as cobalt and iron are increasing algal growth via a nutrient effect Nickel, which has a similar chemistry to cobalt, cannot be oxidized in seawater to Ni(III) hydroxide and is totally ineffective in protecting against copper toxicity. Similarly, Zn(II) is ineffective. The protective effect of manganese and cobalt is enhanced by their ability to scavenge damaging O_2^- and H_2O_2, respectively

Conclusions

The effect of copper ions on cell division, photosynthesis, respiration, ATP production, electron transport and cellular ultrastructure of *Nitzschia closterium* suggests that the main toxic effect of copper is to act within the cytosol, possibly by lowering the intracellular thiol concentration, thus inhibiting cell division but not affecting the other cellular functions. Contributory toxicity may result from the ability of copper to inhibit the enzymes catalase and glutathione reductase A different toxic mechanism is apparently operative in *Asterionella glacialis* and *Chlorella pyrenoidosa,* since for these algae, and unlike *N closterium,* both cell division *and* photosynthesis are affected by copper For all three algal species, lipid-soluble organo-copper complexes were much more toxic than ionic copper.

The addition to the algal growth medium of trivalent metal ions such as Al, Fe, and Cr, or divalent metals (Mn and Co) that can be oxidised by algae to trivalent species, afforded considerable protection against copper toxicity by forming a layer of hydrated metal(III) oxide around the cell, which adsorbs copper ions before they can penetrate the cell A coating of hydrated metal oxide on the cells is unlikely to form in synthetic growth medium containing strong chelators such as EDTA or NTA, because these ligands would complex trivalent metal ions This probably explains why Sunda and Huntsman (1983) found a different protective mechanism (competitive binding) to be operative in a chelating medium.

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Paper 7

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THE DETERMINATION OF TRACE METALS IN SWEAT BY ANODIC STRIPPING VOLTAMMETRY*

J.L. STAUBER and T.M. FLORENCE

CSIRO, Division of Energy Chemistry, Private Mail Bag 7, Sutherland, NSW 2232 (Australia)

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ABSTRACT

Sweating was induced on the forearm of subjects by pilocarpine iontophoresis, and the sweat collected on a small membrane filter. Zinc, cadmium, lead and copper were then determined in the filter by anodic stripping voltammetry (ASV) using one of two alternative techniques: (a) leaching the filter with a supporting electrolyte (0.1 M $NaNO_3$/0.002 M HNO_3), followed by ASV of the leachate; or, (b) direct ASV analysis of the trace metals in the membrane filter using a novel thin-layer cell. Average values found for zinc, cadmium, lead and copper in sweat from males were ($\mu g\,l^{-1}$): 720, < 3, 15 and 80, respectively. Copper in sweat from females was lower (23 $\mu g\,l^{-1}$) than for males, although use of oral contraceptives appeared to increase copper concentration.

INTRODUCTION

With the increase throughout the world of blood-related communicable diseases, non-invasive techniques for biological monitoring are receiving more attention. Non-invasive samples include urine, feces, hair, nails, deciduous teeth, saliva, tears, and sweat.

Analysis of chloride in sweat is used extensively for the detection of cystic fibrosis in newborn babies [1, 2], but there has been little interest in the determination of trace metals in sweat, mainly because of the difficulties in collecting an uncontaminated sample of adequate size. Methods used previously for sweat collection include: (a) placing the subject in a sauna bath [3]; (b) enclosing the subject from feet to neck in a plastic bag in a heated room [4]; (c) after heavy exercise [5]; (d) enclosing forearm and fingers in a plastic glove and applying heat [6, 7]; and (e) placing a hot metal billet or gel-pack on the forearm [2]. These techniques are not only traumatic (and sometimes dangerous [1]!) to the subject, but also involve great risk of contamination of the sweat sample by metals.

The technique of pilocarpine iontophoresis for sweat collection [1, 2] involves passing a small constant current (1–5 mA) across a pair of electrodes in contact with the skin. The positive electrode is covered with a pad soaked in a solution of the alkaloid pilocarpine, while the negative electrode is wet with sodium bicarbonate solution. Pilocarpine, under the influence of the electric

*Dedicated to the memory of Professor Dr Hans Wolfgang Nürnberg.

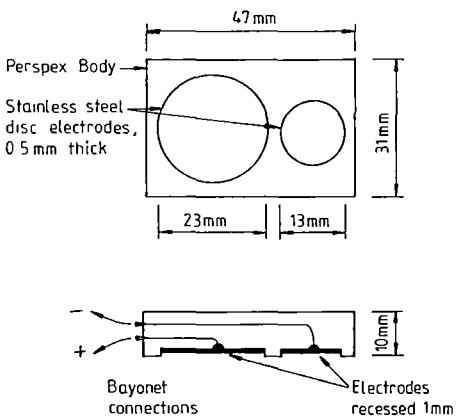

Fig 1 Electrode for pilocarpine iontophoresis

current, is forced into the sweat glands and, after removal of the electrodes, the area under the positive electrode sweats profusely for 30–60 min. In this study we placed a membrane filter over the treated area, and covered it with plastic film. After 30–45 min, the filter was removed and analyzed for zinc, cadmium, lead and copper by anodic stripping voltammetry (ASV), either directly in the filter using a novel type of electrochemical cell, or after leaching the filter with an electrolyte. Choice of the correct type of membrane filter is critical to the formation of well-shaped ASV waves and to the avoidance of contamination.

EXPERIMENTAL

Apparatus

The iontophoresis electrode is shown in Fig. 1. The positive and negative stainless steel electrodes were covered with double thicknesses of Millipore type AP20 pre-filters, 25 and 13 mm diameter, respectively. The positive electrode was wet with 0.35 ml of 0.2% aqueous pilocarpine nitrate (Sigma), while the negative electrode was wet with 0.15 ml of 0.25 *M* $NaHCO_3$. The current source consisted of 4 × 9 V dry cells and a potential dividing circuit with a 0–5 mA milliammeter [1, 2].

The ASV electrochemical thin-layer cell for direct trace metal analysis in the filter paper [8, 9] is shown in Fig. 2. This cell was interfaced [10] to an EG and G Princeton Applied Research Model 384 Polarographic Analyzer. Anodic stripping voltammetry analysis of the membrane filter leachates at a hanging mercury drop electrode (HMDE) was made using the Model 384 with the Model 303 Static Mercury Drop Assembly.

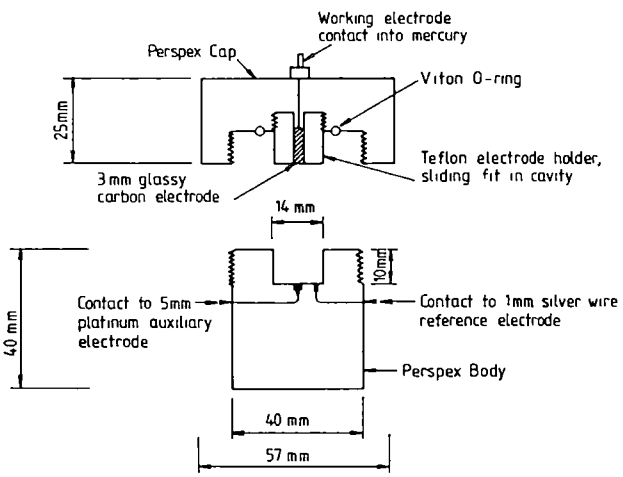

Fig. 2. Electrochemical thin-layer cell for anodic stripping voltammetry in membrane filter discs.

General Procedure

The inner forearm of the subject was washed with 1% Extran-300 (BDH) detergent, rinsed with high purity water (Millipore Milli-Q), then dried with Whatman 542 filter paper. The iontophoresis electrode complete with pilocarpine- and $NaHCO_3$-soaked pre-filters, was pressed firmly on the washed area. The voltage was slowly increased until a current of 1.0–1.2 mA was passing between the electrodes, which was then maintained for 2 min. The current produces a slight tingling or burning sensation in the skin. The applied voltage needs to be periodically reduced during this period to limit the current to 1.0–1.2 mA. The resistance of skin varies considerably, so the necessary voltage varies with the individual. The electrode was then removed, the area under the electrode washed thoroughly with water, then dried completely by blotting with Whatman 542 papers. If ASV at a hanging mercury drop electrode (HMDE) was to be used for analysis, a pre-weighed acid-washed Millipore 25-mm HATF (0.45 μm pore size) membrane filter was placed on the circle of skin which was under the pilocarpine electrode, the paper covered with a square of acid-washed Parafilm (American Can Co.) and the whole area wrapped tightly with clear plastic household lunch-wrap. After 30–45 min the Parafilm was removed and the HATF filter, wet with sweat, was weighed in a small acid-washed plastic vial to determine the weight of sweat collected (typically 0.05–0.07 g). Supporting electrolyte (5 ml of 0.1 *M* $NaNO_3$/0.002 *M* HNO_3), prepared from Merck Suprapur reagents, was then added to the vial which was then shaken to extract the sweat. The electrolyte was decanted into the cell of the polarographic analyzer and ASV analysis carried out. Blanks were determined by treating a blank membrane filter in the same manner, and standardization was achieved by standard addition.

When the thin-layer cell (Fig. 2) is used for ASV analysis, the sweat collection procedure is the same, except that two 13-mm HATF membrane filters were placed under the Parafilm. The two filter discs were then dropped into the cavity of the cell bottom, and the cell screwed together to compress the papers between the electrodes. The glassy carbon electrode was covered with an in situ-formed mercury film. Anodic stripping voltammetry analysis with 1 min deposition at -1.4 V versus Ag/AgCl was then performed to yield labile metal. To determine total metal, one of the discs was replaced by another HATF filter to which 0.05 ml of 0.1 *M* $NaNO_3$/0.002 *M* HNO_3 had been added. Compression mixes the solution in the two discs. Standardization was carried out in a similar manner by using a disc treated with a standard solution.

Contamination of the sweat sample during collection was minimised by careful preliminary washing of the forearm with a metal-complexing detergent (Extran-300) and the use of acid-washed Parafilm to cover the membrane disk during sweat collection. To provide some evidence that contamination was not occurring from exogenous sources or from flakes of skin, an experiment was carried out with one filter, then replaced with a fresh disc and collection carried out in the same spot for another 30 min. If significant contamination was occurring from the skin or the covering, the first membrane disc may have been expected to have a higher trace metal content. However, no significant differences were found.

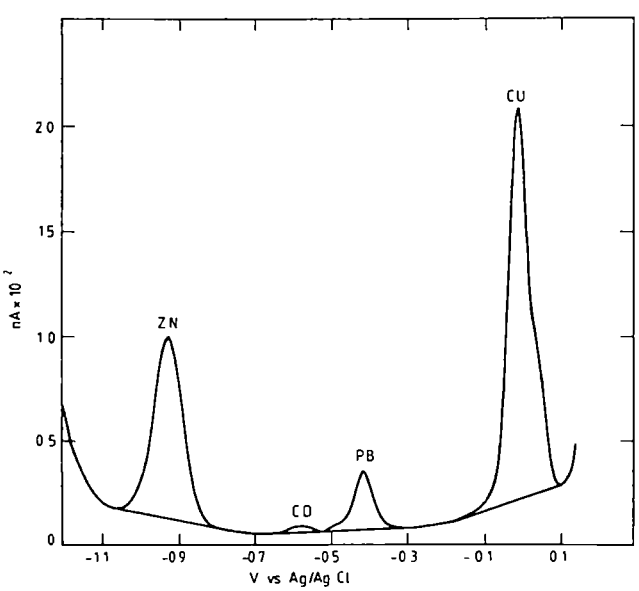

Fig. 3. Anodic stripping voltammogram of sweat using the 0.1 *M* $NaNO_3$/0.002 *M* HNO_3 leaching procedure.

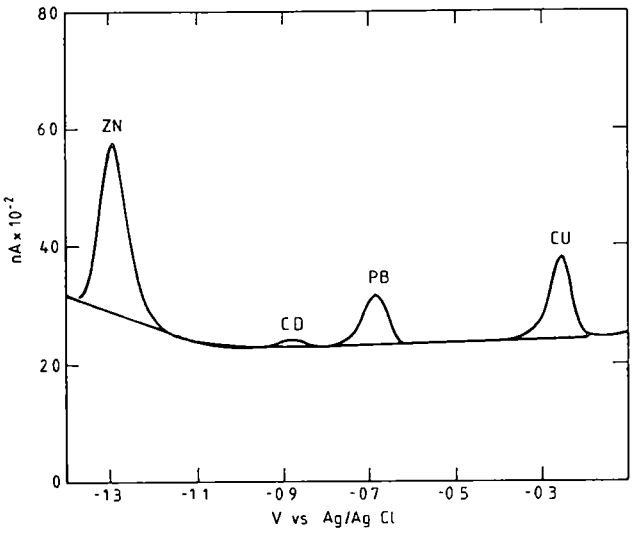

Fig. 4. Direct anodic stripping voltammetry of trace metals in sweat using the thin-layer membrane filter electrode.

RESULTS

Typical ASV voltammograms obtained for sweat using the leaching procedure and the thin layer cell are shown in Figs 3 and 4, respectively. Results for zinc, cadmium, lead and copper in sweat using the leaching method are given in Table 1. Ten measurements on one male, over a period of 5 months gave values of 650 ± 250, < 3, 21 ± 9, and 70 ± 22 $\mu g\,l^{-1}$ for zinc, cadmium, lead and copper, respectively. The depression of zinc, cadmium, lead and copper spikes by sweat (0.05 g) in the 0.1 *M* $NaNO_3$/0.002 *M* HNO_3 electrolyte were (%): 88; 30; 8; 12, respectively. It is likely that the depression of the ASV waves is

TABLE 1

Zinc, cadmium, lead and copper in sweat

Sex	Number of subjects	n[a]	Concentration ($\mu g\,l^{-1}$)			
			Zn	Cd	Pb	Cu
M	9	27	720 ± 270	< 3	15 ± 10	80 ± 39
F[b]	6	14	730 ± 210	< 3	8 ± 5	23 ± 9
F[c]	7	11	750 ± 320	< 3	25 ± 11	85 ± 45

[a] n = total number of measurements.
[b] Not using oral contraceptives.
[c] Using oral contraceptives.

due to protein, since human serum albumin depressed the metal waves in a similar pattern. The complexing capacity of sweat was $6 \times 10^{-6} M$ for copper, and $> 2 \times 10^{-4} M$ for zinc, although the high zinc value is probably due to protein fouling the electrode, rather than by actual zinc complexing [11]. Wet ashing of the membrane filter, followed by ASV analysis, showed that the $NaNO_3$–HNO_3 electrolyte extracted more than 90% of the trace metals from the sweat collected.

The size distribution of the metal species in sweat was investigated by extracting the sweat from the filter with $0.1 M$ $NaNO_3$, then ultrafiltering it through an Amicon Centricon-30 micro concentrator (30000 M.W. cut-off) and carrying out ASV on the ultrafiltrate. Analysis of single sweat samples in this manner from two male subjects showed that the ultrafilterable fractions (%) of zinc, lead, and copper were 85 ± 10, 42 ± 8, and 100 ± 8, respectively. Similar results were obtained using a Centricon-10 tube (10000 M.W. cut-off).

Choice of membrane filter is critical to ASV analysis of sweat. Hydrophobic materials (e.g. Teflon) treated with wetting agent (e.g., Triton X-100) cannot be used because the wetting agent usually causes severe depression of the metal waves. Other materials, such as cellulose and cellulose nitrate, strongly adsorb metal ions, even in the $NaNO_3$–HNO_3 electrolyte, whereas some filter materials were so high in metal impurities as to be useless. Of many membrane filters tested, by far the best was Millipore "Polyvic" (BSWP), a polyvinyl chloride membrane made hydrophilic during the manufacturing process. The Polyvic membranes had good water absorption, were mechanically strong, did not adsorb metal ions, and had no effect on the ASV waves. Unfortunately, however, Millipore recently ceased production of these filters. The only other filter found to be acceptable was the Millipore type HATF, a cellulose acetate membrane. It does not absorb water as rapidly as the polyvic, and so requires 45 min sweat collection time, but in all other respects is equal to Polyvic.

Insufficient ASV data on sweat have been accumulated with the thin-layer

TABLE 2

Composition of sweat (pH 5 2)

Compound	Conc. ($mg\,l^{-1}$)
NaCl	800
KCl	910
$CaSO_4 \cdot 2H_2O$	200
$MgSO_4 \cdot 7H_2O$	88
Lactic acid	1470
Urea	300
Histidine HCl	240
Alanine	920
Glycine	280
Citrulline	152
Urocanic acid	76
Protein	350

TABLE 3

Reported values for zinc, cadmium, lead and copper in sweat

Sex	Concentration ($\mu g\,l^{-1}$)				Ref
	Zn	Cd	Pb	Cu	
M				58 ± 13	13
M	1150 ± 300				6
M		84 ± 53			3
M	500 ± 480		51 ± 42	550 ± 350	7
F	1250 ± 770		118 ± 72	1480 ± 610	7
M			12		12

cell to compare the results with the leaching/HMDE technique, although preliminary indications are that the two methods produce similar metal concentrations.

DISCUSSION

The approximate composition of sweat [4] is shown in Table 2, and literature values for zinc, cadmium, lead and copper in sweat are given in Table 3 [12, 13]. Although there is reasonable agreement between the zinc values found in the present study (Table 1) and by previous investigators (Table 3), we found lower values for cadmium and lead, and much lower copper than reported by Hohnadel et al. [7]. In addition, unlike Hohnadel et al., we did not find a sex-based difference for zinc and lead in sweat. These authors also reported that oral contraceptives had no significant effect on trace metals in sweat. Our results suggested that copper in the sweat of females, which was significantly lower than the male copper level, was elevated to the male concentration by taking oral contraceptives (Table 1). However, more data would be required to establish this point.

The most likely source of trace elements in sweat is blood serum; labile

TABLE 4

Trace metals in whole blood, serum and sweat

Metal	Concentration ($\mu g\,l^{-1}$)		
	Blood	Serum	Sweat[a]
Zn	8500	950	720
Cd	0.8		< 3
Pb	200	10	15
Cu	950	1050	80

[a] Males, this study.

metals dissociate from proteins under the influence of the concentration gradient existing across the blood capillaries, and diffuse through the capillary walls into the sweat glands [14]. Table 4 compares trace metal levels in whole blood, blood serum, and sweat [15]. It is apparent that the concentrations of zinc, cadmium and lead in serum and sweat are very similar, whereas copper is much lower in sweat than in serum. Copper in serum is mainly (95%) present as the protein ceruloplasmin, in which copper is tightly bound [16]. Exchangeable copper in proteins and low molecular weight copper complexes represent only 5% of serum copper [16, 17]. In addition, it has been suggested that 10–15% of copper in ceruloplasmin is moderately exchangeable [16]. Our ultrafiltration experiments on sweat show that nearly all of the copper and zinc are present as low (< 10000 M.W.) molecular weight species. The lower sweat copper values found for females was surprising, since serum copper for males and females is very similar [15]. It is possible, however, that there is a sex-based difference in labile copper in serum.

Abnormal copper/zinc ratios in blood serum are indicative of certain disease states, including leukemia [18]. If it could be shown that there is a correlation between copper and zinc in blood and sweat, analysis of sweat samples would be a convenient, non-invasive technique for diagnosis. Also, lead in sweat may be useful for occupational hygiene monitoring of factory workers handling lead, again if there is indeed a relationship between whole blood lead and lead in sweat. A comprehensive survey is at present being undertaken to compare trace element concentrations in blood serum and sweat taken from individuals on the same day.

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The Use of Scalp Hair to Monitor Manganese in Aborigines from Groote Eylandt

JENNIFER L. STAUBER, T. MARK FLORENCE AND WILLIAM S. WEBSTER[1]

CSIRO Division of Energy Chemistry, Private Mail Bag 7, Menai, NSW 2234, Australia; [1]Department of Anatomy, University of Sydney, NSW Australia

INTRODUCTION

Aborigines at Angurugu, a small township on Groote Eylandt (G.E.) in the Gulf of Carpentaria, Australia, have a high incidence of the neurological disturbances referred to as the Groote Eylandt Syndromes (Cawte, 1984). One syndrome, probably a form of motor neurone disease, is characterized by weakness in the legs and onset occurs in early childhood. The second syndrome is characterized by a cerebellar syndrome and oculomotor disturbances, with onset in middle age (Kiloh *et al.*, 1980; Kilburn, 1986).

It has been suggested that this endemic disease may be caused by high environmental manganese, as similar neurological symptoms have been found in South American miners suffering from manganese poisoning (Cotzias *et al.*, 1968). Manganese, absorbed through the lungs and gastrointestinal tract is cleared quickly from the blood and deposited in tissues, particularly the liver, kidney, pancreas and brain. Manganese is excreted in the feces via bile, with very little excreted in the urine (Piscator, 1979; Keen *et al.*, 1984). Because manganese has a short biological half-life - 4 days for the fast fraction (30%) and 40 days for the slow fraction - monitoring manganese at the low levels found in blood and urine can only indicate relatively recent exposure.

The most convenient record of manganese exposure over a few months is the manganese concentration in scalp hair, which grows at a rate of 1-2 cm per month (Hopps, 1977). Problems in using scalp hair include pretreatment of the hair before manganese analysis, and distinguishing between endogenous (manganese that enters the hair via blood supply to the hair follicle) and exogenous sources of manganese (including dust and sweat) (Chittleborough, 1980).

We have monitored manganese in scalp hair in more than 100 Groote Eylandters in an attempt to determine if the Aboriginal population of Angurugu has a significantly higher manganese intake than other populations. Manganese in blood, sweat and urine samples from affected and non-affected individuals, and in hair, blood and tissues from dogs and melomys (a bush mouse) living in Angurugu have also been determined.

METHODS

Scalp hair samples were collected from the nape of the neck, cut and then washed in 25 ml of non-ionic detergent (0.5% Triton X-100) in an ultrasonic bath for 15 minutes. Hair samples were rinsed with distilled water through a pierced Whatman 542 filter paper, rinsed with methanol and air dried before manganese, copper, zinc, lead, cadmium and iron determination by neutron activation analysis (NAA) and anodic stripping voltammetry (ASV). To ensure that all the dust was removed, G.E. roadside dust containing up to 4% manganese, was rubbed into the hair, shaken overnight and the hair washed in the same manner. The washed hair was also analyzed for aluminum, which is present at high concentrations in the dust. The washing procedure effectively removed all surface dust and gave reproducible results for multiple hair samples from the same person.

Please send requests for reprints to Jennifer L. Stauber.

Manganese in dust trapped in the hair before sampling may be solubilized in sweat, incorporated into the hair shaft and measured as endogenous manganese. Experiments were conducted to determine how much manganese in hair was from this exogenous source, and whether it could be selectively leached from the hair. Hair was shaken overnight in a large excess of roadside dust in synthetic sweat ± protein ± sebum and in sauna-collected sweat, and manganese uptake measured. Hair was shaken for periods of up to one month in sweat containing radiolabeled ^{54}Mn to determine manganese incorporation into hair from sweat.

Manganese in whole blood was determined by atomic absorption spectroscopy (AAS) at the Prince of Wales Hospital, N.S.W. Manganese in urine and sweat was determined by inductively coupled plasma emission spectroscopy (ICP). Tissue manganese concentrations in dogs and melomys were analyzed by NAA. Two dogs from Angurugu were flown to Sydney, washed and partly shaved. Blood, hair and liver biopsies were taken immediately on arrival in Sydney and at monthly intervals. After three months, the dogs were killed and tissue manganese concentrations compared to those of a control dog from Sydney.

RESULTS AND DISCUSSION

Manganese in the scalp hair of Aborigines and Caucasians living in manganese and non-manganese areas is shown in Table 1. Aborigines at Angurugu had much higher hair manganese (means of 34 and 77 ppm for males and females, respectively) than Aborigines in non-manganese areas, *e.g.*, Gove Peninsula, where the mean was only 2 ppm Mn. Although Caucasians at Angurugu had lower hair manganese than the Aborigines, it was much higher than Caucasians living in Sydney. Scalp hair concentrations in Sydney controls (0.6-1.2 ppm) were in agreement with hair manganese values reported previously (Gordus, 1973; Sky-Peck and Jospeh, 1983). We have no evidence to suggest that black hair concentrates manganese more than white hair as reported by Cotzias (1964). Samples of grey and black hair from an elderly Aborigine had the same manganese content, as did black and white hair from a Groote Eylandt dog. Individuals affected with Groote Eylandt Syndrome did not always have high hair manganese. The manganese exposure which caused the neurological damage may have occurred many years previously and was, therefore, not detectable in a few months' growth of hair.

Lead, copper, cadmium, iron and zinc were determined in a number of Aboriginal hair samples and were all within the normal range: lead 1-11 ppm, copper 8-28 ppm, cadmium < 0.1-0.5 ppm, iron < 16-94 ppm and zinc 30-177 ppm.

Manganese uptake into hair was examined to determine whether high manganese in the hair of Angurugu Aborigines was endogenous, reflecting the mobile body store of manganese, or exogenous from dust solubilized in sweat and incorporated into the hair. We attempted to find a selective leaching agent which would remove exogenous manganese without leaching endogenous manganese from hair. The most effective leaching agents were 0.1 M HCl (pH 1.0) and 0.1 M cysteine-HCl (pH 1.34), followed by 0.025 M HCl (pH 1.34), 0.1 M DCyTA (pH 5.5), 0.1 M EDTA, 0.1 M oxalic acid, 0.1 M ascorbic acid, 0.1 M penicillamine and 0.1 M diethyldithiocarbamate

TABLE 1. Manganese in Scalp Hair.

	Mn(ppm)		
Subjects	n	x	Range
Male Aborigines - Angurugu	28	34	3-120
Female Aborigines - Angurugu	15	77	21-240
Male Caucasians - Angurugu	8	9.4	0.6-24
Female Caucasians - Angurugu	7	14	5-28
Aborigines - Gove	10	2.0	< 1-7
Caucasians - Sydney	5	0.9	0 6-1.2

which was least effective. Although nearly 100% of sweat-induced manganese was removed by 0.1 M HCl and cysteine-HCl, these reagents also extracted some of the endogenous manganese. Ultrasonication with 0.1 M DCyTA for 1 hour did not remove any endogenous manganese, but extracted only 15-40% of the exogenous manganese, with the percentage extracted decreasing with increasing exposure time to manganese. We were unable to find a leaching agent which was suitable to pretreat all hair samples before analysis.

Lactic acid in sweat was found to reduce manganese in dust (present as Mn(III) and Mn(IV)). The maximum amount of manganese that could be dissolved from roadside dust (containing 4% Mn) was 1120 μg Mn L^{-1} of sauna sweat (Table 2). Incorporation of manganese into hair from sweat was a linear function of the manganese concentration in dust (Fig. 1) with most manganese being incorporated within the first four days of shaking. Uptake of manganese was unaffected by addition of a fresh manganese solution each day, or by shampooing the hair each day. The average concentration of manganese in air dust collected from Angurugu was 1.7%. At most, this could lead to 500 μg Mn^{-1} dissolved in sweat and from Fig. 1, an incorporation of up to 15 μg Mn g^{-1} hair. This suggests that the elevated hair manganese of Caucasians living in Angurugu (9.4 and 14 ppm for males and females, respectively), originates entirely from this exogenous source. Exogenous manganese, however, cannot account for hair manganese levels of up to 240 ppm in Angurugu Aborigines. The Aboriginal diet of damper, bush tucker and billy-boiled tea (which contains 2000 μg Mn L^{-1}), may contribute to high hair manganese levels in Angurugu Aborigines.

TABLE 2. Solubility of Manganese from Groote Eylandt Roadside Dust in Sweat.

Solution	μg Mn L^{-1}
Distilled Water	80
Synthetic Sweat	800
Synthetic Sweat + Protein	640
Synthetic Sweat + Sebum	840
Sauna Sweat	1120

Manganese in hair, sweat, urine and whole blood from three brothers from Angurugu, two of whom are affected by G.E. Syndrome, is shown in Table 3. Hair manganese was elevated in all three brothers, whereas manganese in urine and sweat were within the normal range (Piscator, 1979). Manganese was elevated in the blood of both affected

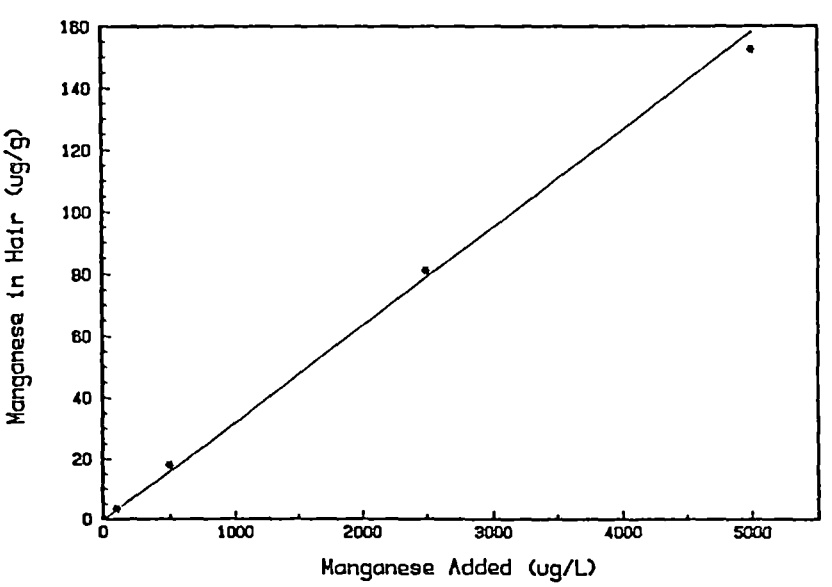

FIG. 1. Uptake of Manganese from Synthetic Sweat into Hair.

TABLE 3. Manganese in Hair and Body Fluids for Three Brothers from Angurugu.

Subject	Manganese			
	Whole Blood ($\mu g\ L^{-1}$)	Urine ($\mu g\ g^{-1}$ Creatinine)	Sweat ($\mu g\ L^{-1}$)	Hair ($\mu g\ g^{-1}$)
Unaffected	25	< 0 5	3.0	32
Mild G.E. Syndrome	41	1.1	11.1	29
Severe G.E. Syndrome	42	< 0.3	5.2	15

Aborigines, compared to the unaffected control, although all three values were higher than the normal range of 7-12 μg Mn L^{-1} (Piscator, 1979). Manganese in blood appears to be the best indicator of individuals affected by G.E. Syndrome.

Tissue manganese concentrations may also be useful to determine body burden of manganese. We measured manganese in Aboriginal teeth, tissues of bush mice (melomys) and dogs from Angurugu. Very little manganese accumulates in teeth: 1-3 μg Mn/g tooth and < 0.43 μg Mn/g root were found in teeth from three Angurugu Aborigines. Tissue manganese concentrations in melomys trapped around Angurugu were compared to control laboratory rats (Table 4). In this mammal, manganese appears to concentrate in the spleen, heart, pancreas and brain.

TABLE 4. Manganese in Tissues of Angurugu Melomys Compared to a Laboratory Rat.

Tissue	Mn (ppm fresh weight)	
	Melomys	Rat
Liver	2.2	2.1
Kidney	1.2	1.2
Pancreas	3 3	1.6
Spleen	1.7	0.3
Heart	0.78	0.28
Lung	0.65	0.45
Cerebellum	1.2	
Cerebral Cortex	0.50	0.38 (Brain)

Manganese in hair, serum and tissues of two dogs from Angurugu were compared to manganese in a Sydney control dog. Both dogs initially had high hair manganese, particularly dog 2. However, hair which regrew after the dogs were removed from the manganese environment for one month was much lower in manganese (Table 5). This suggests that manganese available for uptake into the hair was cleared from the blood very rapidly. Serum manganese in both dogs one month after removal from Angurugu, was as low as that in the control dog. After three months in Sydney, higher manganese concentrations were still found in the adrenal glands, lungs and duodenum of both dogs than in the Sydney control dog (Table 6). Dog 2 with initially high hair manganese, also had high manganese in the liver and brain (choroid plexus and corpus striatum). It has been suggested that manganese may cause neurological lesions because it catalyzes the auto-oxidation of neurotransmitters, *e.g.*,

TABLE 5. Manganese in Hair and Serum in Two Dogs from Angurugu Immediately After Arrival in Sydney and One Month Later.

Tissue	Date	Mn (ppm)		
		Dog 1	Dog 2	Control
Hair	13 3.86	11	259	-
Hair	23.4.86	1.1	0 6	0.5
Serum	13.3.86	5.9	13.9	-
Serum	23.4.86	4.7	6.1	9.5

TABLE 6. Manganese in Tissues of Two Dogs, Three Months After Removal from Angurugu, Compared to a Sydney Control Dog.

	Mn (ppm dry weight)		
Tissue	Dog 1	Dog 2	Control
Liver	7.9	11.1	7.7
Lung	1.24	1.55	0.98
Pancreas	4.09	4.24	4.78
Duodenum	2.04	2.33	1 42
Adrenals	1.52	1.63	0.30
Spleen	1.27	0.90	1.43
Kidney Cortex	6.38	7.79	6.79
Choroid Plexus	< 17	32 9	3.7
Corpus Striatum	1.78	5.4	1.87

dopamines in the brain (Donaldson, 1980).

While scalp hair may be useful in determining a population's overall exposure to manganese, interpretation of the data is difficult without careful study to separate endogenous and exogenous sources of manganese. We are, at present, monitoring manganese along the length of scalp hair and comparing this to manganese in pubic hair, to understand the contribution of exogenous manganese. Blood and tissue manganese levels may be more useful to determine body burden of manganese and to indicate individuals affected with Groote Eylandt Syndrome. We hope to obtain autopsy samples from both affected and unaffected Angurugu Aborigines to gain further insight into the relationship between manganese and Groote Eylandt Syndrome.

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Paper 11

RESEARCH ON -

MANGANESE AND METABOLISM -

GROOTE EYLANDT, NORTHERN TERRITORY

Proceedings of a conference
held at Health House, Darwin, N.T.
on 11 June, 1987
at the invitation of
Dr Keith Fleming, Secretary for Health,
Northern Territory Department of Health

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Ecological Studies of Manganese on Groote Eylandt

T.M. Florence, J.L. Stauber and J.J. Fardy,

CSIRO Division of Energy Chemistry,
Lucas Heights, N.S.W.

1. Introduction

Most manganese salts have low acute toxicity, and manganese was thought for a long time to be one of the most innocuous of elements [1]. The long-term (chronic) toxicity of manganese was recognized later, as a result of neurological disorders with symptoms similar to Parkinson's Disease appearing in some manganese miners, particularly those in Chile [2].

Groote Eylandt has extensive and rich manganese deposits which are mined and provide a valuable export for Australia. The main Aboriginal village of Angurugu is situated in one area of manganese mineralization, with high soil and plant manganese. A small number of Aborigines have developed unusual neurological problems which are somewhat similar to those reported for miners suffering from manganese intoxication, and it was important to determine if these problems (the "Angurugu Syndrome") were associated with excessive manganese intake.

In this respect, an obvious question is: "If all the inhabitants of Angurugu are uniformly exposed to high levels of manganese, why are only 1-2% of the population affected?" This question can be answered on the basis of individual susceptibility. In the Chilean manganese mines, less than 5% of the miners developed chronic manganese toxicity; one miner may have been severely affected while his workmate, working beside him for the

same length of time, and exposed to the same concentrations of manganese in air, showed no toxic symptoms whatsoever [3]. This phenomenon of individual susceptibility has been observed with other metals such as lead, mercury and beryllium, and also with tobacco smoking. Most people know of a relative or friend who has smoked heavily all his life yet has not developed lung cancer or any of the other smoking-related diseases. Others, with a much lower tobacco usage, succumb quickly. The differences in ability to cope with toxicants is believed to result from individual variations in uptake rate and clearance rate of the toxicant, and the inducibility of enzyme systems which detoxify the foreign compound. In case of manganese, the most important factor is believed [4] to be the differences in the excretion ability of the liver and kidneys.

This study was undertaken to establish manganese concentrations in the environment and diet of Groote Eylandt Aborigines, and to compare the results with world average concentrations.

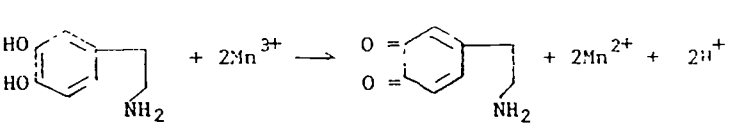
2. Toxicology of Manganese

Donaldson and co-workers [5] have shown how the neurotoxicity of manganese could arise as the result of Mn(II)/Mn(III) catalysis of dopamine oxidation in the brain. Our own studies at Lucas Heights indicate that the relevant reactions are:

$$Mn^{2+} + O_2 \longrightarrow Mn^{3+} + O_2^{\bullet -} \qquad (1)$$

$$Mn^{2+} + O_2^{\bullet -} + 2H^+ \longrightarrow Mn^{3+} + H_2O_2 \qquad (2)$$

net, (1) + (2); $2Mn^{2+} + O_2 + 2H^+ \longrightarrow 2Mn^{3+} + H_2O_2$ (3)



(4)

dopamine dopamine quinone

Reaction (1) proceeds only in the presence of a manganese(III)-complexing ligand (such as pyrophosphate, citrate or xanthine) and dopamine. The products of manganese and dopamine oxidation, ie, hydrogen peroxide and dopamine quinone, are strongly neutrotoxic. Catalase, the enzyme responsible for destroying H_2O_2 is low in the brain, and superoxide dismutase, the enzyme which dissociates superoxide radical ($O_2^{\bullet -}$), is unevenly distributed [6]. Manganese(II), however, efficiently catalyzes the dissociation of $O_2^{\bullet -}$. Superoxide radical, normally considered a dangerous species in biological systems, is nevertheless essential in the brain for the synthesis of the neutrotransmitters, norepinephrine and serotonin [7]. A certain level of manganese may also be essential to participate in vital redox reactions, but too high a concentration could lead to serious toxic effects.

The neurotoxic effects of excess manganese may be, (i) reduction of dopamine concentrations, (ii) production of toxic dopamine oxidation products (quinones and semi-quinones), (iii) production of hydrogen peroxide (iv) destruction of superoxide radical. Other metal ions, such as Cu^{2+} and Fe^{2+}, can also catalyze these reactions, but they are usually tightly bound in enzymes, and not free to participate in reactions such as (1)-(4).

3. Blood Analyses

The determination of manganese in blood is of little use for the diagnosis of chronic manganese poisoning [3]. Manganese miners with severe symptoms of manganism, after removal from the workplace, usually had normal manganese-in-blood concentrations [3]. On the other hand, apparently healthy miners, working in the mine, had high blood manganese. Manganese in the human body is characterized by two half-lives of 4 and 40 days, so blood manganese can only give an indication of recent exposure, and provides no information about the body store of manganese [2]. Nevertheless, for equally exposed individuals, high blood manganese may

indicate those with enhanced uptake mechanisms and/or deficient clearance mechanisms.

Results for catheter-collected (Jan 1987) blood from Groote Eylandt inhabitants are given in Table 1. The manganese values were determined by Mr Graham Hams, Clinical Chemistry Department, Prince of Wales Hospital. Neutron activation analysis was also carried out at Lucas Heights on some of the bloods and, in general, good agreement was obtained.

The average blood manganese (µg/L) for the four groups (Table 1) were: GEMCO workers (omitting No. 1), 8.4; Caucasians in Angurugu, 7.3; affected Aborigines (omitting No. 7), 36.1; unaffected Aborigines, 18.9. The normal range for manganese in blood is 6 to 12 µg/L, with a mean (Sydney) of about 8.5 µgMn/L. The Caucasian inhabitants of Groote Eylandt have blood manganese values close to this mean, but Aborigines unaffected by the Angurugu Syndrome have double the Caucasian blood manganese, and those affected, four times. The affected Aborigines also had low hemoglobin (normal range 12-18 g/dL) and low ferritin (normal 25-150(F), 75-260(M) µg/L). Therefore, in addition to high blood manganese, they have a low iron status (anemia).

One sample of cord blood from an Angurugu baby delivered in Gove had 41 µgMn/L, even though the mother's blood was normal (7.2 µgMn/L) and the concentration of manganese in the placenta was low (0.12 µg/g dry wt.).

4. Factors that Exacerbate Manganese Toxicity

The following factors are known to increase the toxic effects of manganese [8].

(a) Low iron(anemia). Iron and manganese have a similar uptake mechanism: anemic individuals have enhanced absorption of both iron and manganese, and are known to be more susceptible to the toxic effects of this element [2].

(b) Chronic infections. Infections cause an increase in the production of H_2O_2 and free radicals in the body, putting greater strains on the ability

of the cellular and extracellular antioxidants to scavenge these toxic substances, already produced in excess by the catalytic action of excess manganese. Some natural antioxidants are vitamins C and E, glutathione and free radical-dissociating enzymes.

(c) High alcohol intake. The metabolism of ethanol liberates H_2O_2 and free radicals, and depletes the liver of the antioxidant glutathione.

(d) Low dietary calcium. Manganese can displace calcium from nerve endings and hence disrupt the central nervous system. This is more likely to happen in an individual with high manganese and low calcium status.

(e) Low zinc status. Zinc protects sulfhydryl groups (e.g., glutathione) from oxidation by H_2O_2 and free radicals. These compounds are therefore more susceptible to manganese toxicity when zinc is depleted.

5. Manganese in sweat and urine

Three Aboriginal brothers from Angurugu, one deemed as severely affected by the Angurugu Sydrome, one moderately affected, and one unaffected, were brought to Sydney for medical tests. In all three cases, the urine (<0.3-1.1 μgMn/g creatinine) and sauna sweat (3-11 μgMn/L) were within the range found for Lucas Heights controls.

6. Chelation Therapy

The three brothers mentioned above were treated at Prince Henry Hospital with calcium ethylenediaminetetraacetic acid (EDTA) in an attempt to remove excess body manganese. Urine analysis showed, however, that after correction for the manganese blank in the Ca-EDTA, no manganese was removed by the chelation therapy.

7. Manganese in hair

Hair concentrates trace elements from blood supplying the hair follicle, and hence manganese in hair should be an indicator of blood levels of this element. In addition, since scalp hair grows at the rate of 1.0-1.5 cm/month, changes along the length of a hair should be a record of changing blood concentrations during that period.

This ideal situation is complicated by external contamination of the hair - from dust, shampoos, and hair treatment which may add or subtract elements from the hair. Manganese is one of a group of elements known to increase along the length of a hair even when blood concentration of the element is constant. This was believed to be due to increased environmental exposure to the element as the hair grew, but our research has shown that the increase results from sweat elution. Sweat glands near the hair root produce sweat which travels up the hair, dissolving manganese from dust particles on the hair, and concentrating the element towards the tip of the hair. Under these conditions, hair is acting as a wick or chromatographic column, with sweat as the eluant. This phenomenon occurs only with those elements that form lipid-soluble complexes with sweat. To overcome this exogenous effect, a plot of manganese concentration in washed hair versus length from scalp was constructed for each hair sample, and the graph extrapolated to zero length. This "zero-length" manganese concentration should represent the hair manganese unaffected by external factors.

A summary of results is shown in Table 2. All Groote Eylandt inhabitants had hair manganese values higher than Sydney residents, probably as a result of a continuous, albeit low, intake of excess manganese. Aborigines had much higher manganese in hair than Caucasians, although there was no significant difference between affected and unaffected subjects.

8. Manganese in Air and Water

Manganese in air in Angurugu (average about 5 $\mu gMn/m^3$) is 100 x the Sydney and European average (Table 3). However, manganese in air would contribute only about 0.1 mg manganese/day to the intake of an Angurugu resident, and so would be an insignificant percentage of total intake unless, of course, respired manganese is particularly toxic. Manganese in Angurugu air is well below the occupational limit of 200 $\mu g/m^3$.

Manganese in the village tapwater and the Angurugu River appears to vary seasonally, up to 10 x the Sydney and world average. However, manganese from this source would amount to, at the most, 0.3 mgMn/day, and so would be insignificant. Calcium in the Angurugu River (0.2 mg/L) was very low, only 0.01 x world average.

9. Manganese in Traditional Food Sources

Some traditional ("bush tucker") food samples were collected from the Angurugu old village garden areas (used extensively before 1970) and analyzed for manganese (Table 4). Some of the foods were exceptionally high in manganese, and contained 3-100 x the world average for these items. A peeled yam, for example, had 660 µgMn/g (fresh weight), compared with 5 µgMn/g in a Sydney carrot. On this basis, one 20-g yam would supply 13 mg of manganese, or 3 x the recommended daily allowance (RDA) of this element (4 mg).

Boiled ("billy") tea extracts about 5 times the amount of manganese from tea leaves as does brewed tea. One litre of billy tea would contain 6-7 mg manganese.

10. Manganese in Soils

Soil samples in the old Angurugu garden areas (Table 5) were exceptionally high in manganese (up to 100 x world average) and very low in calcium (0.04 world average). Soil samples taken near Aboriginal houses in Angurugu were about 10 x world average in manganese. Umbakumba soil was very low in manganese.

11. Manganese in Organs of Experimental Animals

Organs of animals captured near Angurugu varied considerably in manganese content, but some were particularly high, e.g., thymus, adrenals, spleen, and the brain (Table 6).

12. Conclusions

I have reached the following preliminary conclusions from these results.

1. Angurugu Aborigines have a high intake of manganese, but the actual amount is very difficult to determine. Air and water contribute insignificantly to their total intake but, because of their "close to the earth" living, relatively poor hygiene, and the high manganese content of Angurugu soils, ingested manganese may be considerable.

2. Before the store was opened in 1970, Angurugu Aborigines used about 80% "bush tucker" in their diet. This bush tucker consisted largely of vegetables and fruit grown in the garden area, which has extremely high soil manganese. Their dietary manganese from this source alone could easily have been 100-200 mg manganese/day (25-50 x RDA). At present, bush tucker constitutes only 10-20% of their diet, and may not be as significant as damper cooked in the earth and billy tea.

3. There is no known way of measuring, in vivo, the total body burden of manganese. Blood and hair only indicate manganese in readily exchangeable pools such as soft tissue, and chelation therapy appears to be ineffective. The availability of autopsy samples would help considerably.

4. Anemia, chronic infections, high alcohol intake, low dietary calcium, and low zinc status are factors that exacerbate manganese toxicity, and are commonly found in Angurugu Aborigines.

5. Hair analysis suggests that Caucasians in Angurugu, and GEMCO workers, have a higher manganese intake than non-exposed persons, but much less than the Aborigines.

13. Future Work

I believe that the following work is necessary to clarify the question of excessive manganese exposure in Angurugu.

1. Additional blood analysis. Although it is well established that blood manganese analyses indicate only very recent exposure, they may pinpoint individuals with a defective clearance mechanism and/or enhanced uptake mechanism. Every attempt should be made to collect cord blood and hair samples from babies born to Angurugu women, because it is vital to know if

manganese is concentrated across the placenta. Control cord blood samples would also have to be collected because little is known about the normal manganese concentration in cord blood.

2. Autopsy samples can be rapidly and non-destructively analyzed by neutron activation analysis. As many as possible should be collected.

3. An attempt should be made to estimate the bioavailability of manganese in Angurugu soils. Standard procedures could be used for this.

4. Hair analyses of people who have lived on Groote Eylandt and left should be carried out to determine if, and how rapidly, manganese in hair decreases when exposure to this element is removed. If levels remain high, it may mean that hair can be used as index of body burden of this element.

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TABLE 1 BLOOD ANALYSES ON GROOTE EYLANDT INHABITANTS

Subject	Mn µg/L	Fe µg/L	Hb g/dL	Ferritin µg/L
1. GEMCO workers				
1	22.3	503	16.6	233
2	6.0	454	15.5	280
3	9.9	420	14.2	124
4	9.1	407	14.9	146
5	12.6	498	16.1	165
6(F)	10.7	364	13.3	87
7(F)	6.9	406	14.5	158
8	6.3	495	16.8	93
9(F)	7.7	391	14.1	53
10	6.3	448	15.9	125
2. Caucasians in Angurugu				
1(F)	5.8	419	13.8	-
2	4.9	413	15.1	110
3(F)	8.8	361	12.1	30
4(F)	9.6	359	12.7	67
3. Affected Aborigines				
1(F)	38.7	331	11.3	28
2(F)	36.3	297	10.4	5
3(F)	15.7	356	11.4	0.3
4	42.3	191	8.9	11
5	42.1	-	-	-
6	41.2	-	-	-
7(F)*	9.3	381	13.1	367
4. Unaffected Aborigines				
1(F)	25.5	351	-	-
2	9.9	472	16.4	354
3(F)	16.8	336	12.3	34
4	17.6	453	15.0	96
5	24.9	-	-	-

*Lived in Umbakumba for 15 years.

TABLE 2 MANGANESE IN HAIR

Subjects	Mean manganese, µg/g	
	Scalp*	Pubic
Sydney	0.5±0.2	1.3±0.5
GEMCO workers	2.2±0.8	3.0±0.4
Caucasians in Angurugu	2.5±0.7	7.9±4.1
Unaffected aborigines	15±5	21±5
Affected aborigines	9±3	23±7

*Extrapolated to zero length.

TABLE 3 MANGANESE IN AIR AND WATER

Sample	Angurugu	Sydney	World Average
Tapwater, µgMn/L	4.3(8/85) 70(1/87)	5.8	-
Angurugu River, µgMn/L	27(8/85) 97(1/87)	-	8(for rivers)
Air, µgMn/m^3	23(3 m from road) 1.2(10 m from road)	0.05	0.04(European)

TABLE 4 MANGANESE IN TRADITIONAL FOOD SOURCES COLLECTED FROM OLD GARDEN AREAS*

Sample	µgMn/g fresh weight	
	Angurugu	Sydney
Fish, Angurugu River	36	0.3
Oysters, Mud Cod Bay	0.25	0.05
Yam, young	657	5**
Yam, old	484	5**
Citrus fruit	0.85	0.3
Banana	79	1.5
Billy tea	6.7	-

*U.S. intake: range 2-9 mgMn/day, mean, 3.7 mg/day.
**Root vegetables.

TABLE 5 MANGANESE IN SOILS

Source	Manganese, % dry weight*
Near Angurugu houses	0.2-1.2
Angurugu road	4.1
Old orchard	1.4
Old vegetable garden	4.6
Cassava plantation	0.11
Banana plantation	4.2
Mud Cod Bay sediment	0.33
Emerald River settlement	0.15
Umbakumba	0.002

*World average soil has 0.05% manganese.

TABLE 6 MANGANESE IN ORGANS OF EXPERIMENTAL ANIMALS

Organ	Manganese, µg/g, fresh weight			
	Angurugu Melomys*	Sydney rat	Groote dog	Sydney dog
Thymus	2.5	0.24	-	0.49
Adrenals	4.2	-	0.17	0.05
Spleen	1.7	0.24	0.27	0.33
Cerebellum	1.1	0.45	1.4	0.50
Choroid plexus	1.5	0.59	8.1	0.90

*Bush rat.

Paper 12

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A COMPARATIVE STUDY OF COPPER, LEAD, CADMIUM AND ZINC IN HUMAN SWEAT AND BLOOD

J.L. STAUBER and T.M. FLORENCE

CSIRO Division of Energy Chemistry, Private Mail Bag 7, Menai, NSW 2234 (Australia)

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ABSTRACT

Zinc, cadmium, lead and copper in sweat from 24 male and 39 female volunteers were determined by anodic stripping voltammetry. Sweating was induced on the forearms by pilocarpine iontophoresis Average values found for zinc, cadmium, lead and copper in sweat from males were 181 (range 25–863), 1.4 (< 0.5–10), 41 (6–87) and 103 (< 5–673) $\mu g\,l^{-1}$, respectively. Zinc in sweat from females was significantly higher than in sweat from males (331 $\mu g\,l^{-1}$, range 87–836 $\mu g\,l^{-1}$), while sweat copper and sweat lead in females were lower (29 μg Cu l^{-1}, range < 5–146 μg Cu l^{-1} and 24 μg Pb l^{-1}, range < 5–66 μg Pb l^{-1}). Those taking oral contraceptives showed increased sweat copper concentrations (94 μg Cu l^{-1}, range < 5–480 μg Cu l^{-1}) and sweat lead concentrations (36 μg Pb l^{-1}, range < 5–70 μg Pb l^{-1}). There was no sex-based difference for copper in sauna-induced sweat. Metal concentrations in sweat were compared with ceruloplasmin, alkaline phosphatase, and total and mobile copper and zinc concentrations in serum in males and females.

INTRODUCTION

Determination of trace elements in body fluids by non-invasive techniques has advantages over blood sampling and analysis. Sweat, in particular, may be useful for occupational hygiene monitoring as samples can be collected in the workplace with minimum risk and loss of production. In addition, determination of elements in sweat may be useful in the diagnosis of some diseases. Analysis of chloride in sweat has been used extensively for the detection of cystic fibrosis in newborn babies (Gibson and Cooke, 1959).

Eccrine sweat is produced in tubular coil glands under the skin surface in response to heat/work stress. Sweat contains 99% water and 1% solids, of which half are inorganic salts. While the composition of sweat depends largely on the blood supply to the sweat gland, sweat is not simply an ultrafiltrate of blood plasma. Sodium and chloride are always lower in sweat than serum, whereas potassium, urea, ammonia and lactic acid are higher (Rothman, 1954). The concentrations in sweat are also regulated to some extent by reabsorption in the ductal tubule of the sweat gland (Rothman, 1954).

Results obtained for the composition of sweat vary with the collection technique used. Techniques used include collection of sweat directly from the exposed skin into a beaker (Mitchell and Hamilton, 1949), absorption of sweat onto gauze or cotton wool pad (Shiels, 1954) and collection into a plastic glove

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or bag enveloping the skin (Prasad et al., 1963). Sweating has been induced by heavy excercise (Walker and Griffin, 1976), pilocarpine iontophoresis (Sato et al., 1970), exposing the subject to the sunshine (Prasad et al., 1963) or a sauna (Hohnadel et al., 1973). Determination of trace metals in sweat has been difficult because of the low concentrations of metals in sweat and problems in collecting uncontaminated sweat samples of adequate size.

We recently developed a simple method for the determination of zinc, cadmium, lead and copper in sweat by anodic stripping voltammetry using pilocarpine iontophoresis for sweat collection (Stauber and Florence, 1987). The present study used this method to monitor total metals in the sweat of 63 subjects and compares the results with serum copper, zinc, alkaline phosphatase and ceruloplasmin. Mobile copper and zinc in serum were also determined by extraction with Chelex 100. Metals in iontophoresis-induced sweat and sauna-induced sweat were also compared.

EXPERIMENTAL

Concentrations of copper, cadmium, lead and zinc in iontophoresis-induced sweat, and zinc and copper in serum were determined in 63 volunteers from the Lucas Heights Research Laboratories. Because the use of oral contraceptives raises copper levels in serum (Hambridge and Droegemueller, 1974), the subjects were divided into three groups: 24 males, 13 females taking oral contraceptives and 26 females not taking oral contraceptives. All subjects provided personal histories including dietary mineral supplements taken and type of oral contraceptive used.

Determination of metals in iontophoresis sweat

Sweating was induced on the forearm of subjects by pilocarpine iontophoresis in which pilocarpine was forced into the sweat glands by a small electric current (Stauber and Florence, 1987). The positive electrode was covered with 2 × 25 mm Millipore AP20 prefilters wet with 0.35 ml of 0.2% aqueous pilocarpine nitrate (Sigma Chemical Co.). The negative electrode was covered with 3 × 13 mm prefilters which had been wetted with 0.15 ml of 0.25 M $NaHCO_3$. The current was supplied by 4 × 9-V dry cells.

The inner forearm of the subject was washed with 1% Extran-300 (BDH) detergent and distilled water, and dried with a Whatman 542 filter paper. The iontophoresis electrode was pressed on the washed area and a current of 1.0–1.2 mA was applied for 2 min. The arm was again washed and dried. A preweighed, acid-washed Millipore 25 mm HATF (0.45 μm) membrane filter was placed over the pilcocarpine area, covered with a square of acid-washed parafilm and wrapped tightly in fine polythene domestic wrap.

After 30–45 min, the filter paper, now wet with sweat, was weighed in an acid-washed plastic vial to determine the weight of sweat collected (0.05–0.07 g). The filter paper was shaken for 5 min with 5 ml of 0.02 M $NaNO_3$/

0.002 M HNO_3 (prepared from Merck Supaprur reagents and passed through a Chelex 100 column). The filter paper leachate was decanted into the cell of an EG and G Princeton Applied Research model 384 polarographic analyser, and Zn, Cd, Pb and Cu determined by anodic stripping voltammetry (ASV) at a hanging mercury drop electrode (model 303 Static Mercury Drop Assembly). Metals in a blank filter paper were determined in the same way, and standardisation was by standard addition. When analysed by ASV, the wet-ashed membrane filter showed that the $NaNO_3$-HNO_3 electrolyte extracted more than 90% of the trace metals from the sweat.

Determination of metals in sauna sweat

Copper was also determined in sauna-induced sweat from 41 subjects. After an initial shower, and once the subjects had started sweating, they showered again, rinsed with Milli Q water and dried themselves with Whatman 542 filter papers. They then entered the sauna, and collected sweat dripping from their elbows into acid-washed plastic containers. Samples of sweat from the forearm and underarm were also taken from three male subjects to compare eccrine and apocrine sweat. Consecutive forearm sweat samples from one male and two female subjects were also collected.

All sauna sweat samples were analysed for copper, and some samples for lead and zinc by inductively coupled plasma emission spectroscopy (ICP) and ASV. Analysis of the sweat before and after centrifugation gave identical results. The chloride concentration in sauna and iontophoresis sweat was determined by the method of Florence and Farrar (1971).

The size distribution of the metal species in sauna sweat and iontophoresis sweat was investigated by ultrafiltering it through an Amicon Centricon-10 microconcentrator (10 000 molecular weight cut-off) before ASV analysis of the ultrafiltrate.

Determination of metals in blood and serum

Fasting blood samples from each of 63 subjects were taken on the same day as the sweat collection for determination of serum copper and zinc by ICP. Serum alkaline phosphatase was determined using the Sigma 15 min incubation method (1957). Ceruloplasmin in serum and sweat was measured by its oxidase activity using the *o*-dianisidine dihydrochloride method of Schosinsky et al. (1974).

Lead in whole blood was determined in seven subjects by ASV at a hanging mercury drop electrode (initial potential -0.8 V and final potential -0.2 V). One millilitre of blood was acid digested in $HNO_3/HClO_4$ to strong fumes of $HClO_4$, and diluted with water to 10 ml after the addition of 1 ml of 1 M HCl.

Exchangeable, i.e. loosely bound, copper and zinc in serum was determined in 10 subjects. Two millilitres of serum, diluted to 5 ml with 0.9% NaCl was shaken for 45 h with 20 mg of 50–100 mesh Chelex 100 resin. The Chelex was

washed seven times with 5 ml aliquots of saline, before extraction with 5 ml of 2 M HNO_3 and analysis of the acid extract by ICP. Results were corrected for a saline blank treated in the same way. An acid wash of the Chelex 100 contained $< 4\,\mu g\,Cu\,l^{-1}$ and $1\,\mu g\,Zn\,l^{-1}$.

Determination of copper in urine and hair

Urine samples from two male subjects were collected over a 24 h period in acid-washed plastic containers, acidified with 10 ml of 10% HCl, and analysed for copper by ICP. Copper in urine was expressed per gram creatinine (Tietz, 1982).

Scalp hair samples were collected from the nape of the neck of three male subjects, were cut, and washed with 0.5% Triton X-100 in an ultrasonic bath for 15 min. These samples were then rinsed with distilled water through a pierced Whatman 542 filter paper, rinsed again with methanol and air-dried. Samples were acid-digested in 2 ml of HNO_3 and 0.5 ml of $HClO_4$, and made up to a final volume of 10 ml after the addition of 1.9 ml of 4 M sodium acetate. Copper was determined by ASV (initial potential -1.22 V and final potential 0.14 V).

RESULTS

Metals in sweat

Zinc, cadmium, lead and copper concentrations in sweat from the 63 subjects are shown in Table 1. The concentrations of metals found in sweat were not related to age, dietary mineral supplements or type of oral contraceptive used.

Zinc in female iontophoresis sweat was significantly ($P < 0.05$) higher than in that of males, in agreement with Hohnadel et al. (1973). There was no

TABLE 1

Zinc, cadmium, lead and copper in iontophoresis sweat

Sex	No. of subjects	$\mu g\,l^{-1}$ sweat			
		Zinc	Cadmium	Lead	Copper
Male	24	181 ± 182[a] (25–863)[b]	1.4 ± 2.4 (< 0.5–10)	41 ± 19 (6–87)	103 ± 147 (< 5–673)
Females[c]	26	331 ± 210 (87–836)	2.6 ± 4.9 (< 0.5–18)	24 ± 19 (< 5–66)	29 ± 42 (< 5–146)
Females[d]	13	231 ± 224 (12–767)	2.4 ± 1.5 (< 0.5–5.5)	36 ± 24 (< 5–70)	94 ± 146 (< 5–480)

[a]One standard deviation.
[b]Range.
[c]Females not taking oral contraceptives.
[d]Females taking oral contraceptives.

significant difference in sweat zinc between the females taking and those not taking oral contraceptives, in agreement with Hohnadel et al. (1973), who found no effect of oral contraceptives on levels of nickel, copper, zinc and lead in sauna sweat of 48 healthy subjects.

Cadmium was very low in sweat, as found previously (Stauber and Florence, 1987). The limit of detection for cadmium in sweat using this ASV method is $0.5\,\mu g\,l^{-1}$.

Lead in sweat ranged from < 5 to $87\,\mu g\,l^{-1}$, with averages of 41, 36 and $24\,\mu g\,l^{-1}$ for males and females using/not using oral contraceptives. Lead in the sweat of females not taking oral contraceptives was significantly ($P < 0.01$) lower than lead in male sweat, but was elevated in those taking oral contraceptives.

Results for iontophoresis sweat suggested that copper in the sweat of females ($29\,\mu g\,l^{-1}$) was also significantly lower ($P < 0.05$) than the male copper level ($103\,\mu g\,l^{-1}$), but elevated in those taking oral contraceptives ($94\,\mu g\,l^{-1}$). This sex-based difference for lead and copper in sweat was opposite to that reported by Hohnadel et al. (1973), who found higher lead and copper in sauna sweat of females. In contrast, there was no significant difference between copper in sauna-collected sweat in males and females taking or not taking oral contraceptives (Table 2).

Sauna sweat consistently had a higher copper concentration than iontophoresis sweat, whether analysed by ICP or ASV. If this was due to contamination during collection, we would also expect higher concentrations of other metals. Lead in sauna sweat however was very low ($< 10\,\mu g\,l^{-1}$) and zinc was also lower than in iontophoresis sweat (Table 3). Cadmium in sauna sweat was undetectable.

As thermally-induced sweat may be more concentrated than iontophoresis sweat, chloride was determined in sweat induced by both methods. Chloride concentrations were higher in sauna sweat ($1100 \pm 460\,mg\,Cl\,l^{-1}$) than in iontophoresis sweat ($300 \pm 140\,mg\,Cl\,l^{-1}$). Copper in sauna sweat, even when expressed per mg Cl, was still higher than copper in iontophoresis sweat.

Collection procedures used for sauna sweat were critical in determining the concentration of trace metals. Table 3 shows copper and zinc in successive

TABLE 2

Copper in sauna-collected sweat

Sex	No. of subjects	Copper (μg Cu l^{-1} sweat)
Males	11	325 ± 160[a]
Females[b]	15	540 ± 480
Females[c]	15	700 ± 670

[a]One standard deviation

[b]Females not taking oral contraceptives.

[c]Females taking oral contraceptives.

TABLE 3

Copper and zinc in successive forearm sauna sweat samples

Subject	Sample No.	Chloride ($mg\,l^{-1}$)	Copper		Zinc	
			($\mu g\,l^{-1}$)	($\mu g/mg\ Cl^{-1}$)	($\mu g\,l^{-1}$)	($\mu g/mg\ Cl^{-1}$)
Female A[a]	1	1944	120	0.062	120	0.062
	2	2244	115	0.051	50	0.022
Female B[b]	1	1468	420	0.286	230	0.157
	2	1764	380	0.215	50	0.028
	3	1668	390	0.234	30	0.018
Male D	1	1280	240	0.188	65	0.051
	2	1380	170	0.123	50	0.036
	3	1360	130	0.096	50	0.037

[a]Female A not taking oral contraceptives.
[b]Female B taking oral contraceptives.

samples of forearm sauna sweat from three subjects. In the two females, copper was similar in all samples, while in the male the copper concentration decreased. Zinc decreased in successive sweat samples to a constant level. Chloride in sweat was similar in successive samples.

Table 4 shows copper in sweat collected from three different areas of the arm: the whole arm, the forearm and underarm. Apocrine (underarm) sweat appeared milkier in colour and contained less copper in some subjects than forearm (eccrine) sweat.

Our sweat ultrafiltration experiments showed that all the copper ($100 \pm 8\%$) and most of the zinc ($85 \pm 10\%$) in iontophoresis sweat were present as low molecular weight species (< 10 000 MW), compared with lead, for which only $42 \pm 8\%$ was ultrafilterable. Sauna sweat gave similar results with 100% of copper, 88% of zinc and 30% of lead ultrafilterable.

Ceruloplasmin in the sweat of two females studied was $< 0.5\,IU\,l^{-1}$.

Metals in blood

Table 5 shows copper, zinc, alkaline phosphatase and ceruloplasmin in the serum of the 63 subjects studied. Serum copper and ceruloplasmin for males and

TABLE 4

Copper in sauna sweat collected from whole arm, forearm and underarm of three male subjects

Subject	Copper ($\mu g\ l^{-1}$ sweat)		
	Whole arm	Forearm	Underarm
A	240	170	110
B	240	130	130
C	1360	1590	570

TABLE 5

Zinc, copper, alkaline phosphatase and ceruloplasmin in serum

Sex	No of subjects	Zinc ($\mu g\ ml^{-1}$)	Copper ($\mu g\ ml^{-1}$)	Alkaline phosphatase ($IU\ l^{-1}$)	Ceruloplasmin ($IU\ l^{-1}$)
Males	20	0.89 ± 0.08[a]	1.2 ± 0 3	31 ± 6	104 ± 27
Females[b]	24	0.84 ± 0.08	1 2 ± 0.2	22 ± 8	128 ± 24
Females[c]	12	0 81 ± 0 05	1.9 ± 0.3	20 ± 5	226 ± 67

[a]One standard deviation.
[b]Females not taking oral contraceptives
[c]Females taking oral contraceptives.

females not taking oral contraceptives was similar. In agreement with Hambidge and Droegemueller (1974), serum copper and ceruloplasmin for females taking oral contraceptives was higher than for females not taking them. This increased copper in serum represents an increase in bound copper (to the protein ceruloplasmin) rather than an increase in mobile copper. There was a reasonable correlation ($r^2 = 0.77$) between serum copper and ceruloplasmin in the 63 subjects studied (Fig. 1). Serum zinc and the zinc-containing enzyme in serum, alkaline phosphatase, were similar in males and females. In contrast, a fall in plasma zinc from 0.96 ± 0.13 $\mu g\ ml^{-1}$ to 0.65 ± 0.08 $\mu g\ ml^{-1}$ in women taking oral contraceptives was found by Halsted et al. (1968).

Loosely bound copper and zinc (i.e. readily exchangeable with Chelex 100) in the sweat of 10 subjects is shown in Table 6. Results obtained for mobile copper and zinc by this method depend on the length of time serum is shaken

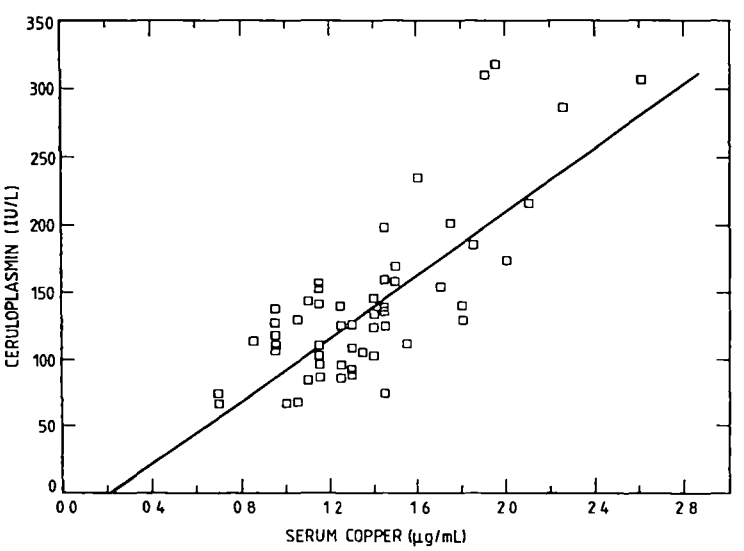

Fig. 1. Ceruloplasmin versus serum copper in 63 subjects studied.

TABLE 6

Exchangeable copper and zinc in serum

Sex	No. of subjects	Serum copper			Serum zinc		
		Total ($\mu g\ ml^{-1}$)	Exchangeable ($\mu g\ ml^{-1}$)	Exchangeable (% of total)	Total ($\mu g\ ml^{-1}$)	Exchangeable ($\mu g\ ml^{-1}$)	Exchangeable (% of total)
Males	5	0.90	0.014	1.7	0.81	0 36	44
Females[a]	3	1.1	0 017	1 5	0.77	0.30	39
Females[b]	2	1.6	0.024	1.5	0.68	0.28	41

[a]Females not taking oral contraceptives.
[b]Females taking oral contraceptives.

with Chelex 100. To detect and compare mobile copper and zinc in the subjects' serum we chose a 45 h shaking time. Loosely bound zinc in serum was significantly higher in males than females ($P < 0.05$), but there was no significant difference between mobile zinc as a fraction of total zinc in males and females taking/not taking oral contraceptives. Mobile copper as a fraction of total copper was similar in all three groups.

To assess the usefulness of sweat as a monitor for occupational exposure to metals, lead in whole blood in seven control subjects and a lead worker was determined (Table 7). The lead worker had the highest lead in blood, 197 $\mu g\ Pb\ l^{-1}$; however, all were within the normal range (Mahaffey et al., 1982). Lead in iontophoresis sweat of the controls was also in the normal range, while lead in the occupationally exposed worker was five times higher. This worker also had very high lead in sauna sweat ($> 2000\ \mu g\ Pb\ l^{-1}$ measured by ASV and ICP). In an attempt to check whether lead contamination from lead dust deep in the pores of the skin was contributing to high lead in sweat, we measured lead in sweat from the unexposed chest of this worker. The lead result was still the same. We also took a sample of lead filings from the workshop and rubbed

TABLE 7

Lead in whole blood and iontophoresis sweat in eight subjects

Sex	Blood Pb ($\mu g\ Pb\ l^{-1}$)	Sweat Pb ($\mu g\ Pb\ l^{-1}$)
F	48	83
M	52	30
F	71	17
M	92	51
F	101	92
M	152	78
M[a]	197	505

[a]Worker occupationally exposed to lead.

them into a control's forearm (under parafilm and fine polythene wrap) for several hours, before taking a sweat sample using the usual skin washing procedure. Lead in this sweat was the same as lead in sweat in the non-exposed arm (189–201 $\mu g\, Pb\, l^{-1}$), but was much higher than usual. This was surprising and suggests lead may diffuse through the skin into the blood very rapidly and be excreted in sweat in a different part of the body within a few hours (Lilley, Florence and Stauber, to be published).

To compare further levels of metals in different body fluids, we studied copper in sweat, blood, urine and hair of three male subjects (Table 8). Subjects A and B had normal sweat copper, while subject C had very high copper in sauna and iontophoresis sweat. Copper in urine of subject C, however, was normal, while serum copper and ceruloplasmin were lower than normal, Subject B also had low serum copper and ceruloplasmin. Hair copper levels ranged from 9 to 54 ppm in the three subjects studied.

DISCUSSION

We found a wide range in concentration of zinc, cadmium, lead and copper in the sweat of the 63 subjects studied. Many factors may influence day-to-day and individual variation in metals in sweat, including diet, fluid intake, exercise, oral contraceptives, atmospheric conditions, occupational exposure and the efficiency of the sweat glands (Robinson and Weiss, 1980).

Widely varying trace metal concentrations in sweat have been reported in the literature (Table 9). We found lower values for zinc, cadmium and copper in sweat than previously reported (Prasad et al., 1963; Hohnadel et al., 1973; Robinson and Weiss, 1980). This may be due either to less contamination or to

TABLE 8

Concentration of copper in body fluids of three male subjects

Copper concentration in body fluid	Subject[a]		
	A	B	C
Iontophoresis sweat (μg Cu mg Cl^{-1})	0.14	0.12	1.05
Sauna sweat (μg Cu mg Cl^{-1})	0.81	0.73	1.62
Urine (μg Cu g creatinine^{-1})	20	ND[b]	22
Hair ($\mu g\, g^{-1}$)	9	42	54
Serum ($\mu g\, ml^{-1}$)	1.4	0.70	0 70
Ceruloplasmin ($IU\, l^{-1}$)	146	67	65

[a]Same subjects as Table 4

[b]Not determined.

TABLE 9

Reported values for zinc, cadmium, lead and copper in sweat collected under different conditions

Sex	Method of sweat induction	Concentration ($\mu g\,l^{-1}$)				Ref.
		Zn	Cd	Pb	Cu	
M	Sauna		84 ± 53			Robinson and Weiss (1980)
M	Sauna				58 ± 13	Mitchell and Hamilton (1949)
M	Sauna	65–1457[a] 31–315[b]			118 ± 100[a] 96 ± 76[b]	Jacob et al. (1981)
M	Sauna	500 ± 480		51 ± 42	550 ± 350	Hohnadel et al (1973)
F	Sauna	1250 ± 770		118 ± 72	1480 ± 610	Hohnadel et al. (1973)
NS[c]	Exercise				1400	Walker and Griffin (1976)
M	Plastic bag	1150 ± 300				Prasad et al. (1963)
M	Plastic bag			7		Rabinowitz et al. (1976)
M	Natural[d]			12		Shiels (1954)
M	Iontophoresis	720 ± 270	< 3	15 ± 10	80 ± 39	Stauber and Florence (1987)
F[e]	Iontophoresis	730 ± 210	< 3	8 ± 5	23 ± 9	Stauber and Florence (1987)
F[f]	Iontophoresis	750 ± 320	< 3	25 ± 11	85 ± 45	Stauber and Florence (1987)
M	Iontophoresis	181 ± 182	1.4 ± 2.4	41 ± 19	103 ± 147	Present work
F[e]	Iontophoresis	331 ± 210	2.6 ± 4.9	24 ± 19	29 ± 42	Present work
F[f]	Iontophoresis	231 ± 224	2.4 ± 1 5	36 ± 24	94 ± 146	Present work

[a]Right arm.
[b]Left arm.
[c]Not stated.
[d]Sweat collected over a few days (not thermally induced)
[e]Females not taking oral contraceptives
[f]Females taking oral contraceptives.

differences in the methods used to induce sweating. We have noted that metals in sweat vary considerably between thermally-induced and pharmacologically-induced sweat. We found higher concentrations of chloride in sauna sweat than in iontophoresis sweat, in agreement with Prasad et al. (1963) who reported that chloride in sweat increased with increasing skin temperature.

Copper in sauna sweat was consistently higher than copper in iontophoresis sweat, even when expressed per milligram of chloride. This is unlikely to be due to contamination as subjects were carefully washed with high purity water before sampling. Further, the females sampled showed similar levels of copper in successive sweat samples. In addition, levels of other metals in all subjects,

for example, zinc and lead in sauna sweat, were lower than in iontophoresis sweat. High copper in sauna sweat could have been caused by different sources of sweat. In the iontophoresis technique, only eccrine sweat from the forearm was collected, whereas in the sauna, sweat was collected from the whole arm, including apocrine sweat from under the arm. However, we analyzed both eccrine and apocrine sweat and found copper to be lower not higher in apocrine sweat. These two different sources of sweat could therefore not account for the higher copper in sauna sweat. Another possibility was that there may be a different size distribution in the copper species in sauna and iontophoresis sweat, for example, more copper in sauna sweat may be associated with colloidal material. However, our ultrafiltration experiments showed that for both sauna and iontophoresis sweat, all the copper is associated with low molecular weight particles ($< 10\,000$ MW). Copper in sweat, like chloride, may increase with increasing skin temperature.

Average lead in sweat is this study was higher than we found previously in a few subjects using the same method (Stauber and Florence, 1987). At present we are evaluating a different method of sweat collection by iontophoresis and have found slightly lower lead values ($\sim 20\,\mu g\,Pb\,l^{-1}$) in three subjects studied so far. Rabinowitz et al. (1976) found $7\,\mu g\,Pb\,l^{-1}$ in sweat using a plastic body bag collection technique. They identified three "pools" of lead in the body — skeleton, blood and soft tissue, the last of which gives rise to lead in sweat.

One important source of trace metals in sweat is blood serum, as labile metals can dissociate from proteins and diffuse through blood capillaries into the sweat glands. However, sweat is not simply an ultrafiltrate of blood plasma, but a product of an active excretory function (Rothman, 1954). The composition of sweat is the difference between what is secreted by the secretory cells and what is reabsorbed by the ductal cells (Sato et al., 1970). Although concentrations of lead and cadmium in serum and sweat are similar, zinc and copper in sweat are much lower than in serum. Exchangeable copper in proteins and low molecular weight copper complexes comprise only 5% of serum, whereas the remaining copper is bound to the protein ceruloplasmin in which 10-15% of Cu is moderately exchangeable. Thirty to forty per cent of plasma zinc is firmly bound to an α_2 macroglobulin, whereas 60–70% is loosely bound to albumin and therefore readily exchangeable. Our ASV results showed that there was a sex-based difference in total zinc and copper in iontophoresis sweat, but not in serum. This suggested that there may be a sex-based difference in mobile zinc and copper in serum. However, when we measured mobile zinc and copper in serum using Chelex 100 we found no significant sex-based difference in mobile zinc (41%) and mobile copper (1.6%).

Abnormal copper/zinc ratios in serum are associated with many diseases, including leukaemia (Underwood, 1977). If there were a correlation between metals in sweat and metals in blood, analysis of sweat samples would be useful in diagnosis. However, we found no correlation between serum copper and sweat copper, nor between serum zinc and sweat zinc. In males, there was evidence that low total copper in serum correlated with high copper in sweat

($r^2 = -0.67$). One male subject in particular excreted large amounts of copper in sweat (possibly due to a high dietary intake of copper from a corroded copper kettle element), while his blood copper level was low.

Sweat may be an important route for excretion of metals, particularly when an individual is exposed to high concentrations. Shiels (1954) showed that lead levels in non-exposed individuals ($\sim 12\,\mu g\,l^{-1}$) rose to $50\,\mu g\,l^{-1}$ when lead was ingested. We found one lead worker with very high levels of lead in forearm and chest sweat, but only slightly elevated levels in blood.

Because high levels of metals were detected in the sweat of exposed individuals when blood metal concentrations were in the normal range, determination of metals in sweat may be useful in screening occupationally exposed workers (Lilley, Florence and Stauber, in preparation). We hope to be able to identify individuals at risk before increases in blood metal concentrations become detectable.

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Paper 13

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THE USE OF SWEAT TO MONITOR LEAD ABSORPTION THROUGH THE SKIN

S.G. LILLEY, T.M. FLORENCE and J.L. STAUBER

CSIRO Division of Fuel Technology, Private Mail Bag 7, Menai, NSW 2234 (Australia)

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ABSTRACT

It is usually assumed that lead can be absorbed through the skin only if it is present as an organolead compound such as tetraethyllead or lead naphthanate. It has been found, however, that finely-powdered lead metal or lead nitrate solution placed on the skin results in rapid absorption of lead, and transport of the metal around the body. The absorbed lead appears in sweat and saliva, but not in blood or urine. The application of 6 mg of lead as 0.5 *M* lead nitrate to the left arm resulted in an increase in lead concentration in pilocarpine-induced iontophoresis sweat samples taken from the right arm, from an initial value of 15–25 $\mu g\ Pb\ l^{-1}$ to $> 300\ \mu g\ Pb\ l^{-1}$ after 2 days. Saliva lead increased from 2.5 to 15 $\mu g\ Pb\ l^{-1}$ in the same period. The rate of lead absorption through the skin increases with increased sweating of the skin. Since no measurable increase in blood lead has been found, the lead must be transported in the plasma and rapidly concentrated into the extracellular fluid pool (sweat and saliva), without significant uptake by the erythrocytes, and with a very low transient concentration in the plasma. Workers occupationally exposed to lead have extremely high levels of lead in sweat even though their lead in blood is only moderately elevated. Lead absorbed through the skin may be eliminated via sweat and other extracellular fluids, and hence not be as great a health hazard as ingested lead, but this will need to be proved by further studies.

INTRODUCTION

Throughout the world there is a growing concern about the use of lead owing to its toxic nature to humans [1–3]. The health effects from lead exposure are well documented, and include alteration of haemoglobin synthesis resulting in anaemia [4], effects on the central and peripheral nervous system [5], and effects on reproduction [6]. The most sensitive indicator for lead exposure is the biochemical change in the haematopoietic tissues in which the biosynthesis of haem is disturbed. At blood lead levels $> 100\ \mu g\ Pb\ l^{-1}$ the inhibition of δ-aminolevulinic acid-dehydratase occurs [7]. At higher blood levels (600–800 $\mu g\ Pb\ l^{-1}$) overall haemoglobin synthesis is affected, resulting in anaemia [3, 4, 8, 9].

Renal damage through lead exposure has been firmly established [8, 9]. The reabsorption of small organic molecules such as amino acids, glucose, uric acid, citric acid and phosphate is decreased. It has been suggested [8, 9] that this is a result of the action of lead on the renal mitochrondria which provide energy for this reabsorption process.

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Lead exposure affects the peripheral and central nervous system. Effects on the former are associated with reduced conduction velocities for motor and sensory fibres [5, 8, 9]. This begins at blood levels between 400 and 500 $\mu g\,l^{-1}$ [5]. The effects on the central nervous system may be varied, but in severe cases the whole brain becomes swollen [9]. Central nervous system effects are most frequent in children, whereas peripheral nervous system effects are associated with adults.

Some other health effects from lead exposure include: (i) endocrine effects [9], where reduced secretion of pituitary gonadotropin has occurred; and (ii) reproductive effects, where lead exposure has been associated with reduced fertility, still-births and neonatal deaths in man [5, 6, 8, 9].

Lead may enter the body through three different routes: inhalation, ingestion and skin absorption. The major routes for intake of inorganic lead are ingestion and inhalation [1, 8, 9, 10]. Skin absorption is usually considered to be minimal, except for organic lead compounds [8]. Most of these conclusions are based on the general population, and not on workers occupationally exposed to lead.

Skin absorption of metal salts varies greatly with different physical parameters [11]. Electrolytes have a higher diffusion constant (up to four orders of magnitude higher) through the skin when the sweat ducts are filled with sweat. This compares with diffusion through the stratum corneum of the skin which occurs when the sweat ducts are dry. These variations in the permeability of the skin may be significant for occupationally exposed workers.

In this study, lead absorption through the skin was investigated. Lead metal or lead salts were placed on the skin of a volunteer and lead concentration in sweat, urine, blood and saliva were monitored.

EXPERIMENTAL

Monitoring exposure to toxic elements via sweat

Apparatus

Electrochemical analysis of sweat, urine, blood and saliva samples was performed with a hanging mercury drop electrode (HMDE) using an EG&G Princeton Applied Research Model 384 Polarographic Analyser, with a Model 303 Static Mercury Drop Assembly, and a Model 305 stirrer.

Reagents

All supporting electrolyte solutions used were prepared from BDH Aristar reagents and water passed through a Milli-Q system.

Analytical procedure

Samples were deaerated for 5 min before anodic stripping voltammetry (ASV). The samples were stirred during deposition and stripped in quiescent solution. The stripping signals were recorded in a differential pulse mode with

the following instrumental settings: deposition potential, −0.750 V versus Ag/AgCl; deposition time 600 s (sweat), 300 s (blood, urine, and saliva); drop time 0.4 s; scan increment 2 mV; pulse height 0.025 V; drop size medium; stirrer speed slow. The lead ASV peak occurred at −0.446 V versus Ag/AgCl. Quantitative analysis of the analyte concentrations was achieved by means of a single standard addition, approximately doubling the analyte concentration of the sample.

General procedure for sweat collection and lead determination

Preliminary washing of the arm with a chelating detergent, the pilocarpine iontophoresis stimulation of sweating, and the collection of the sweat on a membrane filter have been described elsewhere [12].

The Millipore 25-mm HATF (0.45 μm pore size) membrane filter paper, wet with sweat, was placed in an acid washed plastic vial, and shaken for 60 s with 5 ml of 0.2 *M* HCl. The filter paper leachate was emptied into an ASV cell, and the lead concentration was determined via ASV.

Procedure for monitoring lead in sweat and lead in blood

A pilot study was carried out on nine workers occupationally exposed to lead in a lead storage battery factory. Blood and sweat samples were collected and the lead concentration for each sample was determined by ASV. The object of the study was to see if there was a relationship between the lead concentration in sweat and blood.

Procedure for determining lead contamination of the epidermis

Lead powder (20 mg, −100 mesh) was placed on a Millipore 25-mm HATF (0.45 μm) membrane filter and the filter paper was placed on the flexor surface of the *left* arm of a healthy adult male volunteer. It was held firmly in place by wrapping the arm in clear household polyethylene film, and remained on the arm for 6 h each day, for 4 days. At the beginning of each day, the membrane filter was removed, and the area of the skin where the lead powder had been was washed with 5% Extran-300 (BDH) detergent to remove all surface lead present, then rinsed with Milli-Q water, and dried with a Whatman 542 filter paper. Sweat was induced on the area of skin where the lead powder had been, and lead in sweat determined by ASV. An area of skin on the right flexor surface of the arm not exposed to lead was induced to sweat, and lead in the sweat was determined. Sweat samples were taken 1 day before lead was placed onto the skin of the volunteer, then each day up to the fifth day after the lead had been removed.

Procedure for monitoring lead absorption through the skin into various body fluids

One healthy adult male volunteer had 6.2 mg lead applied to his skin. This was achieved by dispensing 60 μl of 0.5 M $Pb(NO_3)_2$ solution onto a Millipore 25 mm HATF (0.45 μm) membrane filter. The filter paper was applied to the *left* arm of the male volunteer and covered with a square of acid-washed parafilm. The filter paper was firmly held to the arm by wrapping the arm in clear household polyethylene film. The lead remained on the arm for 24 h. Sweat samples were collected periodically from the *right* arm and the lead concentration in each sample was determined.

In similar experiments, blood, urine and saliva were sampled in addition to sweat and the lead concentration in each was determined.

Lead in urine was determined directly using a simple clean-up procedure [13] for removing organics in urine to prevent interference with lead determination by ASV.

Lead in blood was determined by a procedure in which 500 W ultra-violet (UV) irradiation was used to destroy organic matter and release total bound heavy metals in blood [14]. Seven hundred microlitres of blood was mixed thoroughly with 100 ml of 0.08 M nitric acid and 0.005 M hydrogen peroxide before UV irradiation.

Lead in saliva was determined using the UV irradiation technique developed for blood with a minor modification. Two and a half millilitres of saliva was added to 25 ml of Milli-Q water containing 0.08 M nitric acid and 0.005 M hydrogen peroxide before UV irradiation.

The use of the Macroduct sweat collection system for lead in sweat

To simplify the sweat collection for the determination of toxic elements in sweat, the Macroduct Sweat Collection System, Model 3700-Sys [15] was tested. In this iontophoresis system pilocarpine induced sweat is collected in a coiled plastic capillary tube above a collection cup. The results were compared with the Stauber and Florence method [12]. The washing procedure developed by Stauber and Florence was used in conjunction with the sweat stimulation and collection procedure developed for the Macroduct [15].

Accurate volumes of sweat (60–80 μl) were collected in the following manner:

(a) the Macroduct containing the blue, water-soluble indicator dye on the collection cup, and (b) Macroduct which has had the dye washed off with Milli-Q water.

The sweat samples were then dispensed into the electrolyte (5 ml of 0.02 M HCl) in an ASV cell, and the lead concentration was determined via ASV.

TABLE 1

Comparison of lead in sweat and lead in blood of occupationally-exposed workers

Pb worker	μg Pb/l sweat[a]	μg Pb/l blood
1	2320	952
2	4080	745
3	4730	621
4	1080	559
5	395	559
6	489	518
7	71	352
8	17700	207
9	307	186

[a] Lead concentration in sweat for a non-exposed person 41 (s = 19) μg Pb/l sweat [19]

RESULTS AND DISCUSSION

Monitoring exposure to toxic elements via sweat

Blood and sweat samples were collected from nine workers occupationally exposed to lead, and the lead concentration for each sample was determined (Table 1). There was no relationship between lead in blood and lead in sweat. The extremely high result for sweat from lead worker 8 may be due to lead contamination in the epidermis, which resisted our normal washing procedure.

A study will be carried out on a large number of lead workers to determine a statistically reliable correlation (if any) between lead in blood and lead in sweat.

Lead contamination to the epidermis

The results of the experiment in which lead metal was placed on the left arm are shown in Table 2. Lead in sweat (from the area of skin exposed to lead) increased over the first 4 days to a level of 4700 μg Pb l^{-1}, compared with the control (area of skin on the opposite arm, unexposed to lead), which yielded 108 μg Pb l^{-1} on the fourth day. This indicates that lead may be absorbed into the internal layers of the skin and sweat ducts, since strong chelating detergent (Extran 300) did not remove all the absorbed lead. This could explain the results for lead worker 8 (Table 1), where absorption of lead into the internal skin layer from external skin exposure to lead metal or lead compounds at his workplace, resulted in high lead in sweat but relatively low lead in blood (compared with the other lead workers surveyed). Other workers may have washed more frequently, preventing lead absorption through their skin.

Table 2 also shows that lead concentration increased in sweat from the

TABLE 2

Lead contamination in the epidermis of a male volunteer

Day	Left arm (μg Pb/l sweat)	Right arm (μg Pb/l sweat[a])	Comments
1	43	34	1 day before Pb left arm
2	339	94	Day 1 Pb on left arm
3	1412	53	Day 2 Pb on left arm
4	2270	< 20	Day 3 Pb on left arm
5	4765	108	Day 4 Pb on left arm
6	1314	44	Day 1 after Pb removed
7	225	42	Day 2 after Pb removed
8	131	37	Day 3 after Pb removed
9	63	28	Day 4 after Pb removed
10	29	14	Day 5 after Pb removed

[a] Average unexposed lead concentrations in the sweat of this volunteer was 26 (s = 15) μg Pb/l sweat.

opposite arm to where the lead was placed. Significantly higher lead in sweat from the control arm was found on days 2 and 5 (94 and 108 μg Pb l^{-1}, respectively). This was almost four times the mean lead in sweat found for this volunteer when he was not exposed to lead. This result indicates that lead enters the body through the skin, and is then transported to different regions of the body through a circulatory system, and appears in body fluids such as sweat.

The amount of lead absorbed into the skin appears to depend on the extent to which the person sweats. When the HATF membrane filter containing the lead powder was moist from the volunteer's sweat (days 2, 3 and 5 of Table 2) the control sweat resulted in a significantly higher lead in sweat. When no moisture was apparent on the HATF filter paper (day 4 of Table 2) the control sweat was not significantly higher in lead. These results may be explained by previous studies where the following was found:

(i) Low-molecular-weight molecules diffuse through different regions of the skin at varying rates. The diffusion coefficients for these molecules in the stratum corneum were four orders of magnitude lower than in the sweat ducts when they were filled with sweat [11].

(ii) The sweat duct contributes ~ 70% of the total skin absorption of strong electrolytes [16].

Monitoring lead absorption through skin via sweat, and other body fluids

To confirm that inorganic lead absorbs through the skin, an experiment was carried out where known amounts of lead as $Pb(NO_3)_2$ solution were placed onto the skin of a volunteer's left arm. Lead absorption was monitored by analysing the lead in sweat on his opposite arm (Fig. 1).

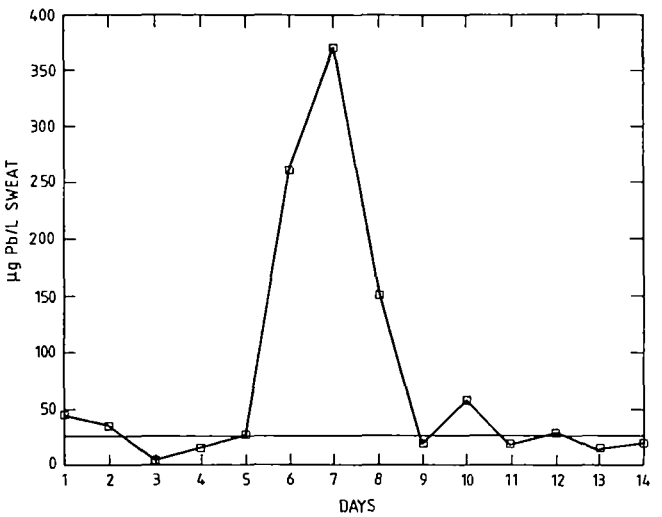

Fig. 1. Lead in sweat. $Pb(NO_3)_2$ placed on the skin of the left arm on day 5 and removed on day 6. Average lead in sweat when not exposed to $Pb(NO_3)_2$, indicated by horizontal line, was 26 $\mu g\ Pb\ l^{-1}$ with a standard deviation of 15 $\mu g\ Pb\ l^{-1}$.

The increase in lead in sweat taken from the right arm from an initial average concentration of 25 $\mu g\ Pb\ l^{-1}$ to 261 $\mu g\ Pb\ l^{-1}$ (day 6, 21 h of lead on the skin of the left arm) and 370 $\mu g\ Pb\ l^{-1}$ (day 7, 1 day after the lead was removed from the skin) indicates that lead is absorbed through the skin and transported around the body via a circulatory system. The dramatic increase in lead in

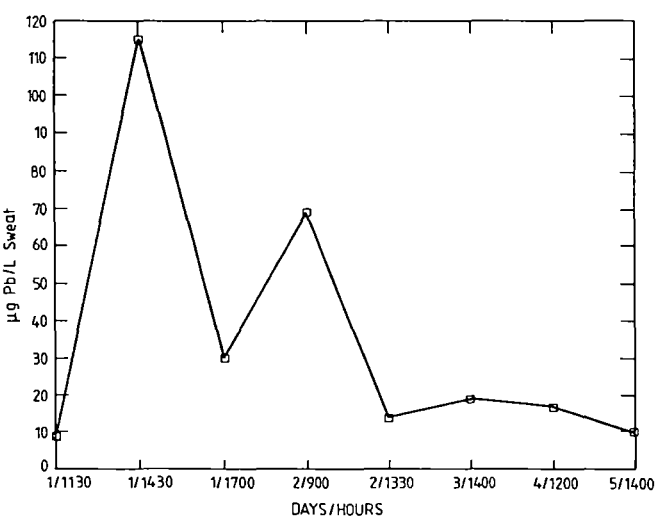

Fig. 2. Lead in sweat. $Pb(NO_3)_2$ placed on the skin of the left arm on day 1/1200 h and removed on day 2/1200 h. Average lead in sweat when not exposed to $Pb(NO_3)_2$ was 14 $\mu g\ Pb\ l^{-1}$ with a standard deviation of 4 $\mu g\ Pb\ l^{-1}$.

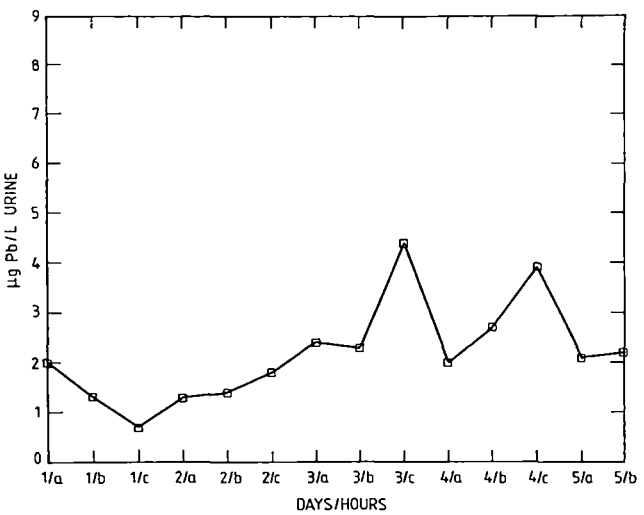

Fig. 3. Lead in urine. $Pb(NO_3)_2$ placed on the skin of the left arm on day 1/1200 h and removed on day 2/1200 h. The average lead in urine when not exposed to $Pb(NO_3)_2$ was 2.2 $\mu g\,Pb\,l^{-1}$ with a standard deviation of 0.9 $\mu g\,Pb\,l^{-1}$. Note: a = 0000–1000; b = 1000–1500; c = 1500–2400.

sweat after the $Pb(NO_3)_2$ was placed onto the skin may indicate that skin absorption of lead occurs rapidly.

Although the lead was on the skin, and a polyethylene film covered the area where the lead was placed, the major transport route for the lead would be through the sweat ducts because of profuse sweating, and the relatively high

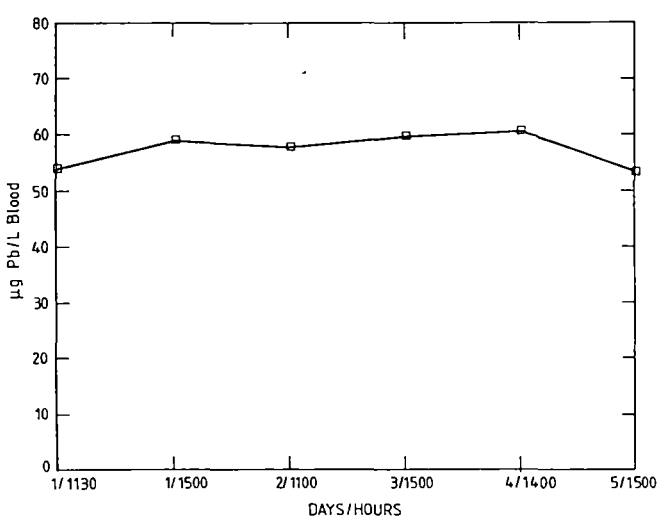

Fig. 4. Lead in blood $Pb(NO_3)_2$ placed on the skin of the left arm on day 1/1200 h and removed on day 2/1200 h. The average lead in blood when not exposed to $Pb(NO_3)_2$ was 58 $\mu g\,Pb\,l^{-1}$ with a standard deviation of 3 $\mu g\,Pb\,l^{-1}$.

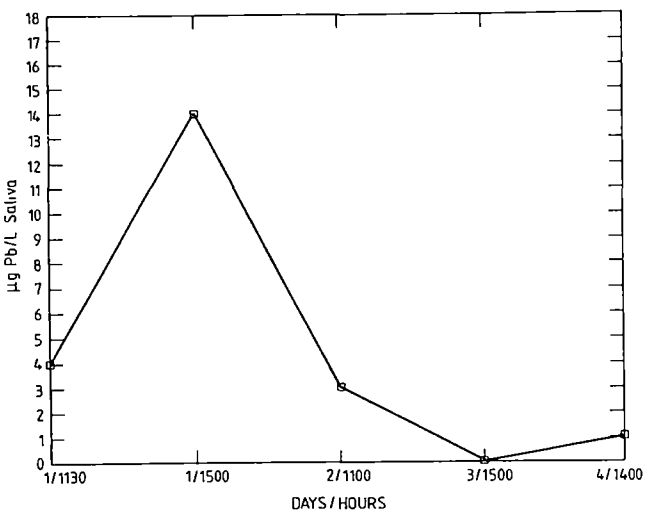

Fig. 5. Lead in saliva. $Pb(NO_3)_2$ placed on the skin of the left arm on day 1/1200 h and removed on day 2/1200 h. The average lead in saliva when not exposed to $Pb(NO_3)_2$ was 2.2 $\mu g\,Pb\,l^{-1}$ with a standard deviation of 3 $\mu g\,Pb\,l^{-1}$.

diffusion coefficient for lead in sweat. The sweat ducts would have been filled with sweat and evaporation of the sweat was prevented because of the covering on the arm. The volunteer confirmed that the area of skin under the polyethylene film was continually moist while it was on the arm, indicating that the sweat ducts would have been filled with sweat. Once the lead and the covering were removed from the skin the transport route through the sweat ducts would have ceased, but slow transport of lead through the stratum corneum would still be active and would account for the higher lead in sweat found 1 day after the lead was removed. This would be due to the "hold up" time of the lead transported across the stratum corneum by its lower diffusion coefficient.

Experiments in which other body fluids including urine, blood, and saliva were monitored together with sweat were carried out to map the progress of lead through the body after it was absorbed through the skin.

Typical results are shown in Figs 2–5 (for sweat, urine, blood, and saliva, respectively). The increase in lead in sweat taken from the right arm from an initial average level of 15 $\mu g\,Pb\,l^{-1}$ to 115 $\mu g\,Pb\,l^{-1}$ after 3 h exposure of the left arm to lead, confirmed that lead can be absorbed through the skin and transported around the body. Lead in urine (Fig. 3) and lead in blood (Fig. 4) however did not increase significantly after placing lead on the skin of the volunteer.

The only body fluid other than sweat to show an increase in lead was saliva (Fig. 5). The increase occurred at the same time that the highest lead in sweat was recorded, i.e. day 1/1500 h or 3 h after the lead was placed on the skin. It was surprising that there was no measurable increase of lead in blood and urine while lead in sweat and saliva increased. A likely explanation is that lead is transported in the plasma, but is removed so quickly into the extracellular fluid

pool (sweat and saliva) that its mean residence time in the plasma is very short, and little lead enters the erythrocytes. Chamberlain [17] found that the transport time for lead from plasma to the extracellular fluid compartment was ~1 min.

The use of the Macroduct sweat collection system for lead in sweat determination

The Macroduct system (Wescor Inc., Utah, U.S.A.) is a sweat collection device for the detection of cystic fibrosis in infants [18]. This instrument was tested to see whether it could be used for the determination of trace levels of lead in sweat. The results are shown in Table 3.

The washed Macroduct gave lower lead concentrations than (i) the Macroduct containing dye (Table 3) (the dye which is used to show the amount of sweat in the collection tubing may contain lead as an impurity); and (ii) the method of Stauber and Florence (Table 3). The larger population surveyed by Stauber and Florence may give a truer reflection of the average lead in sweat of the population than the smaller population surveyed using the Macroduct, or, alternatively, some of the lead in the sweat collected by the Macroduct is lost by surface adsorption to the capillary tube, giving a low result.

Sauna sweat with measured levels of lead was introduced into the Macroduct capillary and allowed to stand for 20–30 min (simulating the time to

TABLE 3

Comparison of lead in sweat via different collection methods

Method	No. of males	Lead (μg/l sweat)
Stauber and Florence [19]	24	41[a] (± 19)[b]
Macroduct (containing dye)	1(3)[c]	57 (± 21)
Macroduct (washed free of dye)	3(13)	13 (± 7)

[a] Average.
[b] One standard deviation
[c] No. of samples

TABLE 4

Comparison between known lead in sauna sweat and sauna sweat placed into a Macroduct capillary for 20 min

Sauna sweat control ($\mu g\ Pb\ l^{-1}$)	Sauna sweat in Macroduct for 20 min ($\mu g\ Pb\ l^{-1}$)
84	89
64	63
67	70
72	77

collect 60–80 μl of pilocarpine iontophoresis induced sweat) before analysis for lead. The results (Table 4) indicate that there are no losses of lead to the capillary walls. This signifies that the Macroduct (washed free of dye) would be useful for collecting sweat for lead determination.

CONCLUSION

Inorganic lead is rapidly absorbed through the skin and enters the cardiovascular system. It may, however, be eliminated via sweat and other extracellular fluids and hence not be as great a health hazard as ingested lead. Further studies are planned using animals to determine where skin-absorbed lead is deposited in the body, and to establish the extent of any health hazard to lead workers.

ACKNOWLEDGEMENTS

The authors are indebted to the Australian National Occupational Health and Safety Commission for providing the financial support for this work. The authors acknowledge Dr Kelvin Wooller and Peter Coomits for their assistance in obtaining the lead in blood results from occupationally exposed lead workers. S.G. Lilley wishes to thank Jesus Christ Yahweh Elohim for his encouragement during this research work.

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Letters to the Editor

SKIN ABSORPTION OF LEAD

SIR,—Skin absorption is not usually considered a significant mode of uptake of lead, unless the lead is present as a lipid-soluble compound such as tetraethyl lead or lead naphthenate.[1] We have found that inorganic lead can be absorbed through skin and rapidly distributed throughout the body. The figure shows the appearance of raised levels of lead in sweat samples taken from the right arm, after a 25 mm diameter membrane filter, to which 60 µl of 0·5 mol l lead nitrate solution (6 mg Pb) had been added, was placed on the left arm, covered with a square of 'Parafilm', wrapped firmly with plastic lunch-wrap, and left on the arm for 24 hours. Pilocarpine iontophoresis sweat samples,[2] taken from the right arm periodically before and after the application of lead, were analysed for lead by anodic stripping voltammetry.[2,3] The sweat lead concentration continued to rise even after the lead was removed, and increased from the normal value of 15–30 µg l to over 350 µg l after 2 days (figure). Similar results were obtained when 100 mesh lead metal or oxide powder were used in place of lead nitrate. Both the increase in sweat lead, and the time taken for skin transport, depended on the extent of skin sweating. If the arm was kept cool and dry, little skin transfer occurred; with profuse sweating, large increases in sweat lead occurred rapidly. These results can be explained by a diffusion model involving simultaneous diffusion of lead ions through a filled sweat duct (rapid) and through the stratum corneum (slow).[4]

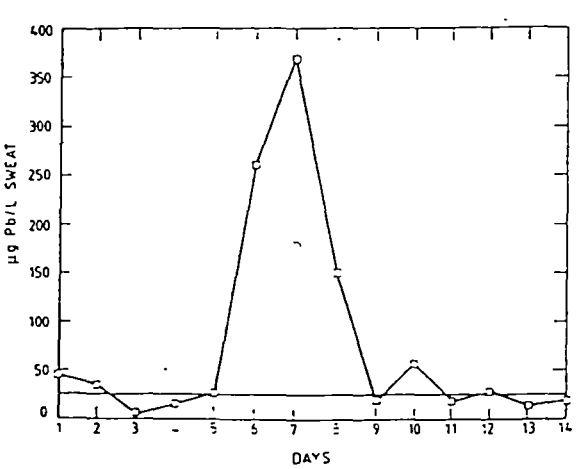
Although the skin absorption of lead gave rise to increased lead in sweat, similar increases in blood and urine were not observed. For the volunteer whose results are shown in the figure neither urine (2 µg l) nor whole blood (55 µg l) lead changed significantly from normal throughout the experiment. Saliva lead concentrations did, however, increase from 2·5 to 15 µg l, and the increase paralleled that of lead in sweat. Since raised sweat lead values were found within 2 h of the application of lead, blood transport must be involved. However, no measurable increase in blood lead was found so the lead must be transported in plasma and rapidly concentrated into the extracellular fluid pool of sweat and saliva without significant uptake by erythrocytes, and with a very low transient concentration in the plasma. The behaviour is very different from that of ingested lead.[5]

Skin absorption of lead may be important in industries such as lead battery manufacture and lead smelting, where the area of a worker's skin covered with lead dust may be orders of magnitude greater than that used in our experiments. Although face masks are usually worn when handling lead powders, few precautions are taken to prevent occupational skin exposure. A survey of workers in a lead battery factory yielded iontophoresis sweat lead values as high as 800 µg l, even after their skin had been scrubbed with a chelating detergent and washed thoroughly before the sweat sample was taken and when they had been away from work for several days. These men had only moderately raised lead in blood (300–400 µg l).

Experiments with a stable lead isotope are underway to find out the fate of skin-absorbed lead and if skin exposure can add significantly to the body burden of this metal.

CSIRO Centre for Advanced Analytical Chemistry, Menai, NSW 2234, Australia

T. M. FLORENCE
S. G. LILLEY
J. L. STAUBER



Lead concentrations in sweat.

Lead nitrate solution placed on left arm on day 5 and removed 24 h later on day 6. Lead was measured in sweat from right arm. Horizontal line represents average background lead-in-sweat value found for this volunteer during the previous 3 months.

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because of less-than-safe handling. Such undetected chronic exposure should have evoked parkinsonism by now, 20 years or more after the introduction of paraquat.

(5) J. D. Parkinson may have been the first to describe the disease fully, but the ancient physicians were well aware of the clinical features; Galen described the main triad of symptoms, relating them to advanced age.

It thus seems unlikely that any postulated environmental agent in IPD is directly related to modern industry or to items such as the banana, potato, or coffee bean that were unknown in Europe before the "New World" was discovered in 1492.

A direct relation between an MPTP-linked exogenous toxin and IPD, as suggested by animal studies, remains tentative therefore. However, because the incidence and prevalence of IPD cannot be explained by a mere genetic aetiology or by age-related deterioration of previously damaged nigrostriatal function alone, the search for risk factors should be extended. Such a search should focus on life styles decades before the onset of disease, and take into account geographic movements, drinking and eating habits, and drugs prescribed, further family and twin studies could also prove helpful.

Neurological Clinic
and Institute for Medical
Statistics and Documentation,
Medical University of Lübeck,
D-2400 Lübeck, West Germany

P. VIEREGGE
D. KÖMPF
H. FASSL

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NEUROTOXICITY OF MANGANESE

SIR,—As part of a study of the possible involvement of manganese[1] in the neurological disorder found in some aborigines living on Groote Eylandt, Australia,[2-4] we investigated the manganese catalysis of dopamine oxidation. Detailed kinetic measurements suggest that, in the presence of manganese, oxidation of dopamine by oxygen proceeds via a manganese (II)/(III) redox couple, the superoxide radical, and a semiquinone intermediate. Neurotoxicity of manganese results from the depletion of dopamine and the production of the neurotoxins dopamine quinone and hydrogen peroxide.

A wide range of compounds were studied for their ability to inhibit dopamine oxidation at pH 7·5 in phosphate buffer (table). Ascorbic acid (vitamin C) and thiamine (vitamin B_1) were the most effective inhibitors, completely inhibiting oxidation in both the presence and absence of manganese. The inhibiting effect of dehydroascorbic acid shows that the mechanism is not simply chemical reduction, although ascorbic acid reduced dopamine quinone back to dopamine. Few of the compounds tested gave a significant reduction in dopamine oxidation rate, and all those that did (table) were either positively charged (eg, thiamine) or had a positively charged group in the molecule (eg, ascorbic acid). This suggests that the inhibitors react with and stabilise the intermediate semiquinone free radical, thus effectively quenching dopamine oxidation. Thiamine-neurotransmitter interaction has been observed in man by Freye and Hartung.[5]

Dopamine loss is known to be associated with manganese toxicity,[1] which causes symptoms similar to those of Parkinson's disease. Since many of the Groote Eylandt aborigines are likely to be deficient in ascorbic acid (poor diet) and thiamine (alcohol intake), it is possible that depletion of these vitamins exacerbates the disease. A detailed study of the vitamin status of the aborigines, and possible vitamin supplementation, is planned for this year.

INHIBITION OF MANGANESE-CATALYSED OXIDATION OF DOPAMINE*

Inhibitor	Concentration (mmol/l)	Reduction in oxidation rate (%)†
Ascorbic acid	0·05	100
	0·01	63
Dehydroascorbic acid	1	100
	0·1	25
Thiamine	1	100
	0·1	47
Folic acid	1	74
Biotin	1	53
Dodecylpyridinium chloride	1	46
Pyridoxine	1	44

*Dopamine 0·3 mmol/l; Mn^{2+} 0·1 mmol/l, phosphate buffer pH 7·5 20 mmol/l, 37°C, air bubbling
†Dopamine concentration measured at 490 nm.

Parkinson's disease may result from early exposure to chemical toxins that deplete dopamine or block dopamine receptors.[6] A curious finding is the inverse relation between Parkinson's disease and tobacco smoking.[6,7] Heavy cigarette smokers have a significantly lower incidence of the disease than non-smokers.[7] Nicotine has two electrophilic nitrogen atoms in the molecule, and perhaps it acts, like ascorbic acid and thiamine, as a free radical acceptor and an inhibitor of dopamine oxidation. This raises the possibility of these vitamins acting as prophylactics towards Parkinson's disease and other diseases involving loss of neurotransmitters.

CSIRO Division of Fuel Technology,
Menai, NSW 2234, Australia

T. M. FLORENCE
J. L. STAUBER

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BROMODIALONE POISONING

SIR,—Dr Greef and colleagues (Nov 28, p 1269) report laboratory findings in two children accidentally poisoned with bromodialone. In the first child, the prothrombin time was greater than 120 s, none of the vitamin-K-dependent factors having an activity of less than 30%; in the second case, the factor assays were more consistent with the prothrombin time reported as well as the partial thromboplastin time. With the factor assays as reported in the first case I would not have expected a prothrombin time longer than 20 s. That the first child was not as severely affected as the second is borne out by the rapid recovery after specific therapy.

Hemostasis and Coagulation Laboratory,
William Beaumont Hospital,
Royal Oak, Michigan 48072, USA

J. N. SHANBERGE

⁂ This letter has been shown to Dr Greeff, whose reply follows.—ED. L.

SIR,—Dr Shanberge's comments on the apparent discrepancy between the initial prothrombin time, partial thromboplastin time (PTT), and the levels of vitamin K factors are correct. However, his view that the first case was not as severe as the second is not borne out by the clinical severity of the generalised bleeding diathesis and the apparently spontaneous bleed into the child's neck, necessitating endotracheal intubation. The response to treatment can be attributed to aggressive clotting factor and vitamin K_1

Paper 17

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MANGANESE IN SCALP HAIR: PROBLEMS OF EXOGENOUS MANGANESE AND IMPLICATIONS FOR MANGANESE MONITORING IN GROOTE EYLANDT ABORIGINES

J.L. STAUBER and T.M. FLORENCE

CSIRO Centre for Advanced Analytical Chemistry, Division of Fuel Technology, Private Mail Bag 7, Menai NSW 2234 (Australia)

(Received November 11th, 1988; accepted December 1st, 1988)

ABSTRACT

The use of scalp hair to monitor manganese was studied as part of an investigation of manganese intoxication amongst a group of Aborigines living on manganese-rich soil on Groote Eylandt, in the Northern Territory of Australia High scalp-hair manganese values were due largely to manganese from exogenous sources. Manganese (IV) dioxide in dust, trapped in hair, was reduced by the components of sweat, leading to the diffusion of manganese (II) into the hair shaft. At least 15 μg Mn g^{-1} hair could be incorporated into hair via this exogenous route.

To overcome the problems of manganese contamination, the ability of a number of leaching agents to remove exogenous manganese selectively from hair was tested. Measurements of manganese along the length of hair strands were extrapolated back to zero length to estimate the amount of manganese in the hair as it emerged from the scalp. Using this extrapolation technique, Aborigines on Groote Eylandt had a mean scalp-hair manganese of 16 ppm. Aborigines in non-manganese areas had 2 ppm manganese in hair. Caucasians living in the same manganese-rich area had 2.5 ppm manganese in hair, compared to 0.5 ppm manganese in non-manganese areas. Measurements of manganese in hair and blood of Groote Eylandt Aborigines showed that the population had a high exposure to manganese, but did not distinguish between those individuals affected/unaffected by the neurological condition, Groote Eylandt Syndrome

INTRODUCTION

Aborigines living at Angurugu, a small township on Groote Eylandt (GE), in the Gulf of Carpentaria, Australia, have a high incidence of neurological disturbances referred to as the Groote Eylandt Syndrome (Cawte, 1984). It has been suggested that GE Syndrome may be caused by environmental manganese, as similar neurological symptoms have been found in South American miners suffering from manganese poisoning (Cotzias et al., 1968). The Angurugu population is exposed to an environment naturally high in manganese. Soil samples collected from the township contain up to 4% manganese as MnO_2 in the mineral pyrolusite (Florence et al., 1987). The Groote Eylandt Mining Company (GEMCO) has mined manganese since the early 1960s.

Manganese, absorbed through the lungs and gastrointestinal tract is cleared

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quickly from the blood and deposited in tissue, particularly the liver, kidney, pancreas and brain. The neurotoxicity of manganese may result from its ability to catalyse the oxidation of dopamine, causing depletion of this neurotransmitter (Donaldson, 1987; Florence and Stauber, 1989). Manganese is excreted in the faeces via bile, with very little being excreted in the urine (Piscator, 1979; Keen et al., 1984). Because manganese has a short biological half-life (4 days for the fast fraction (30%) and 40 days for the slow fraction), monitoring manganese at the low levels found in blood and urine can only indicate relatively recent exposure.

The most convenient record of manganese exposure over a few months is the manganese concentration in scalp hair, which grows at a rate of 1–2 cm per month (Hopps, 1977). Problems in using scalp hair to monitor exposure include pre-treatment of the hair before manganese analysis, and the need to distinguish between endogenous (manganese that enters the hair via the blood supply to the hair follicle) and exogenous sources of manganese, including dust and sweat (Chittleborough, 1980).

To determine whether the Aboriginal population of Angurugu had a significantly higher manganese intake than other populations, we monitored manganese in scalp hair of over 100 inhabitants (Stauber et al., 1987). We found that Aborigines living at Angurugu had much higher hair manganese than those from non-manganese areas, e.g., Gove Peninsula. Although Caucasians at Angurugu had lower hair manganese than Aborigines, it was much higher than hair manganese for Caucasians living in Sydney. To interpret these scalp-hair manganese results, it was necessary to determine the contribution of exogenous manganese to the total manganese measured in hair. In this paper we examine sources of exogenous manganese in hair, including the solubility of manganese from Groote Eylandt roadside dust in sweat and its incorporation into Aboriginal and Caucasian hair. A number of methods to overcome the problem of exogenous manganese in hair were also examined.

EXPERIMENTAL

Hair collection and analysis

Scalp hair (collected from the nape of the neck) and pubic hair were cut and washed in 25 ml of non-ionic detergent (0.5% Triton X-100) in an ultrasonic bath for 15 min. Hair samples were rinsed with distilled water through a pierced Whatman 542 filter paper, rinsed with methanol and air-dried before manganese determination by neutron activation analysis (NAA). Dust washed from the hair was collected and analysed for manganese by inductively coupled plasma emission spectroscopy (ICP). To ensure that all the dust was removed by the washing procedure, GE roadside dust was rubbed into the hair, shaken overnight and the hair washed in the same manner. Analysis showed that the washing procedure effectively removed all surface dust, and gave reproducible results for multiple hair samples from the same person.

Endogenous and exogenous manganese in hair

Manganese in dust trapped in the hair before sampling, may be solubilized in sweat, incorporated into the hair shaft and measured as endogenous manganese. To determine the extent to which this exogenous source of manganese contributes to total manganese in hair, experiments using both Aboriginal and Caucasian hair were conducted.

Solubility of manganese from dust in sweat

Roadside dirt from Angurugu was collected and sifted through a 100 mesh sieve. It contained 4.4% Mn. Sweat from two sources was used in the following experiments:

(i) Synthetic sweat (pH 5.2). The composition of synthetic sweat is shown in Table 1 (Stauber and Florence, 1987). Synthetic sweat was used with/without the addition of 0.4 mg ml^{-1} protein mixture (containing 0.05 g albumin ml^{-1} and 0.03 g globulin ml^{-1}) and sebum (0.025 ml ml^{-1} sweat). The sebum stock solution contained 3% palmitic acid, 5% palmitic acid methyl ester, 2% linoleic acid, 0.1% cholesterol and 0.2% cholesterol hexanoate, dissolved in 10 ml of warm ethanol.

(ii) Sauna sweat. In a sauna, forearm sweat was collected into acid-washed containers (Stauber and Florence, 1988).

The solubility of manganese from dust in sweat was determined by shaking a large excess of Angurugu roadside dust in sweat for 1–6 days. The solution was filtered through an acid-washed 0.45 μm membrane filter and manganese in the filtrate determined by ICP.

As most of the manganese in Angurugu dust was present as MnO_2, the ability of sweat to dissolve manganese from a standard suspension of freshly prepared MnO_2 (Stauber and Florence, 1985) was measured using Milli-Q water, sauna sweat, synthetic sweat, and synthetic sweat without lactic acid. Sweat was shaken overnight with 0.1 ml of 0.1 *M* MnO_2 suspension, followed by centrifugation for 20 min in an Amicon CF-25 Centriflo membrane ultrafilter cone, which retains molecules greater than 25000 MW. Manganese (II) in the ultrafiltrate was determined by ICP. To identify which component of sweat was reducing Mn(IV) to Mn(II), the experiment was repeated using solutions of lactic acid, NaCl, urea, histidine-HCl and citrulline in Milli-Q water (pH 5.2) at concentrations found in synthetic sweat.

Lipid solubility of manganese in sweat

Ten millilitres of synthetic sweat ($\pm$ 0.4 mg protein mixture ml^{-1}) containing 5 μg Mn(II) l^{-1} was shaken for 5 min in 10 ml of n-octanol. After standing for 15 min, the aqueous phase was separated and manganese in the aqueous phase determined by ICP.

TABLE 1

Composition of synthetic sweat[a]

Compound	Concentration (mg l^{-1})
NaCl	800
KCl	912
$CaSO_4 \cdot 2H_2O$	200
$MgSO_4 \cdot 7H_2O$	88
$NaH_2PO_4 \cdot 2H_2O$	31
$FeSO_4 \cdot 7H_2O$	11
Lactic acid	1472
Urea	300
Histidine-HCl	240
Alanine	920
Glycine	280
Citrulline	152
Urocanic acid	76

[a] Synthetic sweat was adjusted to pH 5.2 with 0.2 *M* Na_2CO_3.

Incorporation of manganese from sweat into hair

To determine how much manganese could be incorporated into hair from exogenous sources, Aboriginal and Caucasian hair was shaken for up to one month in sweat containing radiolabelled manganese (^{54}Mn(II) or $^{54}MnO_2$). The total manganese concentration in sweat ranged from < 1 to 5000 μg Mn l^{-1}. After shaking, the hair was washed as usual in Triton X-100, dried and acid digested in 2 ml 15 *M* HNO_3 and 0.5 ml 72% $HClO_4$ (Supaprur) to strong fumes of $HClO_4$. Exogenous manganese incorporated into the hair was determined by counting in a gamma well counter, and compared to total manganese in hair, measured by ICP.

To establish whether more manganese is incorporated into hair when sweat evaporates, hair was shaken overnight in synthetic sweat containing ^{54}Mn (500 μg Mn(II) l^{-1}) and air-dried during the day to simulate evaporation. The procedure was repeated for 5 days, before final washing of the hair, acid digestion and manganese determination by gamma counting. In another experiment, hair was shaken for 3 days in ^{54}Mn(II)-sweat solution, and allowed to stand for 4 days, before measurement of manganese uptake.

Incorporation of manganese from dust via sweat into hair

Hair was shaken for 6 days with 10–500 mg of Angurugu roadside dust in 25 ml of sauna sweat. Manganese uptake into hair was measured by ICP, after washing in Triton X-100 and acid digestion in $HNO_3/HClO_4$.

Manganese incorporation along the length of hair

To examine whether manganese incorporation from sweat was constant along the length of the hair, root-to-tip hair samples were collected and treated as follows:

(1) Hair was shaken with synthetic sweat containing 500 μg Mn(II) l^{-1} (with ^{54}Mn).

(2) Hair was shaken for 6 days in synthetic sweat and 500 μg Mn(II) l^{-1} (with ^{54}Mn), air-dried and the roots dipped in a beaker of synthetic sweat for 6 days.

(3) The roots of the hair were placed in a beaker of synthetic sweat and 500 μg Mn(II) l^{-1} (with ^{54}Mn) for 6 days. After treatment, the hair was cut into 1.5 cm lengths, washed with Triton X-100 and acid digested; the exogenous manganese along the length of the hair was then determined by gamma counting. Manganese in untreated hair, cut into 1.5 cm lengths, was also determined by ICP.

Selective leaching of exogenous manganese from hair

Experiments to selectively leach exogenous manganese incorporated into hair from dust and sweat were carried out. Hair, which had been shaken with radioactive ^{54}Mn (500 μg Mn(II) l^{-1}) in sweat for 1–6 days, was washed as usual with Triton X-100. The hair was then divided into aliquots and washed with 0.1 *M* solutions of HCl, cysteine-HCl, CyDTA,EDTA, oxalic acid, ascorbic acid, penicillamine, diethyldithiocarbamate, hydroquinone and H_2O_2, with ultrasonication for up to 6 h. Other leaching agents included 0.05 *M* dithiodipyridine, 0.02 *M* indomethacin, 0.01 *M* potassium ethylxanthogenate, 0.01 *M* oxine, 50% ethanol and 1% sodium lauryl sulphate. To measure the effect of leaching agents on endogenous manganese, unexposed hair was washed with Triton X-100, followed by various leaching agents and the remaining manganese in hair measured by ICP.

Manganese in blood

Manganese in whole blood from Aborigines and Caucasians on Groote Eylandt was determined by atomic absorption spectroscopy (AAS) at the Prince of Wales Hospital, Sydney, New South Wales, Australia. Iron in blood was determined by NAA. Haemoglobin and ferritin in blood were determined by the Mansfield Pathology Laboratories, Sydney.

RESULTS

Solubility of manganese from dust in sweat

Concentrations of manganese in different sweat solutions after shaking 50 mg Angurugu roadside dust for 1 day in 25 ml of sweat are shown in Table 2.

TABLE 2

Solubility of manganese from Groote Eylandt roadside dust in sweat[a]

Solution	Manganese ($\mu g\ l^{-1}$)
Milli-Q water	80
Synthetic sweat	800
Synthetic sweat + protein	640
Synthetic sweat + sebum	840
Sauna sweat	1120

[a] 50 mg dust + 25 ml sweat, shaken for 1 day. Dust contained 4.4% Mn.

Longer shaking times (up to 6 days) and different amounts of dust (10–500 mg) did not increase the manganese concentrations in sweat. Synthetic sweat dissolved ten times more manganese from dust than Milli-Q water, under the same conditions. The addition of protein and sebum to synthetic sweat had little effect. Slightly more manganese dissolved in sauna sweat.

These results suggest that sweat contains reducing agents that are able to dissolve the Mn(IV) and Mn(III) present in the pyrolusite ore in the dust. The ability of sweat to dissolve manganese from a standard suspension of MnO_2 was measured using synthetic sweat, sauna sweat and Milli-Q water (Table 3). Milli-Q water did not substantially dissolve the manganese, whereas in the sweat solutions there was significant and equal dissolution. Lactic acid, histidine-HCl, citrulline and urocanic acid were the major components of sweat which reduced MnO_2.

TABLE 3

Solubility of MnO_2 in sweat and sweat components[a]

Solution[b]	Manganese ($\mu g\ ml^{-1}$)
Milli-Q water	0.01
Sauna sweat	98
Synthetic sweat	102
Synthetic sweat (without lactic acid)	116
Lactic acid	109
Histidine hydrochloride	120
Citrulline	42
Urocanic acid	50
Sodium chloride	0.26
Urea	0.07

[a] 0.1 ml of 0.1 *M* MnO_2 shaken with 1 ml solution for one day.
[b] Concentration of lactic acid, urea, etc. were the same as that present in synthetic sweat (Table 1). pH was adjusted to 5.2 as for synthetic sweat.

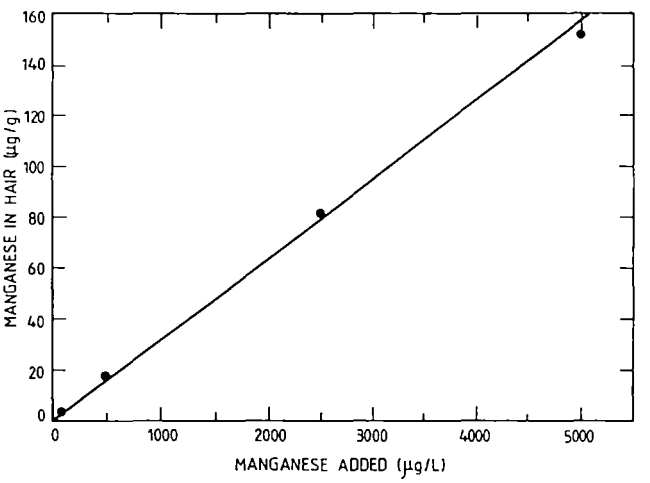

Fig. 1. Uptake of manganese from synthetic sweat into hair.

Lipid solubility of manganese in sweat

Solubility in n-octanol is often used to simulate lipid solubility. However, when a solution of manganese in synthetic sweat was shaken with n-octanol, manganese was not extracted, indicating that manganese in sweat was not in a lipid-soluble form.

Incorporation of manganese from sweat into hair

When hair was shaken for 10 days with synthetic sweat containing 0–5000 μg Mn(II) l^{-1}, the uptake of manganese into hair was proportional to manganese concentration (Fig. 1). When 300 mg of hair was shaken in 25 ml synthetic sweat containing 500 μg Mn l^{-1} (as Mn(II) or MnO_2), about 40% of the manganese was incorporated into hair ($\sim$ 17 μg Mn g^{-1} Triton X-100 washed hair). Aboriginal and Caucasian hair incorporated similar concentrations of manganese. There

TABLE 4

Incorporation of manganese into hair from Groote Eylandt roadside dust, shaken in sauna sweat[a]

Weight of dust (mg)	Manganese incorporated into hair (ppm)
10	54
50	175
150	200
500	380

[a] 10–500 mg dust (4.4% Mn) and 250 mg hair shaken for 6 days in 25 ml sauna sweat.

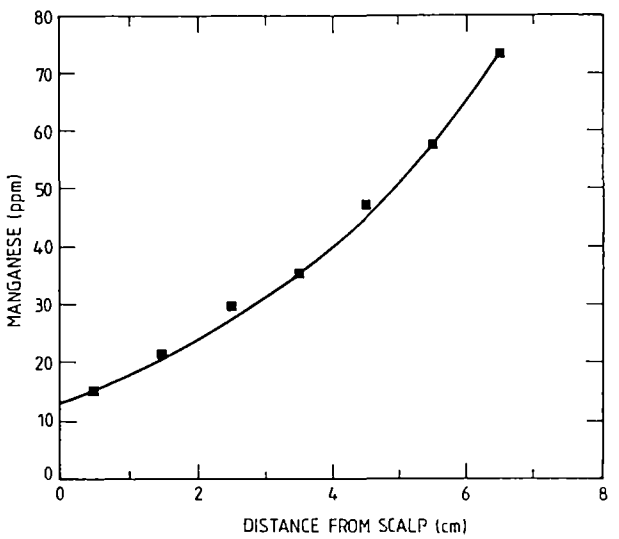

Fig. 2. Manganese along the length of hair from one Aborigine affected by Groote Eylandt Syndrome.

was no increase in uptake when the manganese solution was replaced each day for 10 days. Washing the hair with Triton X-100 after each daily exposure to sweat and manganese (to simulate daily hair shampooing) did not affect uptake of manganese.

Uptake of manganese into hair increased with increased shaking time for 4–5 days, after which additional shaking (up to 1 month) led to only a small increase. Triton X-100 was no more effective than Milli-Q water in removing exogenous manganese from hair after long shaking periods (> 10 days).

Hair shaken with Mn(II) in sweat for 3 days, rinsed with Milli-Q water, allowed to stand for 4 days, then subjected to the standard washing procedure, did not incorporate more manganese than hair shaken for 3 days and measured immediately. Moreover, manganese was just as leachable with Triton X-100 after the 7 day experiment, suggesting that manganese does not diffuse slowly into the hair cortex.

Only slightly more manganese was incorporated into hair (13 μg Mn g^{-1} hair) when Mn(II)-containing sweat was allowed to evaporate on the hair each day for 5 days, compared to hair shaken for 5 days without drying (10 μg Mn g^{-1} hair).

When Angurugu roadside dust, sauna sweat and hair were shaken together for 6 days, incorporation of manganese into hair increased with increasing amounts of dust. This increase was not linear (Table 4). Up to 380 μg Mn g^{-1} hair was incorporated into hair in the presence of 500 mg dust and 250 mg hair. It is unlikely that such large amounts of dust would be associated with Aboriginal hair. Dust collected during the washing of Aboriginal hair with Triton X-100, contributed only $\sim$14 μg Mn g^{-1} hair.

Manganese incorporation along the length of hair

Changes in manganese concentration along the length of hair were measured in a number of Aborigines and Caucasians from Groote Eylandt. Manganese always increased along the length of hair from roots to tips, reaching 80 ppm in the distal ends of some Aboriginal hair. Figure 2 shows a typical plot of manganese along the length of hair from one Aborigine affected by GE Syndrome. This increase in manganese from roots to tips may be due to:

(a) age-related damage to the outer cuticle layer of the hair, which enables more exogenous manganese to be absorbed towards hair tips;

(b) elution of sweat from the scalp, which travels along the hair, dissolves manganese in dust particles on the hair and concentrates manganese towards the tips, i.e. hair acts like a chromatographic column (wick) with sweat as the eluant.

The results of experiments to test the wick effect are presented in Table 5. When the roots only of hair were placed in a beaker of synthetic sweat containing 500 μg Mn(II) l^{-1} with the distal ends hanging over the side of the beaker, it had less manganese along its length towards the tips (treatment 3, Table 5). Manganese increased along the length of hair towards the tips for hair which had previously been shaken with manganese in sweat and the roots placed in a beaker of synthetic sweat (no manganese) (treatment 2, Table 5). However, manganese also increased along the length of hair from roots to tips,

TABLE 5

Incorporation of manganese along the length of hair

Hair section[a]	Manganese (μg/g hair)			
	Untreated	Treatment 1[b]	Treatment 2[c]	Treatment 3[d]
Roots	0.1	4.3	2.6	41.8
	0.2	9.8	6.8	37.0
	0.6	10.7	8.2	30.1
	0.8	10.9	8.8	27.3
	0.7	11.3	8.6	21.7
	1.2	12.0	12.1	20.6
	1.2	13.3	12.8	20.6
	1.2	14.6	14.9	19.4
Ends	1.2	15.6	16.4	22.7

[a] Each hair section was 1.5 cm in length.

[b] 730 mg hair was shaken for 6 days in 25 ml synthetic sweat containing 500 μg Mn(II) l^{-1} (with ^{54}Mn).

[c] 740 mg hair was shaken for 6 days in 25 ml synthetic sweat containing 500 μg Mn(II) l^{-1} (with ^{54}Mn) then roots dipped in a beaker of synthetic sweat for 6 days.

[d] The roots of 737 mg hair were dipped in a beaker of synthetic sweat containing 500 μg Mn(II) l^{-1} (with ^{54}Mn) for 6 days.

when hair was simply shaken in a manganese–sweat solution, and analysed immediately (treatment 1, Table 5). This suggests that increasing manganese towards hair tips is not due to a wick effect, but more likely due to increasing damage to the hair cuticle with age (i.e., distance from the scalp) allowing more exogenous manganese to be absorbed in the cortical cells.

Selective leaching of exogenous manganese

The effectiveness of a number of chemical chelators, thiols, acids and solvents in removing exogenous manganese from hair are shown in Table 6. The most effective leaching agents were 0.1 *M* HCl and 0.1 *M* cysteine-HCl, which removed 71% and 51% of the exogenous manganese, respectively, but also removed much of the endogenous manganese. Ultrasonication for 1 h with 0.1 *M* CyDTA did not remove any endogenous Mn, but only extracted 15–40% of the exogenous manganese, with the percentage extracted decreasing with increasing exposure to manganese. The longer the hair was shaken with manganese in sweat, the less effectively the agents removed manganese. The

TABLE 6

Percentage manganese removed from hair by various leaching agents[a]

Leaching agent	Percentage exogenous manganese removed
HCl	71
Cysteine-HCl (pH 1.3)	51
Cysteine-HCl (pH 1.3) + 50% ethanol	42
CyDTA[b] (pH 5.5)	37
HCl (0.025 *M*, pH 1.3)	34
EDTA	33
Dithiodipyridine (0.05 *M*)	33
Hydroquinone	21
Diethyldithiocarbamate	21
Oxalic acid	21
Sodium lauryl sulphate (1%)	20
H_2O_2 + CyDTA	19
H_2O_2	16
Indomethacin	16
Oxine (0.01 *M*)	12
Ascorbic acid	10
Potassium ethylxanthogenate	4
Ethanol (50%)	3
Penicillamine	3
Cysteine HCl (pH 5.1)	2

[a] Hair was shaken for 1–3 days in synthetic sweat containing 500 μg Mn l^{-1} (labelled with ^{54}Mn). All leaching agents used were 0.1 *M*, unless otherwise stated.
[b] Cyclohexylenedinitrilotetraacetic acid.

anionic detergent, sodium lauryl sulphate (SDS) only removed 20% of exogenous manganese after 1 h ultrasonication at 25°C and 58% at 80°C. Potassium hydroxide (3 *M*), which extracts the keratins (protein components of hair), removed only 60% of exogenous manganese after hair was shaken for 7 days.

DISCUSSION

Large amounts of manganese may be dissolved by sweat from Angurugu roadside dust and incorporated into hair. This source of exogenous manganese in hair may partly account for the elevated manganese levels found in the hair of Aborigines and Caucasians living in Angurugu (Stauber et al., 1987). The average concentration of manganese in hair dust collected from Angurugu was 1.7%. This could lead to 500 μg Mn l^{-1} dissolved in sweat (Table 2) and, from Fig. 1, an incorporation of up to 15 μg Mn g^{-1} hair. Larger amounts of dust in hair, the presence of roadside dust (which is 4% manganese) and evaporation of sweat on hair could all lead to an even greater incorporation of manganese in hair via this exogenous route.

To obtain reliable estimates of manganese taken up from blood in the hair follicle, manganese in hair from exogenous sources must be eliminated. If manganese from exogenous sources is bound only to the outer cuticle layer of hair, and does not penetrate the hair cortex, or is bound to different functional groups on amino acids than endogenous manganese, it may be possible to remove exogenous manganese from hair before analysis. Bos et al. (1985) and Li and Malmqvist (1985), using microbeam particle induced X-ray emission (PIXE), studied the distribution of iron, lead, sulphur, zinc and copper across single hairs. They found that some elements, including zinc, sulphur and copper, have a homogeneous distribution across the hair, whereas endogenous iron and lead have higher concentrations at the hair periphery.

Although the distribution of manganese across the hair has not been determined, there is some evidence from acid staining wool, that manganese binds preferentially to orthocortical cells, perhaps to the amino acid tyrosine (Corbett and Yu, 1964). If this is the case, selective leaching of exogenous manganese from hair will be very difficult. We were unable to find a leaching agent that could remove exogenous manganese, without affecting endogenous manganese. Buckley and Dreosti (1984) were unable to find a washing procedure that removed only exogenous zinc from hair. They concluded that the location and binding of endogenous and exogenous zinc in hair may not be significantly dissimilar. Nishiyama and Nordberg (1972) also found that acid and alkaline washes could not distinguish endogenous and exogenous cadmium in hair. Moreover, endogenous cadmium was more easily eluted than cadmium adsorbed to hair from exogenous sources.

Other ways of overcoming the problem of exogenous manganese include sampling the 1–2 cm of hair closest to the scalp or measuring manganese along the length of hair and extrapolating back to zero length at the scalp. The latter

TABLE 7

Manganese in scalp and pubic hair

Subjects	Mean Mn (ppm)	
	Scalp[a]	Pubic
Caucasians - Sydney	0.5 ± 0.2[b]	1.3 ± 0.5
GEMCO workers	2.2 ± 0.8	3.0 ± 0.4
Caucasians - Angurugu	2.5 ± 0 7	7.9 ± 4.1
Affected Aborigines	9 ± 5	25 ± 13
Unaffected Aborigines	16 ± 12	23 ± 6

[a]Extrapolated to zero length.
[b]One standard deviation.

method enables an estimate of the manganese concentration in hair as it emerges from the scalp, before manganese from exogenous sources interferes. We used this method to reassess manganese in scalp hair from Groote Eylandters. Scalp-hair manganese, extrapolated back to zero, and pubic-hair manganese in Angurugu Aborigines affected/unaffected by GE Syndrome, Caucasians living at Angurugu and some Caucasian GEMCO workers are shown in Table 7. Manganese in both scalp hair and pubic hair of Angurugu Aborigines was about 20 times the non-exposed values of Sydney Caucasians. There was no significant difference in hair manganese between Aborigines with GE Syndrome and those without the disease. Scalp-hair manganese for both the Caucasians at Angurugu and the GEMCO workers were three–six times the Sydney values. These results suggest that the Angurugu population, particularly the Aborigines, have a high intake of manganese.

Higher hair manganese in Aborigines than Caucasians may be due to their different lifestyles, e.g., Aborigines "close-to-the-earth" living or due to differences in their diets. We measured manganese in some traditional food samples, including yams and fruit from the old Angurugu garden areas (Florence et al., 1987). Some of the foods were exceptionally high in manganese, e.g., one 20 g yam would supply 13 mg of manganese; three times the recommended daily allowance of this element. One litre of boiled billy tea, which is part of the Aborigines' staple diet, contained 6–7 mg manganese, five times more than brewed tea drunk by Caucasians.

To determine whether manganese in scalp hair gives a reliable estimate of manganese intake, manganese in blood from the same groups was determined (Table 8). The Caucasians living at Angurugu and the GEMCO workers had blood manganese values within the normal range for Sydney controls (6–12 μg Mn l^{-1}). Angurugu Aboriginals had elevated levels of manganese in blood, although there was no significant difference between those affected and unaffected by GE Syndrome. The affected Aboriginals also had low haemoglobin (normal range 12–18 g dl^{-1}) and low ferritin (normal range 25–150 (F) and 75–260 (M) μg l^{-1}), indicative of low iron status (anaemia). Anaemia,

TABLE 8

Mean manganese, iron, haemoglobin and ferritin in blood of Groote Eylandt inhabitants

Subjects	Mn ($\mu g\ l^{-1}$)	Fe ($\mu g\ l^{-1}$)	Hb ($g\ dl^{-1}$)	Ferritin ($\mu g\ l^{-1}$)
GEMCO workers	8.4 ± 2.3	431 ± 46	15.7 ± 0.9[a] 14.0 ± 0.6[b]	146 ± 68
Caucasians, Angurugu	7.3 ± 2.3	388 ± 32	15.1[a] 12.9 ± 0.9[b]	69 ± 40
Affected Aborigines	36.1 ± 10.2	294 ± 73	8.9[a] 11.0 ± 0.6[b]	11 ± 11
Unaffected Aborigines	18.9 ± 6.5	403 ± 69	15.7 ± 1.0[a] 12.3[b]	161 ± 161

[a]Males.
[b]Females.

chronic infections, high alcohol intake and low dietary calcium and zinc all increase the toxic effects of manganese (Schager et al., 1974; World Health Organization 1981).

Measurement of manganese in blood and hair probably only indicates manganese in the body that is in readily exchangeable pools, such as soft tissue. Because hair and blood determinations can only indicate relatively recent manganese exposure, we were unable to distinguish between those individuals affected/unaffected by GE Syndrome. The exposure which caused the neurological symptoms may have occurred many years previously, perhaps in early childhood. A sample of umbilical cord blood taken from a young Angurugu mother had 40 $\mu g\ Mn\ l^{-1}$. Determination of manganese in scalp hair does show, however, that the Angurugu population has a high intake of manganese. Only 2% of the population are susceptible; perhaps only those individuals with a genetic difference in manganese metabolism which leads to enhanced manganese uptake or reduced manganese clearance from the body.

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MANGANESE CATALYSIS OF DOPAMINE OXIDATION

T.M FLORENCE and J.L STAUBER

CSIRO, Centre for Advanced Analytical Chemistry, Division of Fuel Technology, Private Mail Bag 7, Menai, NSW 2234 (Australia)

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ABSTRACT

Manganese catalysis of the oxidation of dopamine by air was studied as part of an investigation of possible manganese intoxication amongst a group of Aborigines living on manganese-rich soil on Groote Eylandt, in the Northern Territory of Australia. Manganese significantly increased the oxidation rate of dopamine, and the manganese complexes with some purines were especially efficient catalysts. An oxidation mechanism, involving a manganese(II)/(III) redox couple and a semiquinone free radical intermediate, is proposed. Stoichiometric hydrogen peroxide was produced by the oxidation, and the oxidation products of dopamine were highly toxic to the marine diatom *Nitzschia closterium*. Hydrogen peroxide and the superoxide radical did not oxidize dopamine at physiological pH. Some electrophilic compounds, including ascorbic acid, dehydroascorbic acid and thiamine, effectively inhibited dopamine oxidation. The Groote Eylandt Aborigines are likely to be deficient in ascorbic acid (vitamin C) and thiamine (vitamin B_1), and these deficiencies, as well as their lifestyle, may predispose them to manganese intoxication

INTRODUCTION

Groote Eylandt, an island in the Gulf of Carpentaria, in Australia's Northern Territory, is an Aboriginal reserve on which there are extensive deposits of manganese. The main Aboriginal village, Angurugu, is situated on a rich manganese outcropping, with average manganese soil concentrations of 4–5% in the village [1–4].

About 2% of the Aboriginal population of the village have developed a neurological disorder (The "Angurugu Syndrome") with symptoms of manganism similar to those reported for some Chilean manganese miners [1,5–7], and with many of the manifestations of Parkinson's disease. Fruit and vegetables grown in the vicinity of the village are extremely high in manganese, and some of the Aborigines have elevated concentrations of manganese in blood and hair [1].

The neurotoxicity of manganese is believed to result from the ability of this metal to catalyze the oxidation of dopamine (3-hydroxytyramine) by oxygen [8–12], causing depletion of this neurotransmitter. The oxidation products, dopamine quinone and hydrogen peroxide, are also neurotoxic [1, 12].

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Dopamine oxidation appears to involve the superoxide radical and the manganese(II)/(III) redox couple, although the exact reaction mechanism has yet to be elucidated [8].

As part of a detailed study of the health effects of environmental manganese in Angurugu, we have carried out in vitro measurements of the ability of manganese to catalyze the oxidation of dopamine by oxygen, hydrogen peroxide, and superoxide radical. Reaction mechanisms are proposed, and it is shown that the oxidation can be completely inhibited by certain electrophilic compounds, including ascorbic acid (vitamin C) and thiamine (vitamin B_1).

EXPERIMENTAL

Oxidation of dopamine by air

Phosphate buffer (23 ml of 0.02 *M*, pH 7.5) plus metal (0.25 ml of 0.01 *M*) and ligand (0.25 ml of 0.025 *M*) in a 25-ml volumetric flask, was pre-incubated at 37°C for 15 min, then the reaction started by the addition of 0.5 ml of 0.015 *M* dopamine, diluting to 25 ml with phosphate buffer, and mixing. Air was bubbled through the solution continuously. Aliquots were taken at 5 min intervals for the spectrophotometric determination of dopamine quinone at 490 nm and hydrogen peroxide.

Spectrophotometric determination of hydrogen peroxide

To the sample aliquot (1 ml) in a 25 ml volumetric flask was added 5 ml of 1 *M* ammonium trichloroacetate buffer, pH 8.2, and 2.5 ml of Ti-PAR reagent (1:1 mixture of 1.5×10^{-3} *M* potassium titanium oxalate and 1.5×10^{-3} *M* 4-(2-pyridylazo)resorcinol). After dilution to volume, the solution was heated at 45°C for 75 min. The absorbance at 508 nm was measured against an identical solution to which 1 mg catalase was added 10 min before the Ti-PAR reagent. The molar absorptivity of H_2O_2 by this method was 3.6×10^4.

Oxidation of dopamine by hydrogen peroxide

Phosphate buffer (23 ml of 0.02 *M*, pH 7.5) plus metal (0.25 ml of 0.01 *M*) and ligand (0.25 ml of 0.025 *M*) was deaerated at 37°C with nitrogen for 15 min. Dopamine (0.5 ml of 0.015 *M*) was added, and the reaction started by the addition of 1.00 ml of 0.5 *M* H_2O_2 and mixing. A blank was prepared by substituting 1 ml of water for the H_2O_2. After 30 min at 37°C, the absorbance of dopamine quinone was measured at 490 nm.

Oxidation of dopamine by superoxide radical (O_2^-)

Phosphate buffer (23 ml of 0.02 *M*, pH 7.5) plus metal (0.25 ml of 0.01 *M*) and ligand (0.25 ml of 0.025 *M*) was incubated at 37°C for 15 min. Dopamine (0.5 ml

of 0.015 M) and freshly prepared xanthine (0.625 ml of 8×10^{-4} M in 0.01 M NaOH) were added, then the reaction started by adding xanthine oxidase (0.25 ml of 0.25 mg ml^{-1}), diluting to 25 ml with phosphate buffer and mixing. After 30 min at 37°C the absorbance of dopamine quinone was measured at 490 nm and compared with the absorbance of a blank prepared by omitting xanthine oxidase. The effect of adding 0.5 mg superoxide dismutase (SOD) (Sigma, bovine liver) was also examined.

Effect of dopamine on algal growth rate

The effect of 1×10^{-5} M dopamine in the absence and presence of manganese(II) and xanthine was measured by the growth rate of the marine diatom *Nitzschia closterium* [20] in seawater for 4 days.

Production of hydroxyl radical during dopamine oxidation

Formation of hydroxyl radical (OH·) during the oxidation of dopamine (2.5×10^{-4} M) at pH 7.5 with O_2, O_2^- or H_2O_2 was measured using the 4-nitrophenol spectrophotometric method [13]. In this procedure, OH· hydroxylates 4-nitrophenol (yellow) to 4-nitrocatechol (red), which is determined spectrophotometrically at 507 nm.

RESULTS

Using an extrapolation procedure [14] for the rate plot, the rate of oxidation of dopamine by oxygen was found to be pseudo first order. The first order rate constants (k_1) for oxidation in the presence of manganese together with various ligands, and copper and iron alone are shown in Table 1. Rate constants were calculated from the formation of dopaquinone, but very similar values were obtained using H_2O_2 production. The rate constants were independent of dopamine concentration, as expected for a first order reaction. After correction for the H_2O_2 formed from the Mn–xanthine catalyst alone (0.58×10^{-4} M H_2O_2), 3×10^{-4} M dopamine oxidized by air produced 2.97×10^{-4} M H_2O_2, i.e. an equimolar concentration.

The effect of various inhibitors on the rate of the Mn-catalyzed reaction are given in Table 2. No inhibition was found for the following compounds (1×10^{-3} M): 1,10-phenanthroline, 2-nitrophenol, choline, riboflavin, folic acid, oxalate, taurine, tyrosine and vitamin B_{12}. Thiamine and ascorbic acid also inhibited the uncatalyzed (Mn-free) oxidation of dopamine, and ascorbic acid (but not thiamine or dehydroascorbic acid) was able to reduce dopaquinone back to dopamine.

Superoxide dismutase (1 mg/25 ml) reduced the Mn-catalyzed oxidation rate of dopamine by 50%; the heat-denatured enzyme was ineffective. Catalase (1 mg/25 ml) had a much smaller effect. The hydroxyl radical scavenger, mannitol (0.05 M), had no effect on the oxidation rate.

TABLE 1

Oxidation of dopamine by air[a]

Catalyst[b]	Pseudo first order rate constant ($\times 10^2 min^{-1}$)
None	0.26
Mn^{2+}	0.72
Mn^{2+} + xanthine	1.20
Mn^{2+} + hypoxanthine	0.94
Mn^{2+} + uric acid	1.00
Mn^{2+} + adenine	0.87
Mn^{2+} + histidine	0.42
Mn^{2+} + orotic acid	0 91
Mn^{2+} + lactic acid	0.43
Mn^{2+} + guanine	0 85
Mn^{2+} + pyrophosphate	0.63
Mn^{2+} + tyrosine	0.86
None, 0.02 *M* buffer[c]	< 0.10
Mn^{2+} + 0.02 *M* buffer[c]	0.50
Fe^{2+}	4.05
Cu^{2+}	(1.3)[d]

[a] 3×10^{-4} *M* dopamine in 0.02 *M* phosphate buffer pH 7 5, 37°C.
[b] 1×10^{-4} *M* metal + 2.5×10^{-4} *M* ligand.
[c] PIPES buffer in place of phosphate.
[d] Copper appeared to catalyze formation of an indole polymer in the later stages of the reaction.

The k_1–pH data are shown in Table 3. The pH range was 7.0–7.7; at lower pH values the rate was considerably slower, and at more alkaline values (pH > 7.8) the dopaquinone was converted to a dark-coloured indole [11].

Dopamine was oxidized by neither H_2O_2 nor $O_2^{\cdot-}$ in the presence of the catalysts free Mn^{2+}, Mn–xanthine, or Mn–ATP. However, Fe–EDTA was an effective catalyst for the dopamine–H_2O_2 reaction. Two $O_2^{\cdot-}$ generating systems, xanthine–xanthine oxidase and acetaldehyde-xanthine oxidase, were used. The cytochrome *c* spectrophotometric procedure [15] showed that a flux of $O_2^{\cdot-}$ was generated from both systems, but no dopamine oxidation occurred, other than that from dissolved oxygen.

The production of hydroxyl radical during the oxidation of dopamine by air is shown in Table 4. No OH· was produced in deaerated solutions when H_2O_2 or $O_2^{\cdot-}$ were used as reductants. Any interference by the indole polymer was eliminated by preparing a blank reaction solution that was identical to the sample solution, but with 4-nitrophenol omitted.

The effects of dopamine and dopamine oxidation products on the growth rate of *Nitzschia closterium* are shown in Table 5. Dopamine and/or its oxidation products are toxic to *Nitzschia*. It is likely that the oxidation products are the toxic agents, since toxicity is increased in the presence of the oxidation

TABLE 2

Inhibition of manganese-catalyzed dopamine oxidation in air[a]

Inhibitor	Reduction in oxidation rate (%)[b]
Thiamine, $1 \times 10^{-3} M$	100
$1 \times 10^{-4} M$	47
$1 \times 10^{-5} M$	35
Ascorbic acid, $2.5 \times 10^{-4} M$	100
$5 \times 10^{-5} M$	100
$1 \times 10^{-5} M$	56
Dehydroascorbic acid, 1×10^{-3}	100
1×10^{-4}	31
Biotin, $1 \times 10^{-3} M$	40
Morin, $1 \times 10^{-3} M$	55
Dodecylpyridinium chloride, $1 \times 10^{-3} M$	33
4-Nitrophenol, $1 \times 10^{-3} M$	41
Pyridoxine, $1 \times 10^{-3} M$	40
Glycine, $1 \times 10^{-3} M$	50
Reduced glutathione, $1 \times 10^{-3} M$	100
Malachite green, $1 \times 10^{-3} M$	60

[a] $3 \times 10^{-4} M$ dopamine + $1 \times 10^{-4} M$ Mn^{2+} in 0.02 M phosphate buffer, pH 7.5, 37°C.
[b] Reduction in oxidation rate (0.72×10^{-2} min^{-1}) of inhibitor-free solution

catalyst, manganese–xanthine complex, and is reduced by the oxidation inhibitor 4-nitrophenol.

DISCUSSION

In o-phosphate and PIPES buffers, manganese significantly increased the oxidation rate of dopamine by air (Table 1). The rate was faster in phosphate than in the non-complexing PIPES buffer, showing that the manganese phosphate complex is a more efficient catalyst than the hydrated manganese ion. The manganese complexes of some purine derivatives, viz. xanthine, hypo-

TABLE 3

Effect of pH on the rate of dopamine oxidation by air[a]

pH	Pseudo first order rate constant ($\times 10^2 min^{-1}$)
7.00	0.278
7.30	0.440
7.50	0.724
7.70	1.59

[a] $3 \times 10^{-4} M$ dopamine + $1 \times 10^{-4} M$ Mn^{2+} in 0.02 M phosphate buffer, 37°C.

TABLE 4

Production of hydroxyl radical during the oxidation of dopamine by air as measured by the hydroxylation of 4-nitrophenol to 4-nitrocatechol[a]

Catalyst ($1 \times 10^{-4} M$ metal)	4-Nitrocatechol ($M \times 10^6$)
Mn^{2+}	2.5[b]
Mn^{2+} + 0.05 M mannitol	< 0.8
Mn^{2+} + $2 \times 10^{-4} M$ ATP	< 0.8
Fe^{2+} + $2 \times 10^{-4} M$ EDTA	1.8
Fe^{2+} + $2 \times 10^{-4} M$ ATP	1.4
Cu^{2+} + 2×10^{-4} ATP	1.4

[a] $2.5 \times 10^{-4} M$ dopamine in 0.02 M phosphate buffer, bubbled with air for 30 min.
[b] Corrected for inhibition of dopamine oxidation by 4-nitrophenol (Table 2).

xanthine, uric acid, adenine and guanine, were even more effective catalysts than the phosphate complex, although histidine, lactic acid, and pyrophosphate lowered the oxidation rate [8] (Table 1). Copper had a catalytic efficiency similar to that of manganese, but iron was much more effective.

A plot of log k_1 vs pH (Table 3) had a slope of -1.05 (correlation coeff., $r^2 = 0.95$), indicating that one hydrogen ion is produced in the rate-determining step of the oxidation. In addition, stoichiometric hydrogen peroxide was

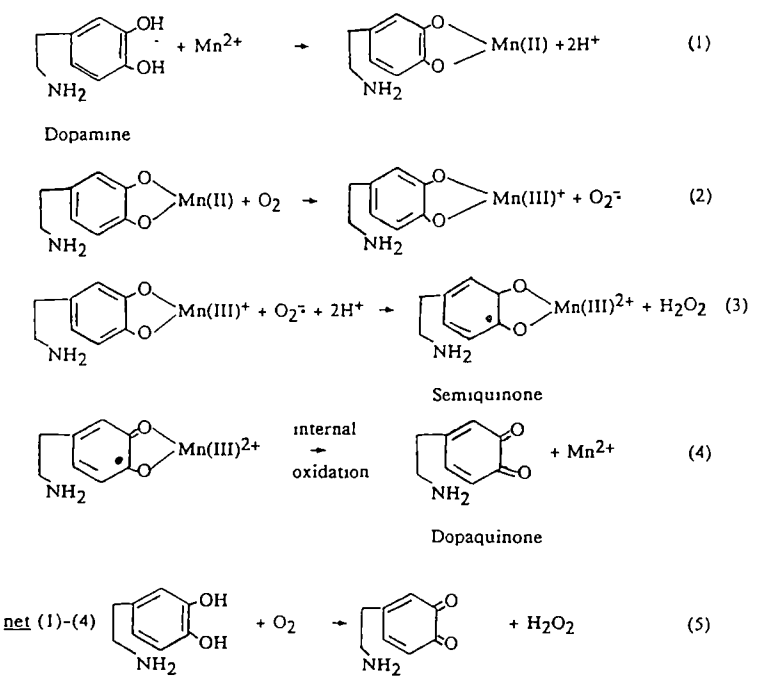

TABLE 5

The effect of dopamine oxidation products on the growth rate of *Nitzschia closterium*[a]

Additions to growth medium	Growth rate (% of blank)[b]
Dopamine, $1 \times 10^{-5} M$	−21
Dopamine, $1 \times 10^{-5} M$, + $2.5 \times 10^{-6} M$ Mn^{2+}	−44
$2.5 \times 10^{-6} M$ Mn^{2+}	96
Dopamine, $1 \times 10^{-5} M$, + $2.5 \times 10^{-6} M$ Mn^{2+} + $2 \times 10^{-5} M$ xanthine	−61
Dopamine, $1 \times 10^{-5} M$, + $2.5 \times 10^{-6} M$ Mn^{2+} + $2 \times 10^{-5} M$ xanthine + $1 \times 10^{-5} M$ nitrophenol	−37
$1 \times 10^{-5} M$ H_2O_2	70

[a] Growth-rate assay carried out in unsupplemented seawater.
[b] Negative growth rate indicates cell death.

produced, but neither O_2^- nor H_3O_3 oxidized dopamine, and only a trace of OH· was generated. Superoxide dismutase strongly inhibited the reaction, but neither catalase nor mannitol had a significant effect on the rate. These data allow the reaction mechanism (1)–(5) to be postulated for the oxidation of dopamine by air at pH 7–8. A minor side reaction, possibly involving the H_2O_2 produced during the oxidation, generates a small concentration of hydroxyl radical (Table 4). This reaction scheme is similar to that proposed by Grinstead [16] and Tyson and Martell [17] for the Mn^{2+}-catalyzed oxidation of 3,5-di-t-butyl-pyrocatechol.

Table 5 shows that the oxidation of dopamine yields products that are very toxic to the marine diatom *Nitzschia closterium* in seawater at pH 8.2. The principal toxic agent appears to be dopaquinone or its polymerized products [12], since the stoichiometric concentration of H_2O_2 has only a low toxicity.

Ascorbic acid (vitamin C), dehydroascorbic acid, thiamine (vitamin B_1) and reduced glutathione were the most effective inhibitors of the dopamine oxidation (Table 2). The observation that dehydroascorbic acid inhibits, shows that inhibition is not simply chemical reduction. Dehydroascorbic acid is unstable in solution, which may explain why it is not as effective as ascorbic acid. Most of the inhibitors are positively charged (e.g., thiamine) or have a strongly electrophilic group on the molecule (e.g., ascorbic and dehydroascorbic acid). 4-Nitrophenol, with an electrophilic nitro group, inhibits the oxidation, but 2-nitrophenol does not, because here the nitro group is hydrogen bonded to the adjacent hydroxyl group [18]. This effectiveness of compounds with a positive charge suggests that inhibition involves reaction with the semiquinone free radical, which would effectively quench the oxidation.

Many of the Angurugu Aborigines have a diet low in vitamin C, and a high alcohol intake. Ethanol not only accelerates the absorption of manganese, but efficiently destroys thiamine [19]. Anemia, common among the Aborigines, also enhances manganese uptake [6]. The Groote Eylandters are therefore

especially at risk from manganism because not only are they exposed to high concentrations of the element (having a close-to-the-earth lifestyle), but they absorb it more efficiently than others, and they are deficient in the vitamins (C and B_1) which may protect against dopamine loss. A vitamin intervention program is being considered as a prophylactic measure.

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THE EFFECT OF CULTURE MEDIUM ON METAL TOXICITY TO THE MARINE DIATOM *NITZSCHIA CLOSTERIUM* AND THE FRESHWATER GREEN ALGA *CHLORELLA PYRENOIDOSA*

J L. STAUBER and T. M. FLORENCE
Centre for Advanced Analytical Chemistry, CSIRO Division of Fuel Technology, PMB 7, Menai, N.S.W. 2234, Australia

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Abstract—The composition of the culture medium and its effects on the toxicity of ionic copper and zinc to the marine diatom *Nitszchia closterium* and the toxicity of copper and lead to the freshwater green alga *Chlorella pyrenoidosa* were examined. The inhibitory concentration of copper giving 50% reduction in cell division rate in *C. pyrenoidosa* increased from 16 μg Cu l^{-1} in synthetic softwater and 24 μg Cu l^{-1} in EPA–AAP medium (minus EDTA) to >200 μg Cu l^{-1} in high nutrient MBL medium. Similarly, marine medium f reduced copper and zinc toxicity to *N. closterium* 10–20 fold compared to seawater. To overcome the problems of metal complexation by chelators such as silicate, iron and EDTA in the assay medium, unsupplemented seawater and buffered synthetic softwater, enriched only with nitrate and phosphate, have been used for the study of metal toxicities to marine and freshwater algae. These low nutrient media are sufficient to support algal growth over the 4-day assay period.

Key words—algae, copper, zinc, lead, toxicity, culture medium, marine, freshwater

INTRODUCTION

To establish realistic water quality criteria for heavy metals in both marine and freshwater environments, algal bioassays have been extensively used (Bartlett and Rabe, 1974; EPA, 1978; Van Coillie *et al.*, 1983). The *Selenastrum capricornutum* Printz algal assay bottle test (EPA, 1971, 1978) and the companion marine algal assay procedure: bottle test (EPA, 1974) were proposed as standard procedures for solving eutrophication problems and testing receiving waters of varying nutrient status. Although both these procedures may be applied to the study of heavy metal toxicty to algae, disadvantages include the use of EDTA which reduces toxicity of metals in the freshwater assay medium (Chiaudani and Vighi, 1978), and the time involved (12–20 days) for each assay.

Although it has been well documented that the toxicity of metals in both marine and freshwaters depends on the presence of chelators such as iron and EDTA (Steeman Nielsen and Kamp-Nielsen, 1970; Erickson, 1972), many workers have used high nutrient culture medium in metal toxicity bioassays (Foster, 1977; Bentley-Mowat and Reid, 1977). In particular, many marine studies have used medium f of Guillard and Ryther (1962), which contains such chelators as phosphate and citrate, and adsorbents such as iron and silicate which can bind the heavy metal ion being tested for toxicity. To overcome this problem, Lumsden and Florence (1983) proposed an algal assay procedure in which the marine diatom *N. closterium* was cultured in modified medium f, but the assays were carried out in raw, unenriched seawater for 4 days. We have used this method extensively in the study of mechanisms of metal toxicity to algae, and its amelioration by metal(III) hydroxides (Stauber and Florence, 1985a, b, 1986, 1987).

It is now well established that the extent of trace metal toxicity to aquatic organisms is governed not by the total metal concentration, but by the free metal ion activity (Sunda and Guillard, 1976; Florence, 1983, 1986; Stauber and Florence, 1987). Complexing agents, by decreasing free metal ion activity, decrease metal toxicity. The only exceptions to this rule are some lipid–soluble metal complexes, which, because of their rapid penetration of the cell membrane, and their ability to transport the ligand as well as the metal into the cytosol, may actually be more toxic than free metal ion (Florence *et al.*, 1983; Ahsanullah and Florence, 1984; Florence, 1986). In simple ionic solutions, or those where EDTA complexes dominate (Jackson and Morgan, 1978) it is possible to compute the free metal activity from chemical models. In most culture media, however, where a variety of trace metal adsorbents, such as hydrated oxides of iron, manganese and silicon are present, such calculations are too inaccurate to be of use.

In this paper we compare the toxicity of copper and zinc to the marine diatom *N. closterium* in unsupplemented seawater and medium f, and copper and lead toxicity to the freshwater green alga, *C. pyrenoidosa* in three different media We propose the use of

unenriched seawater for marine algal assays and a buffered synthetic softwater supplemented with nitrate and phosphate for freshwater assays.

MATERIALS AND METHODS

Seawater

Surface seawater was collected off Port Hacking, N.S.W in carefully cleaned polyethylene bottles and passed immediately through a 0.45 μm filter. Filtered seawater was stored at 4°C until used in the culture medium and algal assays.

Algal cultures

The unicellular marine diatom *N. closterium* (Ehrenberg) W. Smith was cultured in a modified medium f (Guillard and Ryther, 1962). Ferric citrate/citric acid (4.5 mg l^{-1} ferric citrate. $7H_2O$ + 4.5 mg l^{-1} citric acid) replaced iron-EDTA, $Na_2MoO_4 \cdot 2H_2O$ was omitted and the trace metal concentration was halved (Table 1).

The freshwater green alga *C. pyrenoidosa* Chick was maintained in modified MBL medium (Stein, 1973) (Table 2). Both species were obtained from the CSIRO Division of Fisheries Algal Culture Collection. The cultures were maintained at 21°C on a 12:12 h light/dark cycle at 6400 lx. Cells in exponential phase growth were used for all growth experiments

Cell division rate experiments

(a) Chlorella pyrenoidosa. The effects of ionic copper and lead on the cell division of *C. pyrenoidosa* in three different culture media were compared.

Table 1 Composition of f medium

Nutrient	Concentration (mg l^{-1} seawater)
$NaNO_3$	150
$NaH_2PO_4 \cdot 2H_2O$	10
$Na_2SiO_3 \cdot 5H_2O$	25 7
$C_6H_5O_7Fe \cdot 5H_2O$	4 5
$C_6H_8O_7 \cdot H_2O$	4.5
$CuSO_4$ $5H_2O$	0.009
$ZnSO_4 \cdot 7H_2O$	0 022
$CoCl_2 \cdot 6H_2O$	0.010
$MnCl_2 \cdot 4H_2O$	0.180
Thiamine HCl	0.2
Biotin	0 001
Vitamin B_{12}	0 001

(i) Fifty millilitres of enriched synthetic soft water ($NaHCO_3$,48 mg l^{-1};$CaSO_4 \cdot 2H_2O$,30 mg l^{-1};$MgSO_4 \cdot$ $_2O$, 30 mg l^{-1}; KCl, 2 mg l^{-1}) with the addition of 1 ml of 0.5 M Hepes buffer (pH 7.0), 0 5 ml of $NaNO_3$ (2.1 g l^{-1}) and 0.5 ml K_2HPO_4 (0.22 g l^{-1})

(ii) Fifty millilitres of EPA (1971) medium, with modifications by Chiaudani and Vighi (1978) (Table 2). This is the recommended medium for the standard algal assay bottle test using *Selenastrum capricornutum* (EPA, 1978).

(iii) Fifty millitres of modified MBL medium (Stein, 1973) without EDTA (Table 2).

All algal assays were carried out in siliconized 200 ml conical flasks containing 50 ml medium which had not been sterilized. The *C. pyrenoidosa* inoculum was washed three times by centrifugation in the assay medium, before the addition of 4×10^4 cells ml^{-1} to each assay flask. Duplicate assay flasks containing 0–200 μg Cu l^{-1}, were incubated at 21°C at 16,000 lx on a 12.12 h light/dark cycle Cell density was measured initially and on three subsequent days by counting microscopically in a haemocytometer. A regression line was fitted to a plot of $\log_{10}$ (final cell density/initial cell density) vs time (in hours) for each sample, and the cell division rate determined from the slope. The metal toxicity was expressed as the inhibitory concentration which gave a 50% reduction in cell division rate (IC_{50}) after 72 h.

(b) Nitzschia closterium. The effects of ionic copper and zinc on the cell division rate of *N. closterium* in unsupplemented seawater and culture medium f were compared Duplicate assay flasks containing 50 ml seawater or culture medium, 0–200 μg Cu l^{-1} or 0–1000 μg Zn l^{-1} were inoculated with 4×10^4 cells ml^{-1}, according to the method of Lumsden and Florence (1983). The inoculum was washed three times by centrifugation in seawater or culture medium before use Assay flasks were incubated as described above, and cell density determined over 4 days by counting in a haemocytometer.

Determination of ASV-labile lead in enriched synthetic softwater

Electrochemically-labile lead in enriched synthetic softwater was determined by direct current anodic stripping voltammetry (ASV) using an E.G. & G. Princeton Applied Research model 384 polarographic analyser with a model 303 static mercury drop electrode assembly, in a class 100 clean room. Assay flasks containing enriched synthetic softwater, 50–150 μg Pb l^{-1}, with no algae were incubated

Table 2 Composition of the three freshwater media

Nutrient	Enriched synthetic softwater	EPA medium	MBL medium
Major elements (mg l^{-1})			
$NaNO_3$	21	26	85
K_2HPO_4	2.2	1 0	8 7
$NaHCO_3$	48	15	13
Ca	7.0	1 2	10
Mg	2.9	2.9	6 6
Cl	0 9	6 3	18
S	9 5	1 9	4 8
Hepes	2383	—	—
Na_2EDTA	—	—	4 4*
Tris	—	—	500
Minor elements (μg l^{-1})			
B	—	32	—
Mn	—	115	50
Zn	—	1 6	5 0
Co	—	0 07	2 2
Cu	—	0 004	2 3
Mo	—	2 9	—
Fe	—	55	152
Si	—	—	1
Thiamine HCl	—	—	200
Biotin	—	—	1
Vitamin B_{12}	—	—	1

*EDTA was omitted from the MBL medium used in the assay

on a light platform at 21°C for 55 days. ASV-labile lead was determined on a 10 ml aliquot, with 120 s deposition at −0.7 V (vs Ag/AgCl) by peak area measurement

RESULTS AND DISCUSSION

Chlorella pyrenoidosa

The effect of copper and lead on the cell division rate of *C. pyrenoidosa* in different media is shown in Table 3. In the absence of copper, cell division rates for *C. pyrenoidosa* were similar in both low nutrient media (enriched synthetic softwater and EPA medium) and in high nutrient MBL medium (minus EDTA) (Table 4). However, the toxicity of copper was reduced in full strength MBL medium, with an $IC_{50} > 200\ \mu g$ Cu l^{-1}, compared to 16 and 24 μg Cu l^{-1} in synthetic softwater and EPA medium respectively (Tables 3 and 5). We found that copper was much more toxic to *C. pyrenoidosa* than reported by Monahan (1976), who found that 500 μg Cu l^{-1} was needed to cause toxicity to the same species assayed a medium containing the chelators EDTA and iron. Foster (1977), using the high nutrient Bolds Basal medium, found that up to 300 μg Cu l^{-1} was required for complete growth inhibition of *Chlorella vulgaris*.

Chiaudani and Vighi (1978) compared the toxicity of heavy metals in EPA medium ±EDTA to *S. capicornutum* after 4 and 7 days growth. They found that the addition of 300 $\mu g\ l^{-1}$ EDTA to the medium decreased copper toxicity 20-fold from an IC_{50} of 1.6 to 34 μg Cu l^{-1} after 4 days. The IC_{50} for zinc, chromium, cadmium, nickel and cobalt also decreased in the absence of EDTA. They proposed omitting EDTA from the EPA medium used in the standard algal assay bottle test (EPA, 1978). We found an even lower IC_{50} for copper to *C. pyrenoidosa* in buffered synthetic softwater containing nitrate and phosphate. This medium, which contains no added trace metals and supports good growth of *C. pyrenoidosa*, may be used as an alternative assay medium for toxicity studies.

Table 3. The effect of copper and lead on the cell division rate of *C pyrenoidosa* in different media

Metal conc. ($\mu g l^{-1}$)	Cell division rate (percentage of blank)		
	Enriched synthetic softwater	EPA medium	MBL medium
Copper			
10	78	—*	—
20	35	58	77
40	13	—	—
50	10	20	87
70	4	—	—
100	0	20	97
200	—	20	97
Lead			
10	103	—	—
20	88	—	—
40	78	—	—
60	73	—	—
100	77	—	—
150	81	—	—
200	83	—	—
400	100	—	—

*Not determined

Table 4. Blank cell division rates for *N closterium* and *C pyrenoidosa* in different media without toxic metal

Algae	Medium	Cell division rate per hour
N closterium	Seawater	0.009*
	Medium f	0 015
C pyrenoidosa	Enriched softwater	0 013†
	EPA medium	0.012
	MBL medium	0 014

*SD 0.002
†SD 0 001

Table 5. Concentrations of copper giving 50% reduction in cell division rate of *C. pyrenoidosa*

Medium	IC_{50} (μg Cu l^{-1})
Enriched softwater	16
EPA medium	24
MBL medium (minus EDTA)	>200

The toxicity of lead to *C. pyrenoidosa* was examined in enriched synthetic softwater (Table 3). Sixty micrograms of lead per litre gave a 73% reduction in cell division rate; however higher concentrations of lead were less toxic. This may have been due to precipitation of lead in the softwater. We measured electrochemically-labile lead by anodic stripping voltammetry, after assay flasks containing 50, 100 and 150 μg Pb l^{-1} in enriched synthetic softwater (with no algae) were stood on the light platform for 5 days (Table 6). We found that after 5 days ASV-labile lead, pH 7, was only 3–6% of the total lead added; with ASV-labile lead only slightly increasing with increasing total lead concentration. This suggests that most of the lead was in complexes which were not electrochemically available and probably non-toxic to *C. pyrenoidosa*. Hepes has been reported (Calbiochem, Calif., personal communication) to form very weak complexes with heavy metals. The compound had no effect on the ASV peak heights or peak potentials of copper, lead, cadmium and zinc in the pH range 5–9 It is difficult to explain the slight decrease in lead toxicity to *C. pyrenoidosa* at high total lead concentrations.

Nitzchia closterium

Cell division rates for *N. closterium* grown in the absence of metals in seawater and medium f are shown in Table 4. As found by Lumsden and Florence (1983), unenriched seawater can support the growth of *N. closterium* for at least 72 h. The cell division rate for this species is faster in nutrient-rich medium f.

Table 6 Electrochemically-labile lead in enriched synthetic softwater after 5 days

Total lead added initially ($\mu g\ l^{-1}$)	ASV-labile lead pH 7* ($\mu g\ l^{-1}$)
50	2 9
100	4 1
150	4 0

*Direct current stripping, peak area measurement

Table 7. The effect of copper and zinc on the cell division rate of *N. closterium* in different media

Metal conc. ($\mu g\ l^{-1}$)	Cell division rate (percentage of blank)	
	Seawater	Medium f
Copper		
10	50	97
20	39	—*
30	33	—
40	—	93
50	19	—
70	0	—
80	—	89
120	—	59
200	—	53
Zinc		
20	86	—
40	74	105
50	71	—
80	30	98
160	—	96
200	—	98
280	—	92
350	—	85
500	—	81
1000	—	38

*Not determined

The toxicity of copper and zinc to *N. closterium* depends on the composition of the assay medium (Table 7). In unenriched seawater, 10 μg Cu l^{-1} gives a 50% reduction in cell division rate, compared to >200 μg Cu l^{-1} in full nutrient medium f. Culture medium f also ameliorates zinc toxicity, with the IC_{50} increasing from 75 μg Zn l^{-1} in seawater to about 850 μg Zn l^{-1} in medium f.

Some reports in the literature of metal toxicity to marine algae have given erroneously high IC_{50} values because assays were carried out in full nutrient medium containing chelators. Samuel (1976) examined copper toxicity to *N. closterium* (previously named *Cylindrotheca closterium*) in medium f and found 50% reduction in growth rate between 50 and 100 μg Cu l^{-1} at initial cell densities of 5×10^4 cells ml^{-1}. Bentley-Mowat and Reid (1977) found concentrations of lead and copper as high as 5×10^{-4} M were required to inhibit the growth of *Phaeodactylum tricornutum*. *Tetraselmis* sp., *Dunaliella tertiolecta* and *Cricosphaera elongata* in S88 medium (Droop, 1968), containing 1.3×10^{-4} M EDTA. Other workers have reduced concentrations of EDTA, trace metals and iron in the assay medium, but neglected to consider silicate as an adsorbent (Mandelli, 1969; Fisher, 1981). By examining the effect of culture medium on electrochemically-labile copper, Lumsden and Florence (1983) showed that medium f has significant metal complexing capacity, largely due to colloidal ferric hydroxide and silicate. By using raw seawater as the assay medium, metal toxicity may be studied without interference from chelators in the culture medium (Kayser, 1976; Florence *et al.*, 1983).

Not only is the composition of the assay medium critical in determining metal toxicity, but also the maintenance culture medium used before the assay. Gentile *et al.* (1973) found up to five-fold differences in response of bioassay organisms maintained under high and natural nutrient levels. Stauber and Florence (1985a, b) showed that the toxicity of copper to *N. closterium* decreased as the concentration of iron, cobalt and manganese in the culture medium used before the assay increased. Although the inoculum was washed by centrifugation in seawater, metal(III) hydroxides adsorbed to the cells are carried over to the assay in seawater. They then adsorb copper and reduce copper penetration into the cell. The EPA (1976) recommends the test alga should go through two complete growth cycles in nutritionally dilute culture medium before use in the algal assay.

Use of dilute media such as synthetic softwater and seawater in short assays (3–4 days) overcomes the problems of metal complexation by chelators present in full nutrient medium and is much more relevant to the natural situation. Our cell division rate experiments with *C. pyrenoidosa* support the current EPA acute water quality criteria, based on 50% mortality to fish, of 12 μg Cu l^{-1} in softwaters (EPA, 1983). In seawater however, we found IC_{50} values of 10 μg Cu l^{-1} and 75 μg Zn l^{-1} for *N. closterium*, which were lower than the current EPA water quality criteria of 23 μg Cu l^{-1} and 170 μg Zn l^{-1}.

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Mechanism of toxicity of zinc to the marine diatom *Nitzschia closterium*

J. L. Stauber and T. M. Florence

CSIRO Centre for Advanced Analytical Chemistry, Division of Fuel Technology, Private Mail Bag 7, Menai, New South Wales 2234, Australia

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Abstract. The effect of zinc on cell division, photosynthesis, ultrastructure, respiration, ATP levels, mitochondrial electron-transport chain (ETC)-activity, total thiols and glutathione in the marine diatom *Nitzschia closterium* (Ehrenberg) W. Smith was investigated. Although 65 μg Zn l^{-1} halved the cell division rate, photosynthesis and respiration were unaffected by zinc concentrations up to 500 μg Zn l^{-1}. Most of the zinc associated with the cells was bound at the cell surface, with only 3 to 4% of this extracellular zinc penetrating the cell membrane. Once inside the cell, zinc exerted its toxicity at a number of sites. Increased ATP production and ETC activity were observed in zinc-treated cells. Zinc also enhanced cellular thiols (SH) and total glutathione, and zinc toxicity was reversible by the addition of thiol compounds such as cysteine. Zinc-thiol binding may be a detoxification mechanism for the cell. It is suggested that increased ATP production may provide the energy required for increased glutathione synthesis at the expense of other energy-requiring processes including cell division. The mechanisms of toxicity of ionic zinc and copper to *N. closterium* were compared.

Introduction

Large quantities of zinc from industrial and domestic sewage are released annually into coastal marine waters. Zinc in seawater is speciated partly as inorganic moieties (Zn^{2+}, $ZnOH^{+}$ and $ZnCO_3^{\circ}$), in contrast to copper which is present almost entirely in the organic form (Van den Berg 1982).

Recent studies have shown that zinc at low concentrations is essential in maintaining the stability of cell membranes, by either binding to structural components or preventing metal-catalysed lipid peroxidation (Chvapil 1973). At higher concentrations, however, zinc can be toxic to phytoplankton, leading to depressed cell-division rates, uncoupling of cell division and photosynthesis (Fisher and Jones 1981) and inhibition of fucoxanthin synthesis (Gillan et al. 1983). Despite these studies, our understanding of the mechanism of ionic zinc toxicity to marine phytoplankton remains poor.

In this paper we examine the effect of zinc on cell division, photosynthesis, ultrastructure, respiration, ATP levels, ETC activity, total thiols and glutathione in the marine diatom *Nitzschia closterium* (Ehrenberg) W. Smith. We also compare the mechanisms of zinc and copper toxicity to this species, and the reversal of toxicity by sulfhydryl compounds.

Materials and methods

Algae cultures

The unicellular marine diatom *Nitzschia closterium* (Ehrenberg) W. Smith (Hasle 1964) was cultured axenically in Medium *f* (Guillard and Ryther 1962) with the trace elements and iron concentration halved. The culture was maintained on a 12 h light:12 h dark cycle (Philips TL 40 W fluorescent white, 6400 lux) at 21 °C. Cells in log-phase growth were used for all experiments.

Measurement of cell-division rate

Toxicity of zinc, diamide and phenazine methosulfate to *Nitzschia closterium* was studied using algal assays in filtered (0.45 μm) unsupplemented seawater as described previously (Lumsden and Florence 1983). Cell density was measured initially and on three subsequent days by counting in a haemocytometer. A regression line was fitted to a plot of log (cell density − initial cell density) vs time, and cell division rate (μ) was determined from the slope.

Reversibility of zinc toxicity

After 48 h exposure to 120 μg Zn l^{-1}, which completely inhibited cell division, cysteine or cystine (5×10^{-5} *M*) was added to the assay flasks and cell-division rates were compared. Cultures not exposed to zinc were used as controls, with and without each test compound.

Preparation of cells for electron microscopy

Nitzschia closterium cells exposed to 500 μg Zn l^{-1} for 72 h were concentrated by gentle centrifugation (1000 × *g* for 1 min), fixed in 3% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.2) and 0.6 *M* sucrose for 1 h at room temperature, washed in buffer, and post-fixed in 1% OsO_4-0.1 *M* phosphate buffer for 1 h. Samples were dehydrated through a graded acetone series and embedded in Spurr's low-viscosity resin. Thin sections were cut using a diamond knife on a Reichert-Jung Ultratome 3, sequentially stained in uranyl acetate and lead citrate, and examined in a Philips 400 electron microscope.

Determination of intracellular and extracellular zinc

Nitzschia closterium cells were grown in seawater containing 100 μg Zn l^{-1} and 200 μg Zn l^{-1}, spiked with radioactive ^{65}Zn.

To determine total zinc associated with the cells (intracellular plus extracellular zinc), a 20 ml aliquot of the growth medium was filtered through a 0.45 μm membrane-filter. The filter was acid-digested in 2 ml of 15 *M* HNO_3 and 0.5 ml of 72% $HClO_4$, to strong fumes of $HClO_4$, and diluted to 5 ml with H_2O. Cellular zinc was determined by counting ^{65}Zn in a gamma-well counter.

To determine intracellular zinc, a second 20 ml cell aliquot was centrifuged and resuspended in 0.02 *M* EDTA in seawater for 20 min (Bates et al. 1985, Stauber and Florence 1985). Preliminary experiments showed that 97% of zinc adhering to the outside of the cells was removed with one wash in 0.02 *M* EDTA in seawater. The cells were filtered, acid-digested, and counted in a gamma-well counter to determine intracellular zinc. Extracellular zinc was calculated by subtracting intracellular zinc from total zinc associated with the cells.

A significant fraction of the zinc adhered to the glass walls of the culture flasks. The flasks were rinsed with 5 ml of warm 1 *M* HNO_3, which was then counted in a gamma-well counter to ensure that all the radioactive zinc added was recovered.

Measurement of photosynthesis

Photosynthesis after 24 h exposure to zinc was determined by uptake of carbon-14 carbonate, followed by removal of excess $H^{14}CO_3^-$ in acid solution, and liquid scintillation-counting in Instagel (Lumsden and Florence 1983). Results were expressed as μmol CO_2 fixed h^{-1} 10^{-6} cells.

Measurement of respiration

Algal dark respiration rates (μmol O_2 h^{-1} 10^{-6} cells) were determined in a sealed biological oxygen-demand bottle using an Orion Research Oxygen Electrode. Respiration was measured initially for 30 min and at 2, 4, 6, 24 and 48 h after exposure to 500 μg Zn l^{-1}.

Determination of ATP

ATP was determined using a modified method of Holm-Hansen and Booth (1966). After a 24 h treatment with 200 μg Zn l^{-1}, ATP was extracted from the cells in boiling Tris buffer (0.025 *M*, pH 7.75). An aliquot of cell extract was added to 4 ml of Sigma firefly extract (reconstituted in buffer) to a final volume of 10 ml in Tris buffer, and light output was measured on a Packard liquid scintillation-counter. ATP in the cells, with correction for a firefly extract blank, was determined from an ATP standard curve (area under the light decay curve vs ATP concentration).

Determination of electron-transport chain (ETC) activity

Mitochondrial ETC activity in *Nitzschia closterium* was determined using an artifical electron acceptor, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), according to Kenner and Ahmed (1975). Cells exposed to zinc for 24 h were centrifuged and homogenised for 2 min in 6 ml cold buffer (0.05 *M* phosphate, pH 8.0, 75 μ*M* $MgSO_4$, 0.2% Triton X-100 and 0.15% polyvinylpyrrolidone). Extracts were centrifuged and assayed at 25 °C using 1 ml extract, 3 ml substrate buffer (0.05 *M* phosphate, pH 8.0, 0.2% Triton X-100, 0.133*M* disodium succinate, 8.35×10^{-4} *M* NADH and 2.4×10^{-4} *M* NADPH) and 1 ml of 2.5×10^{-3} *M* INT. After 15 min, the reaction was stopped with 1 ml of a 1:1 mixture of 1 *M* sodium formate (pH 3.5) and formalin. The absorbance of INT-formazan was measured at 490 nm and corrected for a boiled homogenate blank which was assayed in the same way. ETC activity was expressed as nmol formazan per 10^6 cells.

Determination of cellular thiols (SH groups)

Cellular thiol groups in control cells and in cells after 24 h exposure to 500 μg Zn l^{-1} or 175 μg Cu l^{-1} were determined using the method of Grassetti and Murray (1967), modified by Stauber and Florence (1986). Algae were centrifuged, washed, and resuspended in 3 ml of 1 *M* phosphate buffer (pH 7.2), 3 ml of fresh 2,2'-dithiodipyridine (2×10^{-3} *M*) were added, and the cells were vortexed vigorously to ensure complete cell breakage. After 30 min, cell debris was removed by centrifugation and the absorbance of the supernatant was measured at 342 and 233 nm. 2,2'-dithiodipyridine reacts with thiols to form 2-thiopyridone with a change in the UV absorption spectrum. Absorbance at 342 nm was corrected for an algal blank, without dithiodipyridine treatment, and for a dithiodipyridine blank in phosphate buffer. The absorbance of this blank at 233 nm was used to correct for unused dithiodipyridine in each sample. A freshly prepared cysteine standard (6×10^{-5} *M*) was used for calibration.

Determination of total glutathione

Total glutathione (GSSG + GSH) in control cells and in cells exposed to 500 μg Zn l^{-1} for 24 h was determined using a modification of the enzyme recycling method of Tietze (1969). Cell aliquots (90 ml) were centrifuged and resuspended in a mixture of 1 ml of seawater, 1 ml of 0.02 *M* HCl and 1 ml of 10% 5-sulfosalicylic acid. Each extract was ground in a hand-held tissue homogenizer, vortexed and centrifuged. Supernatant (0.4 ml) was used for the total glutathione determinations. To a 5 ml volumetric flask was added, 0.87 ml of 1 mg ml^{-1} NADPH, 0.5 ml of 0.006 *M* 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and 1 ml of buffer (0.143 *M* NaH_2PO_4/6.3×10^{-3} *M* EDTA, pH 7.5). The mixture was incubated for 5 min at 30 °C before the addition of 0.4 ml supernatant. The volume was made up to 5 ml with buffer, and the reaction was started with the addition of 0.22 ml of 33 U ml^{-1} glutathione reductase (GR) (Sigma Type III from bakers' yeast). The rate of formation of 5-thio-2-nitrobenzoic acid (TNB), which is proportional to the total GSH concentration, was followed spectrophotometrically at 412 nm for 10 min (Anderson 1985). The reactions are described by the following equations:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+, \quad (1)$$

$$2\,GSH + DTNB \rightarrow GSSG + 2\,TNB \quad (2)$$

Both the blank and GSH standards (0.2 to 1 nmol GSH) contained the same amount of sulfosalicylic acid as the samples. An aliquot of each sample was also spiked with 0.5 nmol GSH in order to correct for inhibition/enhancement of TNB formation due to the sample matrix.

Measurement of glutathione reductase activity

The effect of zinc and copper (4×10^{-5} *M*) on glutathione reductase was determined by measuring the rate of disappearance of NADPH spectrophotometrically at 340 nm The assay was carried out in 0 1 *M* phosphate buffer (pH 7.5) with the addition of 10^{-4} *M* NADPH, $3\ 26 \times 10^{-3}$ *M* GSSG, 4×10^{-5} *M* Zn or Cu and 0 05 U ml^{-1} glutathione reductase (Colowick and Kaplan 1955) Results were expressed as percentage inhibition of glutathione reductase in the presence of Zn or Cu compared to controls

Results

Growth

The effect of ionic zinc on the cell-division rate of *Nitzschia closterium*, expressed as a percentage of the rate in the absence of zinc, is shown in Table 1. By interpolation, a zinc concentration of 65 $\mu g\ l^{-1}$ was required to produce a 50% reduction in cell division rate (IC_{50}), similar to the IC_{50} of 60 μg Zn l^{-1} reported by Zhang and Florence (1987) for the same species. Higher concentrations of zinc (850 μg Zn l^{-1}) were required for a 50% reduction in the cell division rate of *N closterium* when assayed in Nutrient Medium *f*, containing chelators (Stauber and Florence 1989). In the absence of zinc, *N. closterium* has a cell division rate per hour of 0.009 ± 0.002 in unsupplemented seawater (Stauber and Florence 1989)

Table 1. *Nitzschia closterium* Effect of zinc on cell division rate over 4 d

Zn conc ($\mu g\ l^{-1}$)	Cell division rate (% of control)
0	100
20	86
40	74
50	71
80	30
100	0

Table 2. *Nitzschia closterium* Accumulation of zinc in cells after 4 d Values $\pm$ 1 SD

Added Zn ($\mu g\ l^{-1}$)	Intracellular Zn (ng 10^{-6} cells)	Extracellular zinc (ng 10^{-6} cells)
100	$8\ 6 \pm 1\ 0$	294 ± 47
200	$24\ 3 \pm 5\ 6$	628 ± 195

Table 3. *Nitzschia closterium* Effect of zinc on photosynthesis after 24 h exposure Values $\pm$ 1 SD

Treatment (μg Zn l^{-1})	Photosynthesis ($\mu mol\ CO_2/h/10^6$ cells)
Untreated cells	$0\ 079 \pm 0.006$
200	0.090 ± 0.006
500	$0\ 092 \pm 0\ 006$

Zinc accumulation

Intracellular and extracellular zinc in *Nitzschia closterium* after 4 d growth in seawater are shown in Table 2 Both intracellular and extracellular zinc increased (2.8 and 2.1 ×, respectively) as the total added zinc concentration was doubled. Most of the zinc associated with the cells was bound at the cell surface, 80% of this extracellular zinc was removed by three successive washes with seawater (each for 20 min) Only 3 to 4% of zinc associated with the cells was not removed by EDTA treatment and was considered to be intracellular zinc

Photosynthesis

The effect of 24 h exposure to zinc on photosynthesis in *Nitzschia closterium* is shown in Table 3. Zinc concentrations up to 500 $\mu g\ l^{-1}$, which also strongly inhibited cell division after 24 h, had no significant effect on photosynthesis. This decoupling of cell division and photosynthesis was also found after exposure of *N. closterium* to ionic copper and copper complexes (Stauber and Florence 1987) and to zinc and copper-treated cells of the diatom *Asterionella japonica* (Fisher and Jones 1981)

Ultrastructure

No effect of zinc on the ultrastructure of the nucleus, mitochondrion or cell membrane of *Nitzschia closterium* was observed. Zinc-treated cells (500 μg Zn l^{-1} for 3 d) were sometimes larger than control cells, and this enlargement was not due to an increase in the size of the cell vacuoles. Chloroplasts of zinc-treated cells were occasionally larger, with more thylakoid membranes. It is possible that cells respond to zinc stress by synthesizing more thylakoids to maintain photosynthetic rates

Attempts to measure where zinc was localized in the cell, using X-ray spectra, were unsuccessful, as intracellular zinc was below background levels

Respiration, ATP and ETC activity

Zinc (500 $\mu g\ l^{-1}$) did not significantly enhance respiration in *Nitzschia closterium* after 24 or 48 h exposure (Table 4). However, zinc did significantly increase ATP levels and ETC activity after 24 h (Table 5). Zinc may alter the activity of the dehydrogenases which funnel electrons into the ETC, or alter components of the ETC itself, leading to greater electron flow to INT, between Cytochrome b and Cytochrome c_1. As ATP formation is coupled to the ETC, ATP levels in the cell also increase Copper-ethylxanthogenate, H_2O_2 and catalase had similar effects on ATP and ETC activity in *N closterium* (Stauber and Florence 1987).

Table 4. *Nitzschia closterium* Effect of zinc on respiration Values ± 1 SD

Treatment (μg Zn l^{-1})	Respiration (μmol O_2 h^{-1} 10^{-6} cells)		
	0 h	24 h	48 h
Untreated cells	0.032 ± 0 003	0 046 ± 0 005	0 071 ± 0 011
500	0 032 ± 0.003	0 054 ± 0 008	0 084 ± 0.011

Table 5. *Nitzschia closterium* Effect of zinc on ATP and on mitochondrial electron-transport chain (ETC) activity after 24 h exposure Values ± 1 SD

Treatment (μg Zn l^{-1})	ATP (μg 10^{-6} cells)	ETC activity (nmol formazan 10^{-6} cells)
Untreated cells	0 018 ± 0 003	17.8 ± 1 1
200	0 026 ± 0 003	42.5 ± 3 4
500	ND	41 9 ± 1 7

Table 6. *Nitzschia closterium.* Effect of zinc and copper on thiols and total gluthathione after 24 h exposure. Values ± 1 SD

Treatment (μg l^{-1})	Cellular thiols (nmol SH 10^{-6} cells)	Total glutathione (nmol 10^{-6} cells)
Untreated cells	1.29 ± 0 04	0 061 ± 0.008
500 μg Zn	4 01 ± 1.27	0 109 ± 0.011
175 μg Cu	0 76 ± 0 18	0.061 ± 0.009

Table. 7. *Nitzschia closterium* Reversal of zinc toxicity to the diatom by cysteine. Cells were exposed to 120 μg Zn l^{-1} for 48 h before addition of cysteine-HCl or cystine (5×10^{-5} *M*). Growth factor is ratio of final cell density after 96 h to cell density at 48 h when cysteine or cystine were added

Zn (μg l^{-1})	Compound added	Growth factor
0	none	1 7
0	cysteine	1 6
0	cystine	1 6
120	none	1 0
120	cysteine	2 7
120	cystine	1 0

Cellular thiols, total glutathione and glutathione reductase

Zinc-treated cells had higher concentrations of cellular thiols (SH) and total glutathione than untreated cells (Table 6). Zinc can bind, but cannot oxidize, SH. The zinc:SH ratio in the cell was ~0.23.

Copper however, oxidized cellular thiols, leading to a lowering of total thiols in the cell. Copper reacts with reduced glutathione (GSH), according to the following reaction (Stauber and Florence 1986):

$$4\,GSH + 2\,Cu^{2+} \rightarrow 2\,Cu\text{-}SG + GSSG + 4\,H^{+} \quad (3)$$

However, copper had no effect on total glutathione in the cell (Table 6) as measured by the glutathione reductase (GR) recycling assay This is because 2 Cu-SG formed in Reaction (3), can still react with NADPH in the presence of GR to form GSH, which then reacts with DTNB in Reaction (2). To be sure this was the case, we reacted 0.4 nmol GSH with 0.2 nmol Cu, and then compared total glutathione produced in the recycling assay with that produced by the same amount of GSH in the absence of Cu Total glutathione in the absence and presence of Cu was the same. Thus, although copper oxidizes and therefore lowers total SH in the cell, it will not affect total cellular glutathione as long as NADPH and GR are available.

Glutathione only accounted for 3 to 4% of total cellular thiols. It is possible that the glutathione assay only measured glutathione in the cytosol and not glutathione associated with the cell membrane, as cells were lysed and removed prior to the assay with DTNB. In the total thiol method, however, 2,2'-dithiodipyridine (which penetrates the cell) was reacted with intact cells, so that both intracellular thiols and thiols associated with the cell membrane were determined Neither zinc nor copper interfered with either method. The addition of EDTA to the glutathione assay prevented any inhibition of GR by zinc or copper

In a separate experiment, we examined the effect of zinc and copper on glutathione reductase. Zinc (4×10^{-5} *M*) did not inhibit GR (1 U ml^{-1}) At lower concentrations of GR (0.05 U ml^{-1}), zinc gave slight inhibition (19%) compared to 80% inhibition by 4×10^{-5} *M* copper.

Reversal of zinc toxicity

Zinc toxicity to *Nitzschia closterium* was reversed by the addition of 5×10^{-5} *M* cysteine, but not by cystine, its oxidized form (Table 7). We assumed previously (Stauber and Florence 1986) that reversal of copper toxicity by cysteine and GSH was due to thiol-disulphide interchange reactions. Zinc is known to form 1:1 chelates with cysteine (Jocelyn 1972). It may be that addition of cysteine to *N. closterium* overcomes zinc toxicity by more strongly binding zinc than cellular non-glutathione thiols and thereby increasing the concentration of free SH in the cell.

Discussion and conclusions

As the total concentration of zinc in the assay medium increased, both intracellular and extracellular zinc increased, and the cell division rate of *Nitzschia closterium* decreased. Davies (1973, 1978) reported that zinc uptake in the marine diatom *Phaeodactylum tricornutum* was due to rapid absorption of zinc on to external cell-binding sites followed by passive diffusion-controlled transport into the cytoplasm at a rate proportional to the concentration gradient between extracellular zinc and zinc inside the membrane. Davies suggested that most of the intracellular zinc was bound to protein, the maximal binding

capacity of which indirectly regulated zinc uptake. Bates et al (1985) suggested that intracellular zinc in *Chlamydomonas variabilis* was bound to polyphosphate, as concentrations of cell polyphosphate increased with increasing zinc concentration in the medium.

Once inside the cell, zinc may exert its toxicity at a number of sites Although 65 μg Zn l^{-1} strongly inhibited cell division in *Nitzschia closterium* after 24 h, photosynthesis was unaffected by zinc concentrations up to 500 μg Zn l^{-1} Photosynthetic products accumulated, leading to enlarged cells unable to divide. This uncoupling of growth from cell division has also been reported for copper-treated cells of *N closterium* (Stauber and Florence 1987), zinc and copper-treated cells of the diatom *Asterionella japonica* (Fisher and Jones 1981), and mercury-treated *Dunaliella tertiolecta* and *Isochrysis galbana* (Davies 1976).

Some zinc-treated cells did contain more thylakoids in the chloroplast than control cells, although photosynthetic rates were similar Zinc has been shown to decrease production of photosynthetic pigments in other species. Zinc inhibited the biosynthesis of fucoxanthin in *Asterionella japonica* grown for 8 d in 60 μg Zn l^{-1} (Gillan et al. 1983). Bates et al. (1985) found that zinc-grown cells contained less chlorophyll *a* than control cells, and that chlorophyll *a* decreased with increasing zinc concentration

Cell division in *Nitzschia closterium* was less sensitive to zinc than ionic copper, with IC_{50} s of 65 μg Zn l^{-1} and 10 μg Cu l^{-1} Whereas copper had no effect on mitochondrial processes (Stauber and Florence 1987), zinc increased ATP production and mitochondrial ETC activity, with no significant effect on dark-respiration rates Zinc has been shown to alter the structure of mitochondria from gill-epithelium cells of the dogfish *Scyliorhinus canicula* by Crespo and Sala 1986, who suggested that these alterations may impair oxidative phosphorylation. Zinc however had no such effect on *N closterium*, in which no ultrastructural changes to mitochondria were detected by transmission electron microscopy, and mitochrondrial processes were enhanced, rather than inhibited.

Metals such as zinc and copper may exert their toxicity by altering total thiols and, in particular, reduced glutathione (GSH), in the cell. Glutathione is the major non-protein thiol in animals, accounting for over 90% of the cell thiol content (Meister 1981) The concentration of glutathione in algae, however, has been less well studied Tel-Or et al. (1985) reported that two blue-green algae, *Synechococcus sp* and *Nostoc muscorum*, had intracellular total glutathione concentrations of 2 to 5 m*M*, with 89% present as GSH and 11% as oxidized glutathione disulphide (GSSG). Slightly lower concentrations of GSH (241 to 958 nmol 10^{-9} cells or 1 to 2 m*M*) and GSSG (25 to 140 nmol 10^{-9} cells) were reported in *Euglena gracilis* Strain *z*, depending on light conditions during growth (Shigeoka et al. 1987) We found not only lower concentrations of total glutathione (60 to 100 nmol 10^{-9} cells) in *Nitzschia closterium*, but that glutathione accounted for only 3 to 4% of the total cellular thiols in this species

Although copper had no effect on total glutathione in the cell, copper did decrease total -SH in *Nitzschia closterium*. We previously proposed that the primary toxic effect of copper was due to a cytosolic reaction between copper and GSH, leading to a lowering of the GSH:GSSG ratio, and suppression of mitosis (Stauber and Florence, 1987) The hypothesis that GSH is critical for cell division is further supported by the fact that diamide (10^{-4} *M*), a thiol oxidizing-agent specific to GSH in living cells, completely inhibited cell division in *N closterium*. Cell division was also completely inhibited by phenazine methosulfate (10^{-5} *M*), a compound which oxidizes NADPH, the cofactor for enzymatic formation of GSH. Nath and Rebhun (1976) also found that diamide and phenazine methosulfate inhibited cell division in eggs of the sea urchin *Strongylocentrotus purpuratus* In an excellent review, they suggested several possible mechanisms by which GSH acts on target systems involved in mitosis Both regulation of calcium uptake (via Ca^{2+}-activated ATPase) necessary for cell division. and the tubulin molecule itself, may be regulated by reduction and oxidation of sulfhydryls. In addition, accumulation of GSSG, when glutathione reductase is inhibited by copper, may shut off protein synthesis which. in turn, inhibits cell division.

Whereas oxidation of thiols, particularly GSH, may be the primary toxic effect of copper, zinc-thiol binding may be a detoxification mechanism (Viarengo 1985) Zinc-treated cells contained more total thiol and more total glutathione than untreated cells This is consistent with induction of synthesis of phytochelatins of the sort described for heavy metals (including zinc) in higher plants (Grill et al 1985, 1987) These small cysteine-rich peptides may be formed from glutathione or its precursor γ-glutamyl cysteine, as phytochelatin formation was markedly reduced when γ-glutamyl cysteine synthetase was inhibited (Grill et al. 1987)

A similar increase in glutathione in cadmium-exposed fish has been reported by Thomas et al (1982). The activity of γ-glutamyl cysteine-synthetase, one enzyme which regulates GSH, is dependent on GSH concentration (Meister 1981) Zinc, by binding GSH, may reduce the amount of free, active GSH, and indirectly stimulate the enzyme to produce more GSH to compensate. Increased ATP production in zinc-treated cells could provide the energy required for increased GSH synthesis, at the expense of other energy-requiring processes including cell division. In addition, the cell can reduce GSSG to GSH via glutathione reductase, which is only slightly inhibited by zinc.

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Fumed silica for the direct determination of lead in urine by differential-pulse anodic stripping voltammetry

J.L. Stauber * and T.M. Florence

CSIRO Centre for Advanced Analytical Chemistry, Division of Fuel Technology, PMB 7, Menai, N S W (Australia)

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Abstract

The use of fumed silica for the direct determination of lead in urine by differential-pulse anodic stripping voltammetry was investigated. Fumed silica, added to urine prior to the nitrogen purge step, completely removed sorption interferences by urinary organic constituents. Values for lead in urine from eight unexposed individuals were 3 ± 2 μg l^{-1} or 3 ± 2 μg g^{-1} creatinine. This method is a fast, simple and effective means for the accurate determination of lead in undiluted urine without pretreatment.

Keywords Lead, Urine, Fumed silica

The determination of lead in urine is a valuable diagnostic tool in occupational health monitoring for assessing lead exposure. In particular, urinary lead provides the only analytical criterion for exposure to alkyllead compounds [1].

Electrochemical methods such as anodic stripping voltammetry (ASV) have been widely used for measuring toxic metals in body fluids, including blood [2], sweat [3] and urine [4,5], owing to their high sensitivity and ability to determine a number of metals simultaneously. However, the direct determination of metals in urine poses considerable problems in electroanalysis owing to its high organic content, which leads to adsorption at the electrode and suppression of ASV waves.

Most methods for urinary lead determination include a pretreatment step to remove the organic interferences. Wet digestion [6] is time consuming, reduces the sensitivity by increasing the blank and involves a high risk of contamination of the sample. Bond and Reust [5] proposed pretreating urine by passing it through a Sep-Pak C_{18} column. However, unless the Sep-Pak column is first acid-washed, contamination of the sample is again a problem.

The direct determination of lead in urine, without pretreatment, is therefore desirable. Lund and Eriksen [4] heated urine to 40 °C by circulating thermostated water in the jacket surrounding the electrochemical cell, then determined lead by ASV. However, this method was less sensitive and less precise than urine pretreatment by wet digestion. Hoyer et al. [7] used a Nafion-coated thin mercury film electrode. Direct analysis of undiluted urine was not possible, but when urine was diluted 1/3 reasonable ASV waves were obtained, although the sensitivity was reduced.

Recently, Kubiak and Wang [8] used fumed silica to remove interferences by organic surfactants from aqueous solutions for ASV. The adsorptive properties associated with the large specific surface area (255–400 m^2 g^{-1}) of the silica microparticles were exploited for the rapid removal of surfactants from solution during the nitrogen purge step. In this paper we describe the use of fumed silica to remove organic and surfac-

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tant interferences from urine so as to permit the direct ASV determination of lead in urine without pretreatment.

EXPERIMENTAL

Apparatus

Electrochemical analysis of urine was done with a hanging mercury drop electrode, using an EG&G Princeton Applied Research Model 384 polarographic analyser, with a Model 303A static mercury drop electrode assembly and a Model 305 stirrer.

Chemicals

All solutions were prepared using Merck Suprapur reagents and water obtained using a Milli-Q system (Millipore). Lead stock solutions were prepared by diluting a lead standard (1.0 mg Pb ml^{-1} in 1.0 M HNO_3; BDH Chemicals Australia). Fumed silica was obtained from Sigma (No. S-5005, particle size 0.007 μm; specific surface area 400 m^2 g^{-1}). All analytical procedures were carried out at 25 ± 0.5 °C in a Class 100 clean room.

Procedures

Mid-stream urine samples were collected in acid-washed plastic beakers. An aliquot (0.2 ml) was diluted to 5 ml with water for creatinine determination using Sigma Kit Cat. No. 555-A. The remaining urine was acidified to 0.1 M HNO_3. A 1-ml volume of 3 M sodium acetate buffer (pH 4.7) and 100 mg of fumed silica were added to 10 ml of acidified urine in the ASV cell. Lead in urine was determined, after deaeration for 8 min, by differential-pulse ASV. The samples were stirred during deposition and stripped in quiescent solution. The instrumental conditions were as follows: deposition potential, −0.8 V (vs. Ag/AgCl), final potential, −0.1 V; deposition time, 5 min; equilibration time, 30 s; pulse height, 0 025 V; scan rate, 2.0 mV s^{-1}; scan increment, 2 mV; drop, medium; and stirrer speed, slow.

Lead in urine was determined quantitatively by the method of standard addition, using 0.1 ml of 10^{-5} M Pb standard solution. The recovery of lead was determined by spiking urine with 0 05 ml of 10^{-5} M Pb solution before addition of acetate buffer and fumed silica. Lead in a blank, containing 10 ml of 0.1 M HNO_3, 1 ml of acetate buffer and 100 mg of fumed silica, was measured in the same way.

Urine samples from eight non-exposed individuals were analysed in triplicate, together with a certified human urine standard (Seronorm Trace Elements Urine, Batch No. 108, containing 88 μg Pb l^{-1}, obtained from Nycomed Diagnostics, Norway). Between samples, the cell was rinsed successively with ethanol, 2 M HNO_3 and water. Each urine sample, including one standard addition, required an analysis time of 35 min, not including replicates.

The direct determination of lead in urine by the fumed silica method was compared with the results for lead in urine after pretreatment using a modification of the method of Bond and Reust [5] A 15-ml volume of acidified urine (0.1 M HNO_3) was passed through a Sep-Pak C_{18} cartridge (Waters) using an acid-washed 20-ml disposable syringe. The cartridge was pretreated with 5 ml of methanol, rinsed with 5 ml of Milli-Q water and then washed with 10 ml of 0.1 M HNO_3 and 20 ml of Milli-Q water to remove metal impurities. The outflow (10 ml) was collected in an acid-washed plastic container and either transferred to the ASV cell or filtered through an acid-washed 0.45-μm filter (Millex; Millipore). Lead in the treated urine was determined by ASV using the same conditions as above.

RESULTS AND DISCUSSION

Without the addition of fumed silica, the direct electrochemical determination of lead in urine poses considerable problems, largely because of adsorption of proteins and other organic constituents on the electrode surface. The signals were often depressed or masked by unidentified peaks, probably due to redox processes or adsorption/desorption of urine constituents (Fig. 1). The addition of 100 mg of fumed silica directly to the ASV cell before the nitrogen purge step eliminated adsorption and surfactant interferences and per-

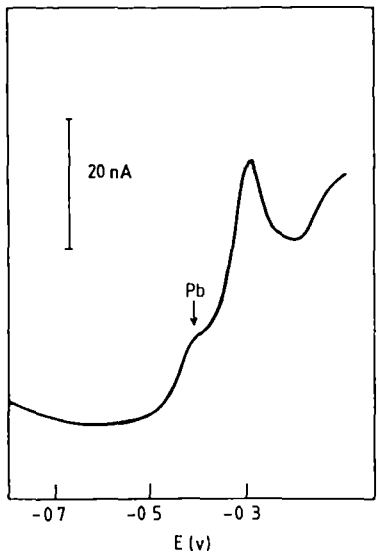

Fig 1 Differential-pulse anodic stripping voltammogram of lead in 10 ml of untreated urine (pH 4 7) without fumed silica

mitted the direct determination of lead in untreated urine. Figure 2 shows a typical voltammogram used for the determination of lead in 10 ml of urine (pH 4.7) after the addition of fumed silica. The lead peak (−0.42 V) was well separated and reproducible; the relative standard deviation

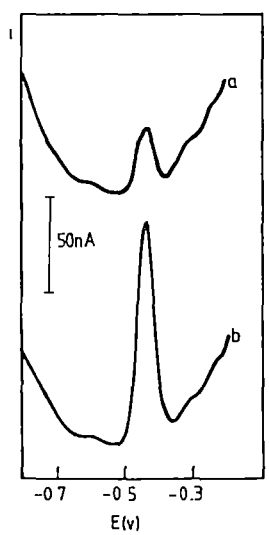

Fig 2. Differential-pulse anodic stripping voltammograms of lead in 10 ml of urine (pH 4 7) plus 100 mg of fumed silica (a) Urine, (b) urine plus 20 μg Pb l^{-1}

for six replicate runs was 7%, at the 6 μg l^{-1} level. The lead peak was independent of the deposition potential from −0.8 to −1.3 V and the peak height increased linearly with deposition time.

When a standard lead solution was added to the urine sample, a linear increase in peak height with concentration was observed. The recovery of lead from a urine sample spiked initially with 5×10^{-8} M Pb was 98–100%.

Blanks (10 ml of 0.1 M HNO_3–0.3 M acetate buffer and 100 mg of fumed silica) gave a value of 0.7 ± 0.1 μg Pb l^{-1}. The fumed silica did not contribute significantly to the blank.

The accuracy of the fumed silica method was further assessed by using the certified 88 μg Pb l^{-1} urine standard. A value of 85 μg Pb l^{-1} was found.

The fumed silica method is a simple, precise and accurate method for the determination of lead in urine at the μg l^{-1} level. It has been used to measure lead in urine from eight unexposed individuals (five males and three females). The average urinary lead value was 3 ± 2 μg l^{-1} (range 1.0–6.5) or 3 ± 2 μg g^{-1} creatinine, with no significant difference between males and females Urinary lead was corrected for creatinine because 24-h urine samples were not collected. Lower values for lead in urine were found than have been reported previously. With the increasing use of unleaded petrol, together with improved analytical techniques, urinary lead values have decreased in recent years. Haeger-Aronsen [9] obtained values of 14 ± 9 μg Pb l^{-1} urine or 8.6 ± 5.6 μg Pb g^{-1} creatinine in unexposed subjects. Tsuchiya et al. [10] found a mean of 12 μg Pb l^{-1} for a group of 2300 policeman in Japan. More recently, Hoyer et al. [7] found 5.2 μg Pb l^{-1} in one urine sample using a Nafion-coated thin mercury film electrode.

In a number of urine samples values for lead obtained with the fumed silica method were compared with those for urine pretreated by passage through a Sep-Pak cartridge. Using the method of Bond and Reust [5], gross contamination of the urine sample occurred owing to lead present in the Sep-Pak cartridge and Millex filter. The values for lead were frequently four times higher than in the fumed silica method. Previously more reliable results were obtained when the Sep-Pak cartridge

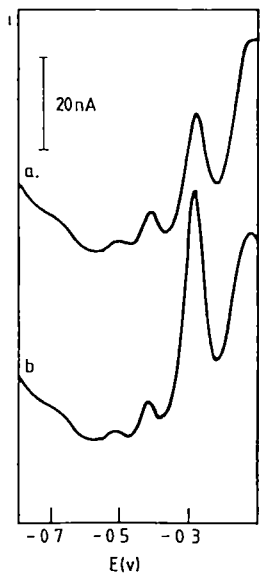

Fig 3 Differential-pulse anodic stripping voltammograms of lead and riboflavin in 10 ml of urine (pH 4 7) plus 100 mg of fumed silica (a) Urine, (b) urine plus 2×10^{-6} M riboflavin.

was washed with dilute acid and the Millex filtration step was omitted [11]. However, even when the Sep-Pak cartridge was rinsed with 0.1 M HNO_3 (which hydrolyses the C_{18} groups but does not affect the ability to remove organics from urine), the lead concentration found was still slightly higher than that given by the fumed silica method. The lead concentration determined in a blank (0.1 M HNO_3 which had been passed through an acid-washed Sep-Pak cartridge) was 1.5 μg Pb l^{-1}, twice that obtained using the fumed silica method.

Initially we hoped that the method might be applicable to the simultaneous determination of other metals such as cadmium and copper in urine. However, at this pH (4.7) no sensible recovery of copper spikes was obtained, possibly owing to solution chemistry effects [7]. Although a cadium peak was often observed, accurate determination of cadium was difficult.

Urine from one subject who was taking large doses of B and C group vitamins gave an additional ASV peak at -0.288 V. A smaller peak at the same potential was also observed in other subjects whether or not they were taking dietary vitamin supplements. The peak was identified as riboflavin and increased proportionally with standard addition of riboflavin (Fig. 3). Dietary supplements of up to 25 mg of riboflavin per day did not interfere with the determination of lead in urine. However, supplements of 50 mg of riboflavin per day led to a large riboflavin ASV peak which interfered with the lead wave. This problem was eliminated if the subject's urine was collected at least 24 h after the last riboflavin dietary supplement was taken.

The application of fumed silica is a fast, simple and effective approach for eliminating matrix interferences and enabling the direct determination of lead in urine without pretreatment. The alternative procedure of direct graphite furnace atomic absorption spectrometry has a detection limit for lead in urine of 5 μg l^{-1}, which is insufficiently sensitive for analysing samples from unexposed individuals: to reach the unexposed level a concentration extraction procedure is required [12]. The application of the technique to the determination of other metals in a variety of body fluids, including serum, is being investigated.

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TOXICITY OF LIPID-SOLUBLE COPPER(II) COMPLEXES TO THE MARINE DIATOM *NITZSCHIA CLOSTERIUM*: AMELIORATION BY HUMIC SUBSTANCES

T. M. FLORENCE[1], H. K. J. POWELL[2]*, J. L. STAUBER[1] and R. M. TOWN[2]†
[1]CSIRO Division of Coal and Energy Technology, Private Mail Bag 7, Menai, NSW 2234, Australia and
[2]Department of Chemistry, University of Canterbury, Christchurch, New Zealand

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Abstract—Algal assays, using the marine diatom *Nitzschia closterium*, have established that humic acid (5 mg kg^{-1}) can ameliorate the toxicity of the lipophilic complex $Cu(oxine)_2$ (3×10^{-8} mol l^{-1} in unsupplemented seawater). The toxicity of $Cu(PAN)_2$ is not ameliorated [PAN = 1-(2-pyridylazo)-2-napthol]. In conjunction with previous visible absorption spectrophotometry and polarographic measurements it was established that humic acid sequesters copper(II) from the hydrophobic complexes, releasing a ligand molecule The copper(II) toxicity may be ameliorated provided the ligand itself is not toxic

Fulvic acid was significantly less effective in ameliorating toxicity. Because of the significant competition from Ca(II) and Mg(II) in seawater, it is inferred that humic substances may be more effective in ameliorating toxicity of hydrophobic copper complexes in fresh water.

Key words—copper complexes, toxicity, algae, humic acid, fulvic acid, hydrophobic, seawater

INTRODUCTION

It is of environmental interest to study the toxic forms of metals and how this toxicity may be ameliorated in a natural system Organisms have evolved mechanisms to maintain low intracellular concentrations of toxic substances, both metals and organic substrates. These strategies include: (i) active expulsion of toxicants after they have entered the cell; (ii) complexation of metals by biologically synthesized ligands; and (iii) oxidation, reduction or chemical modification of the xenobiotic, resulting in precipitation, immobilization or volatilization (Folsom *et al.*, 1986). For example, *Nitzschia closterium* at a cell density of $2–4 \times 10^4$ cells ml^{-1} produces an exudate which can complex about 3.1×10^{-7} mol l^{-1} copper(II). This exudate is only produced in response to aqueous copper(II) ions and the amount excreted increases with the concentration of copper(II) (Lumsden and Florence, 1983). There is evidence that 5×10^{-11} mol l^{-1} is the threshold concentration of free copper ions that algal species try to maintain by excreting chelating agents (van den Berg, 1979).

Copper ion toxicity to *N. closterium* is also reduced by adsorption of copper on Mn(III) and Fe(III) hydroxide coatings on the cell surface (Stauber and Florence, 1985a, b). This reduces Cu(II) penetration into cells. Manganese is more effective than iron, possibly because it can also catalytically scavenge toxic superoxide free radicals. The ability of a range of metal ions to protect against copper toxicity to *N. closterium* was studied by Stauber and Florence (1987), viz. Mn(III), Co(III), Al(III), Fe(III), Cr(III), Ni(II) and Zn(II). The trivalent ions were more effective; indeed, nickel and zinc were unable to reduce copper toxicity. The insolubility of the metal(III) hydroxide provides an indication of its ability to ameliorate copper ion toxicity. However, concentrations of inorganic adsorbers such as Fe and Mn oxyhydroxides are usually very low in open-ocean waters (Hirose, 1990).

It has been assumed that the "free" metal ion is the most toxic form (Magnuson *et al.*, 1979). Thus, complexation of Cu(II) by water-soluble ligands reduces its toxicity. Recently, however, the extreme toxicity of hydrophobic metal complexes to marine organisms has been reported (Florence and Stauber, 1986) (Table 1). Neither algal exudates nor metal hydroxide coatings can inhibit the toxicity of lipid-soluble copper complexes.

A high toxicity index implies that the complex is more toxic than inorganic aqueous copper(II). Lipid solubility and strong chelation with copper are essential for high toxicity. For example, copper(II) complexes of oxine, PAN and TAN were exceptionally toxic to *N. closterium*, whereas the non-chelating isomers of these ligands [4-hydroxyquinoline, 1-(3-pyridylazo)-2-naphthol and 1-(3-thiazolylazo)-2-naphthol] did not enhance copper

*Author to whom all correspondence should be addressed.
†Present address: Department of Inorganic, Analytical and Applied Chemistry, University of Geneva, 30 Quai Ernest-Ansermet, CH-1211, Geneva 4, Switzerland.

Table 1 Toxicity of water-soluble and lipid-soluble Cu(II) complexes to *Nitzschia closterium*

Ligand*	[Ligand]†	[Cu(II)]†	Toxicity index‡§	Percentage solvent extractable Cu(II)¶
NTA	2.0×10^{-6}	3.10×10^{-7}	0.20	<10
LAS	2.0×10^{-6}	3.10×10^{-7}	0.25	13
PAR	5.0×10^{-8}	3.10×10^{-8}	<0.1	<10
TAR	5.0×10^{-8}	3.10×10^{-8}	<0.1	<10
Tannic acid	5.9×10^{-7}	3.15×10^{-7}	0.13	ND
Fulvic acid	1.0×10^{-5}	3.15×10^{-7}	0.08	ND
Humic acid	6.4 mg l^{-1}	3.15×10^{-7}	0.70	ND
Oxine	2.0×10^{-8}	3.20×10^{-8}	13.50‖	ND
	5.0×10^{-8}	3.10×10^{-8}	20	92
PAN	5.0×10^{-8}	3.10×10^{-8}	>25	84
TAN	5.0×10^{-8}	3.10×10^{-8}	>25	92
2,9-dmp	5.0×10^{-8}	3.10×10^{-8}	>25	91

*Abbreviations: NTA = nitrilotriacetic acid, LAS = linear alkylbenzene sulphonate, PAR = 4-(2-pyridylazo)-resorcinol, TAR = 4-(2-thiazolylazo)-resorcinol; 2,9-dmp = 2,9-dimethyl-1,10-phenanthroline, TAN = 1-(2-thiazolylazo)-2-naphthol †mol l^{-1} ‡Relative to Cu(II) = 1.00 §Florence *et al* (1984) ¶20% *n*-butanol in *n*-hexane ‖Ahsanullah and Florence (1984) ND = not determined

toxicity (Florence *et al.*, 1984). Further, addition of substituents to a ligand which increase its water solubility substantially reduce the toxicity of its copper(II) complex (Florence *et al*, 1984).

Many metals enter the environment in the form of hydrophobic complexes or are hydrophobically complexed by ligands entering the environment in industrial effluents. Thus, ethylxanthogenate (used in flotation processes for ore concentration) forms a hydrophobic copper complex [CuL_2], [Cu(II)(oxinate)$_2$] is a common fungicide, and 2,9-dimethyl-1,10-phenanthroline (typical of compounds in oil shale and coal liquefaction process waters) forms a hydrophobic complex with copper(II). As indicated in Table 1, these lipid-soluble complexes are highly toxic to marine amphipods (Ahsanullah and Florence, 1984). Prior to the present work, no toxicity studies had been reported for measurements in the presence of humic substances (HS). These substances have the potential to ameliorate toxicity either by sequestering the copper(II) from the hydrophobic complex (a hydrophilic interaction) or by hydrophobic association.

By using the marine diatom *N. closterium*, we examined the ability of HS to ameliorate the toxicity of the hydrophobic copper(II) complexes formed with oxine (8-hydroxyquinoline) and PAN (1-(2-pyridylazo)-2-naphthol). These very stable Cu(II) complexes were chosen to minimize the chance of ligand displacement due to competitive hydrophilic interaction between the HS and the Cu(II) ion (and to possibly maximize the chance of hydrophobic association)

Evidence is presented for amelioration of the toxicity of Cu–oxine to *N closterium* by humic acid (5 mg kg^{-1} in seawater). Algal assays were unable to establish whether the interaction between HS and the metal complex was of a hydrophilic or a hydrophobic nature. However, parallel studies in our laboratory (Powell and Town, 1991), using visible spectroscopy and anodic stripping voltammetry at a Nafion-coated thin mercury film electrode (TMFE), established that HS interact hydrophilically with Cu–oxine and Cu–PAN resulting in formation of a ternary HS–Cu–L complex. It was inferred that humic acid could ameliorate the toxicity of a wide range of environmentally significant toxic copper complexes provided that the ligand itself is not toxic.

MATERIALS AND METHODS

Seawater

Surface seawater was collected (on 23 February 1989) 2 km offshore in Bate Bay, Port Hacking, Sydney, Australia, in polyethylene bottles and immediately filtered through a 0.45 μm membrane. Filtered seawater was stored at 4°C; it was equilibrated to room temperature before use in algal assays.

To determine the total concentration of copper in the sample, 25 ml of seawater + 10 μl Suprapur H_2O_2 + 83 μl Aristar HNO_3 was irradiated for 2 h with a 500 W u.v. lamp. The concentration of copper in the resultant solution was found to be 2.54×10^{-8} mol l^{-1} (by differential pulse ASV). Labile copper (measured without acidification and u.v. irradiation) was 1.4×10^{-9} mol l^{-1}. The DOC content of the filtered seawater, determined by the method of Matthews *et al* (1990), was 2.5 mg kg^{-1}.

Algal cultures

Nitzschia closterium (Ehrenberg) W. Smith, a marine diatom (CS-5c, obtained from CSIRO Division of Fisheries Algal Culture Collection, Hobart, Australia) was cultured in f medium (Guillard and Ryther, 1962) except that iron–EDTA was replaced with ferric citrate/citric acid (4.5 mg l^{-1} ferric citrate and 4.5 mg l^{-1} citric acid), and all other trace metal concentrations were halved.

Stock algal cultures were illuminated at 6400 lux (fluorescent daylight tubes) on a 12 h light/dark cycle at 21 ± 2°C. Transfers were made under axenic conditions (using autoclaved culture medium, in a laminar flow hood in a class 100 clean room) every week. Stock cultures 10 days after transfer (exponential growth phase) were used for algal assays.

Algal assays

To minimize possible complexation of copper(II) by components of the culture medium, algae were washed 3 times with unsupplemented seawater before use (by centrifugation at 2500 rpm for 8 min using a JOUAN CR 4.11 centrifuge). The resulting washed algal suspension was

homogenized (to disperse clumps) and vortexed to ensure homogeneity Aliquots of algae were added to 50 ml of unsupplemented seawater in silanized 200 ml conical flasks [which had been acetone rinsed and acid washed (10% HNO_3)] to give an initial cell density of 2–5 × 10^4 cells ml^{-1} The flasks were covered with a loose glass cap and illuminated on a light box at 16,000 lux (fluorescent daylight tubes) on a 12 h light/dark cycle at 21 ± 2°C. The higher intensity light used for the assays was to ensure algal division each day It has been shown that unsupplemented seawater can support the logarithmic growth of *N. closterium* for at least 72 h (Lumsden and Florence, 1983)

The density of live cells was measured at $t = 0$, 24, 48 and 72 h by counting microscopically on a haemocytometer (Olympus BH-2 phase contrast microscope, 250 × magnification). Logarithmic growth was maintained over this period All assays involved replicate counts (at least 4) on duplicate flasks. A blank, consisting of algae in unsupplemented seawater, was monitored for each assay

Hydrophobic metal complexes

The copper(II) and ligand concentrations employed in this study (3.1 and 6.2 × 10^{-8} mol l^{-1} respectively) were chosen to be comparable with previous work (Florence *et al.*, 1984; Florence and Stauber, 1986); these values are also environmentally relevant The exogenous copper was significantly greater than labile copper in seawater (1 4 × 10^{-9} mol l^{-1})

To counter possible complications from slow coordination reactions occurring in seawater (Hering and Morel, 1989), and to ensure an identical metal·ligand ratio in each flask, the copper(II)–ligand complex was pre-formed in Analar ethanol. Addition of 50 μl of the stock ethanolic solution to 50 ml of seawater generated 3.1 × 10^{-8} mol l^{-1} Cu(II) and 6.2 × 10^{-8} mol l^{-1} ligand in the final assay solution. Each flask was prepared in duplicate.

PAN (1-(2-pyridylazo)-2-naphthol) was obtained from Sigma and recrystallized from "Spectroscopic" ethanol (BDH) before use. Microanalysis established the composition: C, 72.15%, N, 16 94% and H, 4.62% (calc for $C_{15}H_{11}N_3O$· C, 72 21%; N, 16.86% and H, 4.41%) Oxine (8-hydroxyquinoline), BDH Analar, was used without purification. Stock ligand solutions were prepared in ethanol and stored in the dark.

In assays involving humic substances, CuL_2 and the humic or fulvic acid were allowed to equilibrate in seawater for at least 2 h before addition of the algal suspension (unless otherwise stated).

Determination of K_{ow}

The K_{ow} value for Cu–oxine and Cu–PAN was determined by equilibrating a solution (10 ml) of 2 × 10^{-6} mol l^{-1} Cu(II) and 6 × 10^{-6} mol l^{-1} ligand in 10^{-3} mol l^{-1} acetate buffer (pH 5.5) with 10 ml octanol The solutions were mixed for 12 h. The organic phase was then washed several times with Milli-Q water to remove any colloidal material, followed by extraction with 0 1 mol l^{-1} HNO_3 to release Cu(II) from the octanol. The copper content of the acid extract was measured by DC-ASV at a Nafion-coated TMFE; this corresponded to the CuL_2 in the octanol phase The concentration of CuL_2 in the aqueous phase was obtained by the difference between the amount of Cu(II) added to the solution (2 × 10^{-6} mol l^{-1}) and the concentration of Cu(II) in octanol All experiments were performed in a class 100 clean room

Humic substances

Algal assays were performed using two different concentrations of humic substances, 1 and 5 mg kg^{-1}.

Fulvic acid (FA) was the sample FA4 extracted from the International Humic Substances Society (IHSS) reference peat sample by the acid pyrophosphate–XAD-7 method (Gregor and Powell, 1986) It was completely soluble in seawater (no material retained on a 0 025 μm membrane) A stock solution of FA in Milli-Q water was prepared (1000 mg kg^{-1}), aliquots of this solution were added to each flask to generate the 1 and 5 mg kg^{-1} solutions

Humic acid (HA) was an IHSS reference soil humic acid, Summit Hill humic acid. A stock solution of HA in seawater was prepared (5 mg kg^{-1}) HA was very sparingly soluble in seawater (probably due to the high concentration of calcium ions). To effect dissolution, the HA was "predissolved" in 200 μl of 0 8 mol l^{-1} NaOH, stood for approx 1 h, followed by dilution to 500 ml with seawater Immediately after this dilution, HCl was added to adjust the pH to 8 0 (the pH of the seawater sample). Some precipitation of HA was observed over the time scale of the assays (4 days)

The trace metal impurities in the humic samples used in this work have been reported (Powell and Town, 1991).

Instrumentation

DC-ASV measurements were performed with a Nafion-coated TMFE formed on a laboratory-built glassy carbon electrode (GCE), reference (Ag, AgCl in 1 mol l^{-1} KCl, with vycor junction), and counter (Pt) electrodes coupled to a PAR 174 potentiostat and a PAR RE0074 X-Y recorder Instrumental parameters were nitrogen flush, 10 min, mode, DC, scan rate, 100 mV s^{-1}, deposition potential, −0.60 V; rate of stirring, *c* 700 rpm (Teflon-coated stirrer bar), deposition time, 5 min. To prepare the Nafion-coated TMFE, Nafion 125 (Aldrich) was diluted with "Spectroscopic" grade ethanol (BDH) to yield a solution of 0.5% w/v Nafion. 1 μl of this solution was applied to the GCE (via a Teflon-tipped microsyringe) and the solvent evaporated (60 s) with warm air from a hair dryer Mercury was deposited through the film for 20 min at −1 0 V (vs Ag,AgCl) from a solution containing Hg(II) (7 × 10^{-5} mol l^{-1}).

Differential pulse ASV measurements were performed with a PAR 303A hanging mercury drop electrode coupled to a PAR 384 polarographic analyser and a PAR RE0082 digital plotter Instrumental parameters as given above, except. mode, DP, scan rate 2 mV s^{-1}, deposition time, 15 min

All experiments were carried out in a class 100 clean room

RESULTS

Properties of the copper(II) complexes

Log K_{ow} for Cu–oxine was 1.70, that for Cu–PAN was 0.46. These results (expressed as percentage complex extracted from water) are comparable to those reported by Florence and coworkers (Table 2)

The stoichiometry and stability of the Cu–oxine and Cu–PAN complexes has been reported, spectrophotometric parameters are. CuL, λ_{max} 550 nm, ϵ_{max} 13,500, CuL_2 λ_{max} 550 nm, ϵ_{max} 22,500 (Powell and Town, 1991).

Table 2 Percentage copper(II) complex extractable from water

	20% *n*-butanol in *n*-hexane*	*n*-octanol
Cu–oxine	92	78†, 97‡
Cu–PAN	84	84†, 72‡

*Florence *et al* (1984) ([Cu(II)] = 3 15 × 10^{-8} mol l^{-1}, [ligand] = 5 × 10^{-8} mol l^{-1})
†Florence and Stauber (1986) ([Cu(II)] = 3 15 × 10^{-8} mol l^{-1}, [ligand] = 5 × 10^{-8} mol l^{-1})
‡This work

Algal assays

In all assays, an initial lag in growth was observed with no significant cell division occurring within 24 h That is, the cell numbers after 24 h were the same as the initial cell density. Following this, logarithmic growth was maintained for 48 h It is possible that the algae required a certain time to recover from the assay preparation procedure before cell division could resume. Alternatively the higher light intensity used in the algal assay, compared to that for the algal innoculum, may cause this initial lag in algal growth.

(i) Humic acid and Cu–oxine For these assays the humic acid and Cu–oxine (3.1×10^{-8} mol l^{-1}) were equilibrated for approx. 30 min in the seawater culture medium before addition of algae. The presence of Cu–oxine resulted in cell death within 24 h. With 1 mg kg^{-1} HA some initial cell death occurred during the first 24 h, followed by some cell division by the remaining algae. In contrast, after leaving HA (1 mg kg^{-1}) and Cu–oxine to equilibrate in seawater for 2 h prior to addition of algae, cell numbers remained constant over 4 days Based on this observation, humic substances and the copper complex were equilibrated for 2 h before addition of algae in all subsequent experiments

The toxicity of Cu–oxine was completely ameliorated in the presence of 5 mg kg^{-1} HA, with normal cell division observed, i e the growth rate was the same as that for the blank (seawater). However, with 0.025 μm filtered 5 mg kg^{-1} HA (resulting in a concentration of *c* 1.5 mg kg^{-1}, *vide infra*) cell numbers remained constant in the presence of Cu–oxine (as observed with 1 mg kg^{-1} unfiltered HA). Typical growth curve plots are shown in Fig. 1; additional data are given in Table 3.

(ii) Fulvic acid and Cu–oxine. Fulvic acid (1 mg kg^{-1}) did not ameliorate Cu–oxine toxicity. With 5 mg kg^{-1} FA cell division was inhibited and cell numbers remained constant for the duration of the assay (Table 3).

(iii) Humic and fulvic acids and Cu–PAN. Cu–PAN is extremely toxic to *N. closterium*; all cells died within 24 h of exposure to this complex. Cell death also occurred in the presence of 1 mg kg^{-1} HA or 5 mg kg^{-1} FA; in contrast, cell numbers remained constant with 5 mg kg^{-1} HA (Table 3)

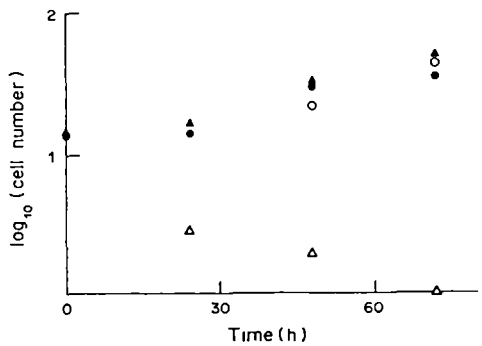

Fig. 1 Growth curve plots for *N. closterium*. Assays conducted in seawater containing· O, no other additives, ▲, 5 ppm HA; ●, 5 ppm HA + Cu–oxine [3.1×10^{-8} M Cu(II), 6.2×10^{-8} M oxine]; △, Cu–oxine

Table 3 Growth data for *Nitzschia closterium*•

	Mean cell count†			
Treatment	0 h	24 h	48 h	72 h
8-Hydroxyquinoline				
Control	13	14	32	44
$Cu(Ox)_2$	13	5	2	0
+ HA (5 ppm)	14	16	28	43
+ HA (1 ppm)	14	15	15	15
+ FA (5 ppm)	10	12	12	10
HA (5 ppm)	14	17	33	51
Oxine	13	15	26	44
1-(2-Pyridylazo)-2-(naphthol)				
Control	20	21	57	86
$Cu(PAN)_2$	20	0	0	0
+ HA (5 ppm)	20	17	9	10
+ HA (1 ppm)	20	1	0	0
+ FA (5 ppm)	20	0	0	0
HA (5 ppm)	20	31	55	120
PAN	20	9	0	0

•[Cu(II)] = 3.1×10^{-8} M; [oxine] = 6.2×10^{-8} M, [PAN] = 6.2×10^{-8} M

†Cell count average for 4 replicates each on duplicate flasks, cell density = (count/4) × 10^4 ml^{-1}

To investigate this latter result, a longer pre-equilibration time was tried, with the 5 mg kg^{-1} HA/Cu–PAN/seawater solution being left for about 60 h before addition of algae. However, assay results were not reproducible (6 replicates) In some cases cell numbers remained constant, while in others cell death occurred.

(iv) Toxicity of PAN and oxine. To measure the toxicity of the ligands themselves, assays were conducted in media containing oxine or PAN with no added copper(II). Normal growth was observed in the presence of 6.1×10^{-8} and 1.2×10^{-7} mol l^{-1} oxine, and also with 6.1×10^{-8} mol $^{-1}$ oxine in the presence of 5 mg kg^{-1} HA. Consistent with the earlier study (Florence and Stauber, 1986), cell death occurred in the presence of 3.1×10^{-8} mol l^{-1} PAN.

Chemical modelling of copper complexes in seawater

The species distribution of copper complexes in seawater was calculated by use of the chemical equilibrium program SIAS (Fardy and Sylva, 1978). Calculations were performed for the algal assay experimental conditions: 3.1×10^{-8} mol l^{-1} Cu(II), 6.2×10^{-8} mol l^{-1} ligand (PAN or oxine), 0.012 mol l^{-1} Ca(II), 0.0532 mol l^{-1}

Table 4 Calculated distribution of copper in seawater in the presence of oxine and PAN

	Percentage of total copper	
Complex	Oxine	PAN
CuL	37 1	66 2
CuL_2	47 6	
$CuSO_4$	6 3	13 8
CuCl	1 6	3 4
CuOH	5 6	12 4
Free Cu	1 8	4 0

Mg(II), 0.536 mol l^{-1} chloride, 0.0276 mol l^{-1} sulphate, 2.32×10^{-3} mol l^{-1} CO_3^{2-}. Hydroxy, sulphate, chloride, carbonate and bicarbonate complexes of Ca(II), Mg(II) and Cu(II) were included, as were the Ca(II) and Mg(II) complexes of oxine and PAN. The calculated distribution of copper(II) complexes in seawater in the presence of PAN and oxine is given in Table 4

DISCUSSION

The composition of both the assay medium and the maintenance culture are critical in determining metal toxicity (Millington *et al.*, 1988). On comparison of copper and zinc toxicity to *N. closterium* in f medium and unsupplemented seawater, Stauber and Florence (1989) recommended that unenriched seawater be used for marine algal assays.

The concentrations of humic substances used in this work (1 and 5 mg kg^{-1}) were environmentally significant Rivers and lakes contain approx 2–10 mg kg^{-1} dissolved organic carbon (DOC), ground waters 0.7 mg kg^{-1} and seawater 0.5 mg kg^{-1} (equivalent to approx. 1 mg kg^{-1} humic substance) (Thurman, 1985). This value for seawater corresponds to the lower concentration used here. The higher concentration used (5 mg kg^{-1}) may be pertinent in near shore, harbour and estuarine waters. The concentration of DOC is greatest in surface seawater and decreases rapidly with depth (Marty *et al.*, 1988)

Measurements on "blanks" (seawater plus HS) indicated that the growth rate of *N closterium* in the presence of HS was the same as that in raw seawater; i.e. HS had no nutrient effect on *N. closterium* growth Thus, results did not need to be corrected for any effects due to the presence of humic substances

The calculated species distributions for Cu(II), Table 4, should be taken as only an approximate guide There are problems with obtaining reliable stability constants. For example, the stability constants reported for the 1.1 Cu–PAN complex are in poor agreement, viz. log $K_1 = 12.60$ (Betteridge *et al.*, 1963), or 16 (Pease and Williams, 1959) Further, although PAN forms a 1:2 complex with Cu(II) (Powell and Town, 1991), to our knowledge no stability constant has been published for this species. In addition, the use of stability constant data to calculate species distributions outside the pH and concentration ranges under which they were determined is open to question. There are other problems also such as the lack of data on the stability of ternary species, as well as the possible presence of unidentified chelating agents (Florence and Batley, 1976).

Amelioration of the toxicity of lipid-soluble copper(II) complexes to N. closterium by humic substances

Three types of response of the algae to the culture medium were observed, viz. normal growth, no growth (cell division inhibited) and decreasing cell numbers (cell death). Fulvic acid did not reduce the toxic effect of either hydrophobic complex. The only situation in which complete amelioration of toxicity was observed was with 5 mg kg^{-1} HA and Cu–oxine. Humic acid could not completely inhibit Cu–PAN toxicity. Even when the HA/Cu–PAN/seawater solution was equilibrated for 60 h before addition of algae the toxicity of Cu–PAN was not completely ameliorated (in some experiments cell numbers remained constant as opposed to cell death)

Algal growth typically has two stationary phases, viz. an initial stationary phase, followed by a period of exponential growth, then a second stationary phase when cell division equals cell death (therefore cell numbers do not change) Some toxicants can lengthen the initial stationary phase by their impact on cell physiology, a percentage of the cells will remain active and normal growth will resume when cells are resuspended in fresh nutrient medium Hence, it is possible that the toxic effect exerted by Cu-PAN was altered in the presence of 5 mg kg^{-1} HA such that inhibition of cell division, rather than cell death, occurred A similar effect was observed with 1 mg kg^{-1} HA and Cu–oxine, and 5 mg kg^{-1} FA and Cu–oxine.

The toxicity of the ligands themselves is also important PAN, in the absence of added copper, was extremely toxic to *N. closterium*. However, oxine itself was not toxic. This result confirms that the concentration of labile copper in unsupplemented seawater (measured as 1.4×10^{-9} mol l^{-1}) was minor in terms of toxicity. However, this result contrasts with earlier work in which oxine was observed to be toxic to *N. closterium* (Stauber and Florence, 1987). It is possible that the seawater used in the earlier study had a higher labile copper content than that used in the present work. Further, the observation that oxine is expelled by *N. closterium* in an unaltered form (Florence and Stauber, 1986) suggests that this ligand does not exert a toxic effect.

The toxicity of copper to biota is greatly increased in the presence of oxine and PAN although the octanol–water partition coefficients for these copper(II) complexes are several orders of magnitude lower than those reported for hydrophobic substrates such as DDT (log $K_{ow} = 6.90$; Nebeker *et al.*, 1989) However, the lipid solubility of the uncharged Al (maltolate)$_3$ complex having log $K_{ow} = 0.67$ is sufficient to induce marked neurotoxicity (Finnegan *et al.*, 1987)

Interpretations

The observation that humic substances can have an impact on the toxicity of hydrophobic copper complexes has important environmental implications. A variety of mechanisms may be responsible for this ameliorating effect; these are now discussed.

(i) Hydrophobic dissolution in a humic phase Results from the algal assays were inconclusive, providing evidence both for and against a hydrophobic mode of interaction. In support of a hydrophobic association is the observation that humic acid was more effective at reducing the toxicity of these lipid-soluble copper(II) complexes than was fulvic acid.

However, the copper(II) complexes used in the present work had similar log K_{ow} values Therefore, if hydrophobic association was involved, a similar amelioration of toxicity was expected for Cu–oxine and Cu–PAN.

(ii) Adsorption on colloidal humic particles. The observation that filtered (0.025 μm) humic acid could not ameliorate Cu–oxine toxicity suggests that it is the colloidal/particulate humic components which interact with non-polar compounds. Alternatively, this phenomenon may be a concentration effect. Thus, for a humic acid solution (14 mg kg^{-1}) prepared in 0.1 mol l^{-1} Tris buffer (pH 8.2) a 27% decrease in absorbance at 300 nm was observed following filtration (0.025 μm). For a parallel experiment in seawater the change was −71% (precipitation of calcium and magnesium salts also inferred). That is, the effective concentration of a filtered 5 mg kg^{-1} HA solution was approx. 1.5 mg kg^{-1}, which may be insufficient to ameliorate toxicity.

(iii) Formation of a protective humic coating on the surface of algal cells. Humic substances may form a protective coating on the surface of the algal cells which acts as a physical barrier to the xenobiotics. This is analogous to the formation of metal(III) hydroxide coatings which reduce copper(II) ion toxicity (Stauber and Florence, 1985a, b, 1987)

To test this hypothesis, algae were equilibrated in a 5 ppm HA/seawater solution for 2 h. The algae were then filtered (0.6 μm), and rinsed with seawater followed by dilute NaOH (0.02 mol l^{-1}) to dissolve any humic coating. The u v.–visible absorption spectrum of the NaOH washings of the humic equilibrated algae, and that of algae not exposed to humic acid, were recorded. No evidence was obtained for a humic coating; however, the amounts of humic coating may have been below detection by this technique.

(iv) Hydrophilic complexation of copper(II) by humic acid. Due to their high affinity for Cu(II) ions, humic substances may complex Cu(II) and displace the hydrophobic ligand, thus detoxifying the lipid-soluble complex by this mechanism. Importantly, complexation of the Cu(II) aqua ion by humic substances did reduce the toxicity of the metal ion to *N. closterium* (Table 1)

As noted above, previous work (Powell and Town, 1991) established a hydrophilic interaction between HS and the hydrophobic copper complexes ($Cu(oxine)_2$ and $Cu(PAN)_2$ resulting in formation of a ternary complex, HS–Cu–L, and accompanied by release of L (i e. $CuL_2 + HS \leftrightharpoons HS–Cu–L + L$) This observation is consistent with the algal assay results If the free ligand is toxic, as is PAN, then an association involving displacement of a coordinated ligand by humic acid will not remove this toxicity. This mechanism requires that humic acid (which did ameliorate Cu–oxine toxicity) complexes Cu(II) more strongly (at pH 8 2 in seawater media) than does fulvic acid Ion selective electrode (ISE) potentiometric studies on the effect of Mg(II) (the dominant divalent metal ion in seawater) on Cu(II) complexation by humic and fulvic acids support this proposal (Town, 1991). Results indicated that in seawater media, at the same concentration (mg kg^{-1}) of HA or FA, HA will form Cu(II) complexes of greater stability

Environmental implications

The results presented here have important environmental implications. They may allow the potential toxic impact of metals in soils and natural waters to be predicted Specifically, this study indicated that the toxicity of a hydrophobic copper(II) complex would be ameliorated if a certain threshold concentration of humic substance is present and if the ligand displaced from the copper complex is not toxic.

Coupled with information from ISE potentiometric studies (Town, 1991), these results may be tentatively extrapolated to freshwaters. In such environments the total concentration of other metal ions competing for humic Cu(II) binding sites is much lower than in seawater; also the concentration of humic substances will be greater. Thus, the ameliorating effect of humic substances on the toxicity of hydrophobic copper complexes is expected to be greater for freshwater aquatic organisms such as *Chlorella pyrenoidosa.* The toxicity of lipid-soluble copper complexes to this freshwater algae has been demonstrated (Stauber and Florence, 1987, 1989). The ability of humic substances to complex Cu(II) is pH dependent, as is the formation of the copper complexes, toxicity of the less stable copper complexes should be ameliorated more readily

Complexes formed by other environmentally significant metals, such as Pb(II), Cd(II) and Zn(II), with oxine and other hydrophobic ligands (e.g. diethyldithiocarbamate) are insufficiently stable to allow their existence at environmental concentrations (Town, 1991) Further, in seawater, competing equilibria are important: lead binds to inorganic adsorbents such as colloidal ferric hydroxide and silica (Florence, 1983), cadmium forms very stable chloro-complexes and zinc exists predominantly as a dihydroxy complex (Florence and Batley, 1976)

In environmental samples several metal ions may simultaneously compete for the humic binding sites, the presence of other competing ligands must also be considered For example, an influx of Al(III) into a natural water (as may arise from acid precipitation) may exert indirect toxicity by displacement of Cu(II)

from hydrophilic (non-toxic) organic complexes (Reuter *et al.*, 1987).

It is noted that the humic substances used in this study are relatively "pure", i.e. they have low ash and trace metal impurities. In contrast, in soils and natural waters the strong binding sites of humic substances may be saturated with metal ions and hence less able to sequester an exogenous contaminant Furthermore, the soil derived humic substances used in this work are unlikely to be representative of marine humic materials (Malcolm, 1990).

This work has demonstrated that the contribution from humic substances must be considered in any assessment of the impact of pollutants on an environmental system. This highlights the complexity of these systems and establishes the importance of speciation measurements as opposed to (often) meaningless analysis of, say, the total metal content of a sample.

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Paper 25

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Determination of trace element speciation and the role of speciation in aquatic toxicity

T M. Florence, G.M. Morrison and J.L. Stauber

CSIRO Centre for Advanced Analytical Chemistry, Private Mail Bag 7, Menai, N S.W. 2234, Australia

ABSTRACT

Knowledge of trace element speciation in waters is essential to an understanding of aquatic toxicity and bioaccumulation, as well as to the partitioning of elements between water and colloidal and particulate phases. In natural waters, only very small percentages of the dissolved heavy metals, such as copper, lead cadmium or zinc, are present as free (aquo) metal ion; most of the metal is adsorbed to colloidal particles or combined in complexes. For aquatic toxicity studies, the aim of the speciation measurement is to determine the fraction of total dissolved metal (the 'toxic fraction') that will react with, and be transported across, a biological membrane such as a fish gill. In this review, a range of trace element speciation techniques is discussed and compared. A simple anodic stripping voltammetric method is recommended for the measurement of the fraction of electroactive metal in a sample, i.e. the fraction of total dissolved metal that can be deposited into a mercury electrode at the natural pH of the sample. The electroactive fraction is believed to approximate the toxic fraction. A rapid ion exchange method, suitable for field use, is proposed for the determination of the toxic fraction of copper in waters.

Key words: speciation; toxicity; metals; bioavailability; anodic stripping voltammetry

INTRODUCTION

Speciation analysis of an element in a water sample may be defined as the determination of the concentrations of the different physico-chemical forms of the element which together make up its total concentration in the sample. The individual physico-chemical forms may include particulate matter and dissolved forms such as simple inorganic species, organic complexes and the element adsorbed on a variety of colloidal particles (Table 1). All these species can coexist, and may or may not be in thermodynamic equilibrium with one another. An ionic metal spike added to a filtered natural water sample may take times ranging from hours to months to equilibrate with the natural pool of metal species [1–4].

TABLE 1

Possible forms of trace elements

Physicochemical form	Possible examples	Approximate diameter (nm)
Simple ionic species	$Zn(H_2O)_6^{2+}$	0 8
Inorganic complexes	$CdCl^+$, $PbCO_3$	1
Differing valency states	Cr(III), Cr(VI)	1
Lipid soluble complexes	CH_3HgCl	1
Organometallic species	$CH_3AsO(OH)_2$	1
Weak complexes	Cu fulvate	2
Adsorbed on colloidal particles	Cu^{2+}/humic acid/Fe_2O_3	10–500
Particulate	Metals adsorbed into or contained within clay particles	>450

For many heavy metals in seawater or river waters, the predominant physico-chemical forms are unknown. Cu in sea water, for example, is believed to exist mainly as undefined, highly stable organic complexes, the principal ligands perhaps being porphyrins, siderophores or metallothioneins [5]. In this situation, where neither the nature nor the concentrations of the dominant ligands are known, it is obviously futile to attempt to apply computer modelling techniques to determine speciation. However, for well defined experimental waters, or for elements at higher concentrations (e.g. Ca and Mg in seawater), chemical modelling can be a powerful tool [6].

There are two main reasons for studying the speciation of elements in waters — to understand either the biological or the geochemical cycling of the elements. Biological cycling includes bioaccumulation, bioconcentration, bioavailability and toxicity, and geochemical cycling involves the transport, adsorption and precipitation of the element in the water system. It is now well established that no meaningful interpretation of either biological or geochemical cycling can be made without speciation information [7]. Each different physico-chemical form of an element (Table 1) has a different toxicity, so analysis of a water sample for *total* metal concentration alone does not provide sufficient information to predict toxicity. For example, two rivers may both contain 40 $\mu g\ l^{-1}$ of total dissolved Cu; if the first has most of the copper adsorbed on colloidal particles there will be little or no effect on aquatic life, but if the second river has free Cu(II) ion as the main species, few organisms would survive. Lipid-soluble metal complexes are particularly toxic forms of heavy metals because they can diffuse rapidly through a

biomembrane and carry both metal and ligand into the cell. Examples of lipid-soluble complexes are copper xanthates (from mineral flotation plants), copper 8-hydroxyquinolinate (agricultural fungicide) and alkylmercury compounds.

Variation in the speciation of an element will also affect its degree of adsorption on suspended matter, its rate of transfer to the sediment and its over-all transport in a water system. Speciation analysis will therefore assist in the prediction of the distance over which a river will be affected by effluent discharged from a point source.

Speciation measurements have been made by a variety of techniques, including electroanalysis, ion exchange, dialysis, ultrafiltration, solvent extraction and computer modelling [1]. If the measurements are made to study aquatic toxicity, then the aim is to determine the toxic fraction of the element, i.e. the fraction of its total concentration in the water sample that is toxic to aquatic organisms. For a metal complex, this aim will be realised if the fraction of total metal that is reactive at a mercury electrode, adsorbed by an ion-exchange resin or measured by some other technique, is similar to the fraction that is dissociated at, and transported across, a biomembrane.

Electroanalysis is a powerful technique for the study of trace element speciation, and has been applied to (or is potentially applicable to) about 30 elements: Ag, As, Au, Bi, Br, Cd, Cl, Co, Cr, Cu, Eu, Fe, Ga, Hg, I, In, Mn, Mo, Ni, Pb, S, Sb, Se, Sn, Tl, U, V, W, Yb and Zn. Four metals of prime environmental concern, Cu, Pb, Cd and Zn, can be determined simultaneously and with great sensitivity. Moreover, the redox potential of an electrode can be varied accurately, precisely and continuously over a wide potential range, and the study of the kinetics of metal complex dissociation at an electrode is supported by well established theory. Of all trace element speciation methods available at present, electroanalysis appears to provide the best opportunity for experimentally modelling the bioavailability of elements and their complexes with organic and inorganic ligands.

MEASUREMENT OF TRACE ELEMENT SPECIATION

At present it is not possible to measure the concentrations of all the individual chemical species that make up the total concentration of an element in a natural water (Table 1). Ion selective electrodes can determine the activity of free metal ions, such as Cu^{2+}, Cd^{2+} and Pb^{2+}, but are usually insufficiently sensitive for use in natural waters and, in addition, interference effects are often severe. Currently available speciation techniques allow the dissolved elements to be divided only into groups of species based on behavioural differences [1]. Some of these techniques are described below.

Anodic stripping voltammetry (ASV)

This electroanalytical technique can be used for the determination of about 15 elements, although, in natural waters, copper, lead, cadmium and zinc are the most commonly determined metals. The ASV procedure can be adjusted to measure either total or labile metal. Labile metal represents the fraction of the total dissolved metal that can be deposited at the electrode under carefully defined conditions, and has often been assumed to be similar to the toxic fraction of the dissolved metal (Table 2). Distinct advantages of the ASV-labile measurements are that no preliminary separations are needed, no blanks are involved, and the opportunity for contamination is minimal.

Ion exchange

Ion exchange is an attractive technique for trace metal speciation because separations can be achieved with little manipulation of the sample. The iminodiacetate chelating resin, Chelex-100 (Bio-Rad), has found wide application for the measurement of labile metal in water. Metal combined in inert complexes will not be retained by a column of Chelex-100 resin, and metal associated with colloidal particles will be only partly removed. The resin may therefore be used to separate inert metal forms from dissociable complexes. However, studies with algae indicate that Chelex-100-labile metal (i.e. the fraction of total metal removed by the resin) is a serious overestimate of the toxic metal fraction (Table 2).

TABLE 2

The relationship between speciation and toxicity to algae

Chemical species	ASV-labile (%)	Chelex-100 labile (%)	Solvent extractable	Toxicity factor (compound/ (metal chloride)
Cu-fulvic acid	1.5	66	<10	0.36
Cu-tannic acid	5.5	100	<10	0 60
Cu-oxine	64	98	70	>10
Cu-oxine-5-sulfonate	100	77	<10	1.7
Cu-ethylxanthate	11	—	86	>10
CH_3HgCl	—	—	80	7
n-C_3H_7HgCl	—	—	98.4	20
n-$C_5H_{11}HgCl$	—	—	99.8	300

ASV, anodic stripping voltammetry.

Solvent extraction

Solvent extraction of water samples with solvents such as *n*-octanol, chloroform, or hexane-butanol, which simulate the solvent properties of lipid material, is used to estimate the metal fraction that is lipid soluble. Synthetic copolymers, such as Bio-Rad SM2 resin, which has a high affinity for large molecules that have both hydrophobic and hydrophilic moieties, may also be useful models for lipid solubility. In seawater and some fresh waters, organically-associated metal can be determined by ASV-labile measurements before and after UV irradiation of the sample to destroy organic matter.

Dialysis, ultrafiltration, and gel permeation chromatography

These techniques separate molecules on the basis of molecular size. No clear relationship has been established between molecular size and toxicity, although, in natural waters, metals associated with most large molecules (e.g. copper-humic acid) are relatively non-toxic. There are significant problems with contamination using all three techniques, blank values are high in gel permeation chromatography and, as in all speciation techniques, the ionic equilibrium in the sample is disturbed.

Computer modelling

By using sophisticated ionic equilibria computer programs and known data for pH, total ion concentrations and equilibrium constants, trace element speciation can be calculated. This approach is adequate for synthetic solutions and for major ions in natural waters, but cannot be used for the speciation of trace heavy metals in natural waters because neither the nature, nor the concentrations, of all the complexing agents present are known.

THE RELATIONSHIP BETWEEN SPECIATION AND TOXICITY

Heavy metal ions, such as Cu^{2+} and Cd^{2+}, are readily adsorbed on the cell surface of algae, or by the gill mucous of fish. The adsorption probably involves metal-protein interactions. The adsorbed metal slowly diffuses through the cell membrane to the interior of the cell, where it may participate in injurious reactions. The toxic effect appears to be related to the rate of diffusion through the membrane. Chromate ion, for example, is much more toxic than chromium (III), because the small, non-hydrated chromium (VI) anion diffuses rapidly through biomembranes. Lipid-soluble metal complexes diffuse very rapidly and, for several metals, toxicity increases with increasing lipophilicity (Table 2). Likewise, the copper complex of

8-hydroxyquinoline (oxine), which is soluble in organic solvents, is remarkably toxic towards algae, while its water-soluble sulfonate derivative has a much lower toxicity (Table 2).

Nature has provided fish and other aquatic animals with effective defences against ingested heavy metals, which are eliminated via the gut and detoxified in the liver, kidneys and spleen by a group of proteins called metallothioneins. These defences allow the animals to cope with quite high levels of heavy metals in the food chain and sediment. Evolution has not, however, equipped them to tolerate free metal ions or toxic lipid-soluble complexes in the water pumped through their gills. Unpolluted seawater or river water contains very low concentrations of these toxic metal forms, most of the dissolved metal being adsorbed on colloidal particles or combined in non-toxic complexes. The situation with dissolved pollutants which interact directly with the gill of an aquatic animal is analogous to the danger posed to terrestrial animals by volatile heavy metal compounds such as lead aerosols from automobile exhausts, which can enter the bloodstream by direct absorption through the lungs. Natural waters have powerful detoxification mechanisms which convert free ionic metal species into non-toxic forms, but considerable damage can be caused close to the source of pollution.

The metallothioneins are a class of low molecular weight, sulfur-rich proteins which occur widely in vertebrate and invertebrate animals, including humans, rats, fish, oysters and mussels. These proteins have a high affinity (via sulfhydryl bonding) for such metals as cadmium, copper, zinc and mercury, and their biological function is to bind, detoxify and store the heavy metals in the liver, kidney and spleen. The synthesis of metallothioneins in these organs is induced when the animal is exposed to heavy metals. Even when fish are raised in water which is highly polluted with copper or cadmium, the fish muscle (flesh) contains extremely low concentrations of these metals because they are bound as metallothionein complexes in the liver (Cd, Cu, Zn and Ag), kidney (Cd, Cu, Hg, Ag) and spleen (Cd). It is believed that heavy metal toxicity in animals manifests itself only when 'spillover' from the metallothionein proteins occurs, i.e. when the amount of metal ingested exceeds the animal's ability to synthesize the detoxifying metallothionein.

The complexing capacity of a water system can be likened to the metallothionein-synthesizing ability of an animal. Complexing capacity is an important water quality parameter because it measures the ability of the water to complex and detoxify added heavy metals. Copper (II) is usually chosen as the heavy metal titrant because it is a common ion, highly toxic to aquatic life. Complexing capacity is defined as the concentration of copper (II) ion (mol l^{-1}) that must be added to a water sample before free Cu^{2+} appears. It reflects the concentration of organic and inorganic substances in the

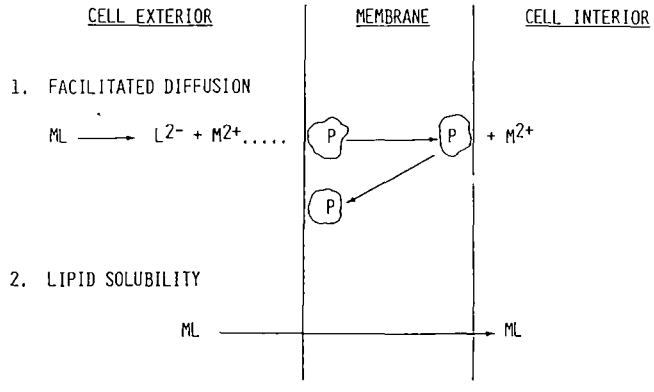

Fig. 1. Metal transport into the cell.

water sample, both molecular and colloidal, that bind copper ions (Table 1). Near-shore surface seawater has a copper complexing capacity of about 2×10^{-8} M, while river waters range from 1 to 50×10^{-8} M.

For the study of aquatic toxicity by metals, the electrochemical and solution parameters should be chosen so that the ASV-labile fraction of total dissolved metal is similar to the toxic fraction. Hydrophilic heavy metal ions are believed to be transported across the hydrophobic space of a biomembrane by the 'shuttle' process of facilitated diffusion (or 'host-mediated transport'), where a receptor molecule (e.g. a protein) on the outer membrane surface binds a metal ion. The hydrophobic metal-receptor complex then diffuses to the interior of the membrane and releases the metal ion into the cytosol where it is trapped, perhaps by reaction with a thiol compound. The receptor then diffuses back to the outer surface of the membrane, ready to collect another metal ion (Fig. 1). Alternatively, if the metal complex is lipid soluble, the much more rapid process of direct diffusion can take place (Fig. 1). Direct diffusion is basically different from facilitated diffusion, not only because it is faster, but because the ligand is also transported into the cytosol.

The fraction of total metal in solution that can be transported across a membrane surface is equivalent to the bioavailable or toxic fraction. This in turn depends on the relative affinity of the metal for solution ligands and the receptor molecule (Fig. 1), or on the solution-membrane partition coefficient for a lipid-soluble complex.

The process of metal accumulation in an organism by dissociation of a

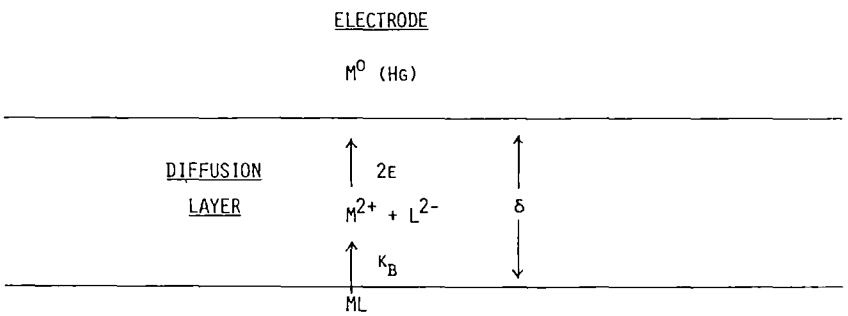

Fig. 2. Electrochemical reduction of metal complexes. The degree of dissociation of the metal complex, ML, at the electrode (and hence the lability of the complex) increases with increasing K_B and increasing δ.

metal complex at a membrane surface, facilitated diffusion of the metal through the membrane and deposition in the cytosol (Fig. 1), has obvious similarities to the process of electrodeposition (Fig. 2), where the metal complex dissociates at the diffusion layer boundary and the metal ion travels through the diffusion layer to the electrode where the metal is deposited.

A key concept in anodic stripping voltammetric (ASV) determination of trace metals in waters is ASV lability, which refers to the fraction of total dissolved metal that is measured by the ASV technique used. The fraction of metal measured depends on the effects of complexing agents and surfactants on both the deposition and stripping steps. If ASV-labile measurements are to be used for the estimation of the toxic fraction of metal, however, it is important to eliminate or determine any effect of dissolved components on the stripping process so that the ASV-labile fraction can be equated to the electroactive fraction, (i.e. the fraction of metal deposited into the electrode during the deposition stage of the ASV measurement).

The contribution of the stripping process to ASV lability can be determined by a medium exchange technique where the sample solution is exchanged for a blank solution after metal deposition has been completed and before stripping is initiated. The deposited metals are stripped into a simple electrolyte or buffer solution that contains no complexing agents or surfactants. Using an acetate buffer (pH 4.7) as an exchange medium, Florence and Mann [8] found significant differences in the ASV lability of copper, lead, cadmium and zinc in both natural and synthetic water samples when measurements were made with and without medium exchange (Fig. 3). They

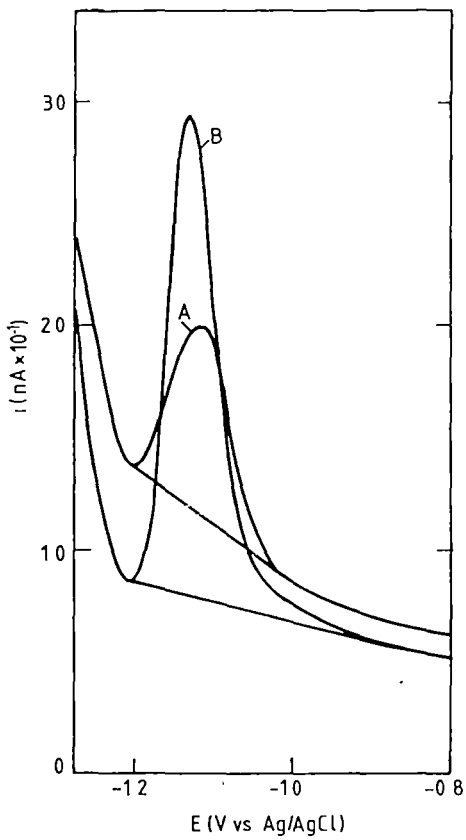

Fig. 3. Differences in the ASV-labile determinations with and without medium exchange.

recommended that all ASV-labile determinations be made with medium exchange.

ASV lability determined by high-frequency techniques such as differential pulse or square wave voltammetry is especially sensitive to the composition of the sample solution because complexing agents and surfactants that affect the rate at which deposited metals are stripped from the electrode will also affect the ASV peak height.

A combination of medium exchange and sample acidification techniques can be used to enable (Table 3) the calculation of the individual effects of complexing agents and surfactants on the ASV deposition and stripping steps [9]. It is apparent from Table 3 that it should be possible to calculate

TABLE 3

Influence of complexing agents and surfactants on DPASV-lability measurements in different sample media

DPASV technique	Complexing agents[a]		Surfactants[a]	
	Deposition	Stripping	Deposition	Stripping
(1) Total metal	No	No	No	No
(2) Labile metal, pH 7.0	Yes	Yes	Yes	Yes
(3) Deposition, pH 7.0; medium exchange, pH 7.0	Yes	No	Yes	No
(4) Deposition, pH 7.0; stripping, pH 1.9	Yes	No	Yes	Yes
(5) Deposition, pH 1.9, stripping, pH 1.9	No	No	Yes	Yes
Effect of complexing agents on deposition = (5) - (4)[b]				
Effect of complexing agents on stripping = (4) - (2)				
Effect of surfactants on deposition = (1) - (5) - (4) + (3)				
Effect of surfactants on stripping = (3) - (4)				

[a]Table shows whether or not complexing agents or surfactants could affect deposition or stripping processes for the various sample media

[b]Numbers refer to percentage of ASV-labile metal determined using the procedure of that number in the table.

the 'toxic fraction' of each metal (i.e. the fraction of total metal that can be electrodeposited) from peak height (5) minus peak height (4), which is equivalent to the effect of complexing agents on deposition. The toxic fraction can therefore be determined in a very simple manner: an ASV measurement is made with deposition at the natural pH, but with the pH of the sample in the cell adjusted to pH 1.9 before stripping is begun, then ASV is carried out again on the same solution, but now both deposition and stripping are made at pH 1.9. The difference in peak heights between the second and first measurements is used to calculate the toxic fraction. Some results for synthetic solutions and actual water samples are shown in Table 4. It is obvious that in most cases, direct ASV measurement seriously underestimates the toxic fraction.

TABLE 4

Estimation of the toxic fraction of metal for the solutions studied in phosphate buffer pH 7 0

Sample	DPASV-labile metal (%)	
	Direct measurement	Toxic fraction[b]
Synthetic solutions[a]		
Cu + fulvic acid		
1 mg l^{-1}	29	60
5 mg l^{-1}	13	36
Cu + humic acid		
5 mg l^{-1}	10	25
Cu + Triton X-100,		
1 mg l^{-1}	35	50
5 mg l^{-1}	49	74
Cd + Triton X-100		
1 mg l^{-1}	12	91
5 mg l^{-1}	9	98
Pb + Triton X-100		
1 mg l^{-1}	24	73
5 mg l^{-1}	9	95
Water Samples		
South Creek, Cu	4	[c]
Toongabbie Creek, Cu	10	[c]
Sewage effluent, Cu	7	[c]
Road runoff		
Pb	41	78
Cu	8	75
Urban runoff		
Pb	33	69
Cu	20	68

[a]Metal concentrations in synthetic solutions = 4×10^{-8} M.
[b]Assuming that toxic fraction = 100 - (5) + (4), using results from Table 3
[c]Large effect of surfactants masks any complexing effects

A novel adsorbent has been developed for the rapid determination of the toxic fraction of copper in natural and polluted seawater and fresh water. The column consists [10] of a small cation exchange resin column in the form of a hydrolysed aluminium salt ($R-SO_3-Al(OH)_2$) which binds copper with an equilibrium constant ($\log K = 9.87$) which is similar to the value found

TABLE 5

Determination of the toxic fraction of copper in synthetic and polluted waters

Sample	Toxic fraction of copper (% of total Cu)		
	Al-resin	ASV	Algal assay
Seawater + 10 mg l^{-1} fulvic acid	20	25	20
Freshwater + 10 mg l^{-1} fulvic acid	35	35	42
Polluted seawater No. 1	71	82	85
Polluted seawater No. 2	50	62	65
Polluted freshwater	10	<20	10

for the binding of copper to an algal membrane. After passing the water sample through the aluminium-resin column, the adsorbed copper (equivalent to the toxic fraction) can be eluted with DTPA. The column can be reused several times after washing with water. A preliminary elution of the column with methanol removes lipid-soluble copper. Several lipid-soluble copper complexes were found to be strongly adsorbed on the column, and could be eluted with methanol.

Toxic fractions of copper in various synthetic and polluted waters were determined by the aluminium-resin column, the double acidification ASV technique, and algal assay as the reference method. Table 5 shows that, in

TABLE 6

Determination of lipid soluble and ionic copper in seawater by successive elution of an aluminium-resin column (total copper = 1×10^{-6} M in all experiments)

8-Hydroxyquinoline (M)	Copper recovered from aluminium-resin column (% of total)	
	Methanol-eluted	DTPA-eluted
Nil	2	95
2×10^{-7}	20	76
5×10^{-7}	47	48
1×10^{-6}	97	2
2×10^{-6}	97	4

general, good agreement was obtained. Table 6 shows that the column can successfully determine the two components of the toxic fraction of copper (lipid soluble and ionic) by successive elution.

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Paper 26

Applications of Plasma Source Mass Spectrometry II

Edited by

Grenville Holland
Department of Geological Sciences, University of Durham

Andrew N. Eaton
VG Masslab, Wythenshawe, Manchester

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A Comparative Study of ICP-MS and TIMS for Measuring Skin Absorption of Lead

L. S. Dale, J. L. Stauber, O. P. Farrell, and T. M. Florence
CSIRO DIVISION OF COAL AND ENERGY TECHNOLOGY, LUCAS HEIGHTS, N.S.W., 2234, AUSTRALIA

B. L. Gulson
CSIRO DIVISION OF EXPLORATION GEOSCIENCE, NORTH RYDE, N.S.W., 2113, AUSTRALIA

1 INTRODUCTION

The major sources of lead exposure are ingestion and inhalation. Exposure by absorption through the skin was previously thought to occur only if the lead was present as lipid-soluble organic complexes such as tetraethyl lead. It has however, been shown that many inorganic forms of lead such as lead metal, lead oxide and lead nitrate are rapidly absorbed through the skin[1]. An absorption mechanism was proposed based on the rapid diffusion of lead ions through the filled sweat ducts and their slower diffusion through the stratum corneum into the blood capillaries[2]. It was found that approximately 1.5 percent of the total lead absorbed through the skin was excreted in sweat. Similar amounts were found in sweat when lead was introduced intravenously[3] or by inhalation or ingestion[4].

The behaviour of skin-absorbed lead is very different from ingested lead. Previous skin absorption studies using the enriched stable isotope ^{204}Pb in which TIMS was used to analyse blood samples, revealed that, while there was a substantial increase in ^{204}Pb in blood, no significant increase in the total lead in blood was observed. Since blood lead is the main criterion by which industry determines lead exposure, skin absorption of lead would remain undetected. The possibility remains that a significant proportion of the lead body burden of workers results from skin absorption. The occupational hygiene implications of this are profound. Although precautions are taken in the lead industry to protect workers from ingestion and inhalation of lead, no attempt is made to avoid skin contact.

To further investigate the fate and transport of skin-absorbed lead, a controlled experiment was carried out using lead enriched in ^{204}Pb. Sweat and urine samples were analysed by both ICPMS and TIMS. The rationale behind the use of both techniques was that ICPMS, because of its high sample throughput and minimal sample preparation requirements, would provide rapid

was not known whether these data would be adequate to provide a satisfactory monitoring profile. On the other hand, analysis of the samples by TIMS, because of its established accuracy and precision, was expected to provide a more definitive indication of the lead transport.

The experiment, therefore, provided the opportunity to compare the analytical performance of ICPMS and TIMS in relation to isotope ratio data and determination of low levels of lead in body fluids.

2 EXPERIMENTAL

Preparation of $^{204}Pb(CH_3COO)_2$

Lead metal (4.8 mg) obtained from Harwell and containing 49 at. percent ^{204}Pb, was dissolved in 0.3 mL concentrated nitric acid (Merck Suprapur) by repeated evaporation to dryness. Milli-Q water (60 µL) and 15 µL 1M acetate buffer (pH 4.7) were added to bring the final solution to pH 4.

Lead Exposure

The left arm of a subject was washed with 5 percent Extran-300 (BDH) detergent, rinsed with Milli-Q water and dried with a Whatman 542 filter paper.

The $Pb(CH_3COO)_2$ solution was pipetted onto a 25mm Millipore HATF (0.45 µm) membrane filter and placed on the flexor surface of the subject's left arm. It was covered with a small piece of acid-washed Parafilm and held in place for 24 hours on the forearm using clear household polyethylene film.

Collection of Sweat Samples

Sweat samples from the opposite arm were collected in a sauna the day before exposure and $1^1/_2$, $3^1/_4$, 6 and 25 hours after the application of the lead. At the onset of sweating the subject washed the right arm with Milli-Q water. Sweat was then collected from this arm into acid-washed Savillex containers, weighed and stored at 4°C prior to analysis.

Collection of Urine Samples

Urine samples were collected in 500 mL acid-washed polyethylene containers before lead exposure and $1^1/_2$, $3^1/_4$, 6, 15, 25 and 47 hours after lead exposure. The samples were split for analysis by ICPMS and TIMS.

Analysis by ICPMS

Sweat samples were diluted five-fold with Milli-Q water. Urine samples were diluted ten-fold. They were acidified with Merck Suprapur nitric acid (1 percent $^w/_w$) and spiked with bismuth internal standard solution at a concentration equivalent to 100 µg L^{-1}. Calibration standards of lead in the range 0.1 to 5 µg L^{-1} were prepared containing the same acid strength and bismuth concentration as the samples.

The samples and standards were run on a VG Plasmaquad PQ2 PLUS (Fisons Instruments) using an isotope ratio peak jump procedure. The conditions are given in Table 1.

Table 1 ICPMS Instrumental Conditions

Measurement Mode	peak jump
Dwell Time	10240 µs
Points per Peak	7
DAC Steps	5
No. of Sweeps	120
No. of Runs	10
Isotopes Measured	204, 206, 207, 208, 209
Sensitivity	10 M cps / ppm equivalent

Analysis of TIMS

Sweat and urine samples were microwave digested with sub-boiling distilled nitric acid. Each sample was spiked with a standard ^{202}Pb solution prior to digestion to enable measurement of lead content and isotope ratios on the same solution. The lead in the samples was separated using a two-stage ion exchange procedure. The initial separation used a 0.5 cc bed of AG1-X4 200-400 mesh anion exchange resin and final separation purification used a 0.05 cc bed of AG1-X8 200-400 mesh anion exchange resin. All operations were carried out in laminar flow work bench stations.

Purified samples were loaded onto outgassed rhenium filaments using silica gel/phosphoric acid as an emitter. The samples were run on a VG Sector 54 Mass Spectrometer (Fisons Instruments) using the conditions given in Table 2.

Table 2 TIMS Instrumental Conditions

No. of Scans	60
Integration Time	1 s per peak, 2 s delay
Run Time	10 min after beam stabilisation
Ratios Measured	$^{208}/_{206}$, $^{207}/_{206}$, $^{206}/_{204}$

3 RESULTS AND DISCUSSION

The results for the concentrations of ^{204}Pb and total Pb in the sweat and urine samples obtained by ICPMS and TIMS are shown in Table 3. Agreement for the sweat samples is considered very satisfactory given that the actual levels measured by ICPMS are one-fifth lower than indicated. The agreement obtained for the urine samples is, in some cases, not as good. The reason for this was later attributed to the presence of mercury which was detected in the residue of one sample after the isotope ratio measurements had been performed. Mercury would have a significant contribution to mass 204 thus affecting the ^{204}Pb determination. This was most

Table 3 Concentrations of ^{204}Pb and Total Pb in Sweat (S) and Urine (U) Samples

SAMPLE	TIME* (h)	ICPMS ^{204}Pb µg L^{-1}	ICPMS Total Pb µg L^{-1}	TIMS ^{204}Pb µg L^{-1}	TIMS Total Pb µg L^{-1}
S1	0	0.46	31	0.63	44
S2	$1^1/_2$	0.17	9.9	0.17	9.5
S3	3	0.61	5.5	0.70	5.4
S4	6	0.51	9.3	0.60	8.3
S5	25	6.7	24	9.1	27
U1	0	0.20	9.3	0.09	6.4
U2	$1^1/_2$	0.17	4.3	0.16	4.8
U3	$3^1/_4$	0.13	6.7	0.08	4.7
U4	6	0.16	8.6	0.12	7.2
U5	15	0.35	18	0.07	4.7
U6	25	0.13	6.0	0.09	5.7
U7	47	0.13	5.0	0.05	3.1

* Time sample taken after application of lead.

evident in the ICPMS results for ^{204}Pb in samples U1 and U5.

Of greater significance is the lead concentration profiles inferred from the ^{204}Pb results. For the sweat, there was only a significant increase in ^{204}Pb after 25 h exposure. For urine, no significant breakthrough of ^{204}Pb was apparent. No significant trends in the sweat and urine samples were observed even when the concentrations of both total Pb and ^{204}Pb concentrations were normalised to chloride and creatinine respectively.

In contrast to this the results obtained for the ^{204}Pb isotope abundances (expressed as atomic percent ^{204}Pb) in the samples shown in Table 4, indicate a sharp breakthrough after 3 hours followed by gradual increase to 25 hours for sweat and a sharp breakthrough after $1^1/_2$ hours for urine. The fact that these profiles were not detectable from ^{204}Pb and total Pb concentrations is indicative of the complex mechanisms of lead transport in the body and highlights the uncertainty in monitoring lead body burden.

A comparison of the ICPMS and TIMS data for ^{204}Pb isotope abundances in sweat and urine are shown in Figures 1 and 2 respectively. The profiles obtained by ICPMS for both the sweat and urine profiles were very similar to those obtained with the TIMS data. The ICPMS data for sweat appeared to exhibit some bias. This is considered of minor consequence since the same profile was obtained.

Of perhaps greater significance is the relative ease with which the ICPMS data was obtained. Using minimal sample preparation and a simple calibration procedure the data was obtained and processed in one day. This is in contrast to the need for lengthy sample pretreatment

(3-4 days) and instrument setup time for TIMS. Thus the ICPMS technique is extremely cost-effective.

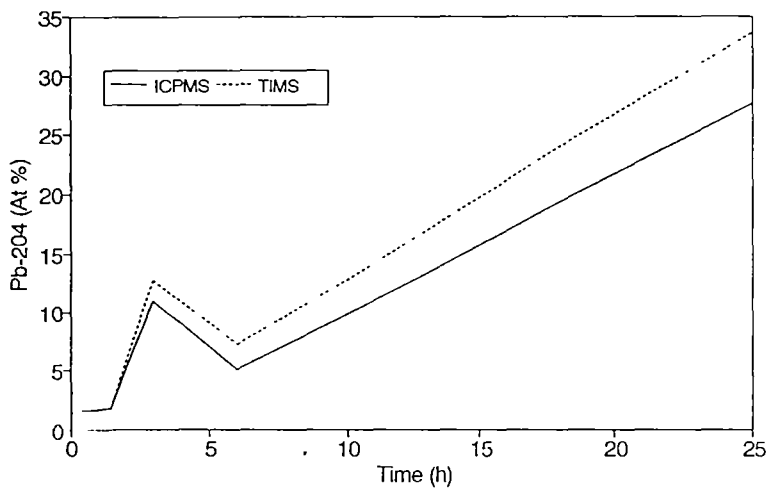

Figure 1 Comparison between ICPMS and TIMS results for ^{204}Pb (at. percent) in sweat.

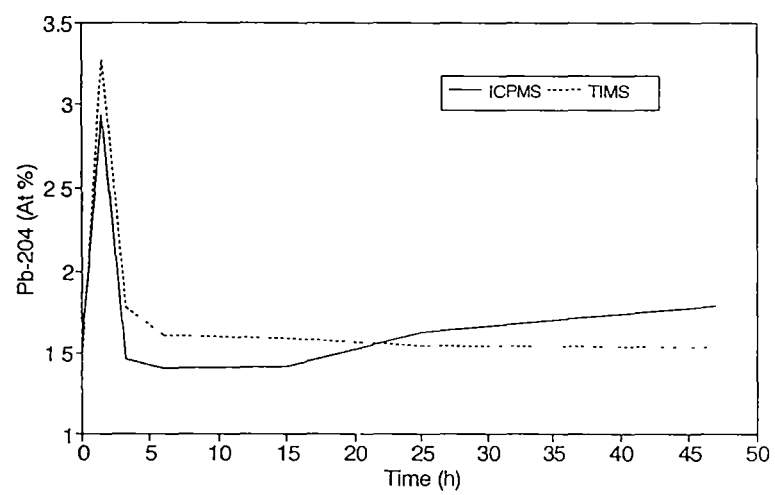

Figure 2 Comparison between ICPMS and TIMS results for ^{204}Pb (at. percent) in urine.

TABLE 4 ^{204}Pb Abundances in Sweat and Urine Samples

SAMPLE	TIME (h)	^{204}Pb (at. percent) ICPMS (±2σ)	TIMS (±2σ)
S1	0	1.481±0.027	1.4473±0.0022
S2	$1^1/_2$	1.754±0.054	1.7674±0.0027
S3	3	11.06±0.31	12.850±0.019
S4	6	5.15±0.39	7.249±0.011
S5	25	27.66±0.066	33.738±0.051
U1	0	1.55±0.16	1.457±0.002
U2	$1^1/_2$	2.94±0.20	3.279±0.005
U3	$3^1/_4$	1.46±0.08	1.775±0.003
U4	6	1.40±0.14	1.606±0.002
U5	15	1.41±0.07	1.587±0.002
U6	25	1.63±0.13	1.541±0.002
U7	47	1.79±0.07	1.527±0.002

4 CONCLUSIONS

The use of lead enriched in ^{204}Pb coupled with isotope ratio measurements has provided important data on the transport of lead absorbed in the skin. It has been shown that ICPMS has the capability of providing data adequate for monitoring lead transport at the very low levels of concentration encountered in body fluids. Although ICPMS lacks the accuracy and precision achieved by TIMS, its application described here is enhanced by the minimal sample preparation required and the high sample throughput than can be achieved.

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Percutaneous absorption of inorganic lead compounds

J.L. Stauber*[a], T.M. Florence[a], B.L. Gulson[b], L.S. Dale[a]

[a]*CSIRO Centre for Advanced Analytical Chemistry, PMB 7, Menai, NSW, 2234, Australia*
[b]*CSIRO Division of Exploration Geoscience, North Ryde, NSW, 2113, Australia*

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Abstract

In vivo experiments with the stable lead isotope, ^{204}Pb, have confirmed that inorganic lead compounds can be absorbed through the skin. Three different analytical techniques — thermal ionization mass spectrometry, inductively coupled plasma mass spectrometry and anodic stripping voltammetry — showed that lead, as lead nitrate or lead acetate, was rapidly absorbed through the skin and detectable in sweat, blood and urine within 6 h of skin application. Of the 4.4 mg of lead applied to the skin in one experiment, 1.3 mg was absorbed within 24 h. Initial rapid uptake was probably via sweat glands and hair follicles, followed by slower absorption via the transepidermal route. While increases in ^{204}Pb concentration and abundance were observed, no increase in total lead in blood or urine was found It is possible that the physicochemical form of skin-absorbed lead partitions strongly into extracellular fluid, but has a low affinity for erythrocytes. There was no significant difference in uptake of lead into erythrocytes (in vitro) from normal saline, synthetic sweat or sauna sweat. Ultrafiltration of sweat showed that up to 70% of lead in sweat was associated with >30 000 MW particles. It is possible that percutaneous absorption of lead could contribute significantly to lead body burden, particularly from occupational exposure to lead in dust. Moreover, because lead absorbed through the skin was only just detectable in blood, and blood lead is the main criterion by which industry determines exposure, skin-absorbed lead may remain undetected

Key words: Lead, Skin absorption; ICPMS, TIMS; Sweat

1. Introduction

The major sources of lead exposure to humans are considered to be via inhalation or ingestion. However, considerable quantities of lead from environmental sources, such as soil, water and dust, and workplace exposure, are in continuous contact with the skin (Ferguson, 1990). Lead, therefore, has the potential to be absorbed on the stratum corneum and diffuse through and between epidermal cells into the dermis for uptake into blood capillaries.

Skin absorption of lead is not usually considered to be a significant mode of uptake unless the lead is present as lipid-soluble organic complexes, such as tetraethyllead (Rastogi and Clausen, 1976). The limited evidence for cutaneous absorption of inorganic metal compounds has been larg-

* Corresponding author

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ely derived from animal studies (Laug and Kunze, 1948) or in vitro experiments using cadava or animal skin (Wester et al., 1992).

Laug and Kunze (1948) found that lead acetate, a highly polar lead salt, could be absorbed through the skin of the rat, resulting in a rise in lead concentration in the kidney. However, the skin of many laboratory animals, including the rat, is known to be much more permeable than human skin (Wester and Noonan, 1980). In addition, skin absorption depends on many experimental parameters including the applied dose, surface area, site of application, occlusion, skin damage, carrier compounds, pH, temperature and lead speciation.

Moore et al. (1980) examined the uptake of lead acetate from two hair-darkening cosmetics through the skin of eight human volunteers. They found only minute quantities of lead (0–0.3% of the applied dose) were detectable in blood, with a slight increased absorption when the skin was damaged. However, the presence of colloidal sulphur in the lead acetate solution applied to the skin, would have led to the formation of insoluble lead sulphide, which would be unlikely to be skin-absorbed.

More recently, Lilley et al. (1988) and Florence et al. (1988) have shown that many inorganic forms of lead, including lead metal, lead oxide and lead nitrate, were rapidly absorbed through human skin in vivo. An absorption mechanism was proposed whereby lead ions diffused rapidly through the filled sweat ducts, followed by a slower diffusion through the stratum corneum. Skin absorption of lead caused elevated levels of lead in saliva and in sweat from the opposite arm to which the lead was applied, but no significant concomitant increase in lead in blood or urine was detected using anodic stripping voltammetry. This inability to detect a rise in blood lead was of major concern because the rapid circulation of skin-absorbed lead around the body (< 1 h) suggested that plasma transport had to be involved.

The aim of this study was to monitor the uptake and transport of skin-absorbed inorganic lead compounds in sweat, urine and blood of human volunteers using a stable lead isotope enriched in ^{204}Pb. To improve the detection of small increases of lead in body fluids, total lead and ^{204}Pb concentrations, together with lead isotope ratios, were determined using thermal ionisation mass spectrometry (TIMS) and inductively coupled plasma mass spectrometry (ICPMS).

2. Experimental

2.1. Collection of sweat

Forearm sweat was chemically induced by pilocarpine iontophoresis or thermally induced in a sauna.

2.1.1. Iontophoresis The inner forearm of the subject was washed with 1% Extran-300 (BDH) detergent and Milli-Q water, and dried with a Whatman 542 filter paper. Sweat samples were collected using the Macroduct Sweat Collection System Model 3700-Sys, after removal of the blue, water-soluble indicator dye.

2.1.2 Sauna Prior to sauna entry, volunteers scrubbed their forearms with 1% Extran-300 detergent and Milli-Q water. After commencement of sweating, volunteers' forearms were again washed with Milli-Q water and dried with a filter paper Sweat was collected from the forearm into acid-washed Savillex teflon containers, weighed and stored at 4°C prior to analysis. Chloride in sweat was determined by the method of Florence and Farrar (1971)

2.2 Collection of urine

Urine samples were collected into 500-ml acid-washed polyethylene containers. Subsamples were stored at 4°C prior to creatinine determinations using Sigma Diagnostics Kit 555-A and lead determinations by inductively coupled plasma mass spectrometry (ICPMS) and thermal ionization mass spectrometry (TIMS).

2.3. Collection of blood

Venous blood samples were collected before, during and after lead exposure in a Class 100 clean room, using either heparin vacutainers for whole blood or pre-cleaned Savillex teflon beakers for separation of serum after clotting.

2.4 Analysis of total lead by ASV

Accurate volumes of sweat (60–80 μl) were

dispensed into 5 ml of 0.02 M Merck Suprapur HCl. Lead in sweat was determined by anodic stripping voltammetry (ASV) at a hanging mercury drop electrode, using an EG & G Princeton Applied Research Model 384 Polarographic Analyser with a Model 303 static mercury drop assembly (Stauber and Florence, 1988; Lilley et al., 1988).

2.5. Analysis of lead by ICPMS

Sweat samples (diluted 1:5) and urine samples (diluted 1:10) were acidified with Merck Suprapur HNO_3 (1% w/w) and spiked with a bismuth internal standard solution to 100 $\mu g \cdot l^{-1}$. Samples and calibration standards were analysed on a VG Plasmaquad PQ2 PLUS (Fisons Instruments) using an isotope ratio peak jump procedure. Isotopes at mass 204, 206, 207, 208 and 209 were measured (Dale et al., in press).

2.6. Analysis of lead by TIMS

Sweat, urine, serum and whole blood samples were spiked with a standardised ^{202}Pb solution prior to microwave digestion with sub-boiling-distilled HNO_3. The lead in the samples was separated using a two-stage ion exchange procedure. The initial separation used a 0.5-ml bed of AG1-X4 (200–400 mesh) anion exchange resin and the final separation/purification used a 0.05-ml bed of AG1-X8 (200–400 mesh) resin. All operations were carried out in laminar flow workbench stations.

Purified samples were loaded onto outgassed rhenium filaments using silica gel/phosphoric acid as an emitter. Ratios of 208/206, 207/206 and 206/204 were measured on a VG Sector 54 Mass Spectrometer (Fisons Instruments).

2 7. In vivo lead exposure experiments

Quantitative lead absorption. Lead (4.4 ± 0.1 mg, as 0.5 M $Pb(NO_3)_2$) was pipetted onto a 0.45 μm Millipore HATF 25-mm diameter membrane filter and placed on the flexor surface of the left forearm of Subject A for 24 h. It was covered with a square of acid-washed Parafilm (American Can Company, Dixie/Marathon, Greenwich) and held in place using clear household polyethylene wrap. Two iontophoresis sweat samples from the flexor surface of the right arm were taken before and 1 h after the lead was placed on the left arm and analysed for total lead in sweat by ASV.

After 24 h, the filter paper was removed and placed in a large beaker. The Parafilm and polyethylene wrap were rinsed with Milli-Q water into the same beaker. The subject's arm was wiped twice with Whatman 54 filter paper segments soaked in 2 M Suprapur HNO_3, and these, together with Milli-Q rinses of the arm, were placed into the same beaker. A blank containing an HATF membrane filter, Whatman filter paper segments and rinsed Parafilm and wrap, was made up to the same volume (120 ml) with Milli-Q water Both solutions were evaporated in a clean air cupboard, followed by digestion with 5 ml HNO_3 plus 0.5 ml $HClO_4$ (Suprapur) to strong fumes of $HClO_4$. Lead was determined by ASV, after dilution and addition of 3 M sodium acetate buffer (pH 4.7), and by inductively coupled plasma atomic emission spectroscopy (ICPAES).

Lead uptake experiments Lead (5 mg) as 0.5 M $Pb(NO_3)_2$, was placed on an HATF membrane filter on the forearm of Subject C for 24 h and total lead in iontophoresis sweat was determined hourly for 24 h by ASV.

Further experiments with Subjects A and D investigated the uptake of lead through the skin with and without plastic covering, and lead in successive sweat samples from the same site on the arm.

Lead-204 experiments. Lead carbonate, containing 66 atom percent (at. %) ^{204}Pb was obtained from Oak Ridge National Laboratory, TN. Preliminary experiments showed that $PbCO_3$ was not absorbed through the skin. The enriched $PbCO_3$ (6.6 mg) was therefore dissolved in 0.3 ml HNO_3 (Merck Suprapur) and excess acid removed by repeated evaporation to dryness Milli-Q water (60 μl) was added to the $Pb(NO_3)_2$ residue to obtain a final pH of 3–4. In later experiments, because $PbCO_3$ enriched in ^{204}Pb was unavailable, lead metal (4.8 mg), containing 49 at. % ^{204}Pb (obtained from Harwell Chemistry Division, Oxfordshire, UK) was dissolved in the same way and the pH adjusted to 4 7 with the addition of 15 μl of 1 M acetate buffer.

In the first two experiments, 5 mg Pb (as 0 4 M

$Pb(NO_3)_2$, enriched in ^{204}Pb) was pipetted onto a Millipore HATF membrane filter and placed on the left arm of Subject A. It was covered with Parafilm and held in place with polyethylene wrap for 24 h (Experiment 1) or 48 h (Experiment 2)

In the first experiment, sweat samples were collected by iontophoresis from the opposite arm before (3 replicates) and during lead exposure. A further sweat sample was obtained after the lead was removed. Total Pb, ^{204}Pb and ^{204}Pb abundance were determined by ICPMS.

In Experiment 2, iontophoresis sweat samples were collected before and during lead exposure and analysed for total lead by ASV. Venous blood samples were also collected and total Pb, ^{204}Pb and ^{204}Pb abundance were determined by TIMS.

The ^{204}Pb experiment was repeated with 5 mg Pb as 0.4 M $Pb(CH_3COO)_2$, enriched in ^{204}Pb, with a 24-h exposure using Subject B. Iontophoresis sweat samples and urine samples were collected before, during and after the lead was placed on the arm and analysed by TIMS. Two additional urine samples were collected 14 and 15 days after the lead was removed. Blood samples were collected, in a Class 100 clean room, before, during and after exposure. Subsamples were allowed to clot and the serum separated by centrifugation. Serum and whole blood samples were stored at −15°C prior to TIMS analysis.

In the fourth experiment, 5 mg of Pb as 0.4 M $Pb(CH_3COO)_2$, enriched in ^{204}Pb, was placed on Subject B for 24 h, and sweat samples collected in a sauna before, during and after lead exposure, to provide larger volumes of sweat than could be obtained by iontophoresis for analysis by TIMS and ICPMS. Urine samples were also collected before, during and after exposure and were analysed by both ICPMS and TIMS.

2.8. In vitro experiments

Ultrafiltration of sauna sweat A 3-ml aliquot of sauna sweat was centrifuged for 30 min in a 30 000 MW Amicon Centricon microconcentrator, which had been prewashed and centrifuged with 2 ml of 0.02 M HCl, followed by Milli-Q water. One millilitre of the ultrafiltrate was retained for lead analysis and the remainder centrifuged through a 10 000 MW microconcentrator. Lead in the sauna sweat, before and after ultrafiltration, was determined by ASV after UV irradiation in 0.015 M HCl and 0.005 M H_2O_2.

Lead dissolution in sweat. To determine the solubility of lead in sweat, 50 mg of lead metal powder (< 200 μm) was shaken in either 50 ml of Milli-Q water, synthetic sweat (pH 5.3) (Stauber and Florence, 1988), sauna sweat, NaCl (0.8 mg ml^{-1}, pH 5.2) or lactic acid (1.5 mg ml^{-1}, pH 5.2) for 16 h. Each solution was filtered through a 0 45 μm disposable membrane filter, which had been prewashed with 2 M HNO_3 (Suprapur) and Milli-Q water. Lead in the filtrates and in a blank subjected to the same procedure was determined by ICPAES.

The experiment was repeated with 50 mg Pb (as PbO) and with ultrafiltration by centrifugation through a prerinsed Amicon Centricon microconcentrator (30 000 MW cutoff).

Uptake of ^{210}Pb into Erythrocytes. The in vitro uptake of lead from saline (0.9%), synthetic sweat and sauna sweat into erythrocytes was determined.

Blood was collected from 4 male volunteers, pooled, and the hematocrit determined. Total lead in blood was analysed by ASV, after UV irradiation in 0.1 M HNO_3 and 0.005 M H_2O_2. Total lead in a Seronorm reference standard (Nycomed AS, Norway) was determined using the same method.

Aliquots of blood (2 ml) were dispensed into plastic centrifuge tubes and incubated at 37°C for 30 min. ^{210}Pb (20 μl of 1 mCi/5 ml stock) was added to 1 ml of 0.9% saline and neutralised by the addition of 12 μl of 5 M NaOH. Aliquots (0.1 ml) were added to the blood tubes at 37°C. At various time intervals, tubes were removed from the waterbath and 0.2 ml blood diluted to 5 ml with saline for counting in a gamma well counter. The remaining 1.8 ml blood was centrifuged, 0.2 ml plasma removed and diluted to 5 ml for a gamma count to determine Pb in plasma. Lead in erythrocytes was determined by difference.

The experiment was repeated using ^{210}Pb in synthetic sweat and sauna sweat, in place of saline. ^{210}Pb (20 μl) was added to 1 ml of synthetic sweat and the pH adjusted to 5.3. Sauna sweat was spiked with 20 μl of ^{210}Pb and the pH adjusted to 4.7. Both solutions were equilibrated for 24 h before use.

The rate of leaching of ^{210}Pb from erythrocytes

was also examined. Four centrifuge tubes, each containing 2 ml blood and 0.1 ml of ^{210}Pb in either saline or synthetic sweat, were incubated at 37°C for 30 min. Lead in whole blood, plasma and erythrocytes was determined at zero time. At various time intervals, 0.2 ml of blood from each tube was counted in a gamma well counter. The remaining blood was centrifuged and ^{210}Pb in the supernatant (plasma) determined by gamma counting. The plasma was removed and the erythrocytes washed twice with saline or synthetic sweat, before resuspension. ^{210}Pb in each washing and the final solution was determined.

3. Results

3.1. In vivo experiments

Preliminary experiments showed that lead as $Pb(NO_3)_2$, $Pb(CH_3COO)_2$, PbO and elemental Pb were absorbed through the skin and detected in sweat on the opposite arm within a few hours. Only lead as $PbCO_3$ was not absorbed through the skin on the forearm.

Lead in sweat measured hourly by ASV during a 24-h skin lead exposure to Subject C is shown in Fig. 1. The average lead in sweat before exposure was 6 ± 4 $\mu g \cdot l^{-1}$. Lead in sweat from the right arm reached a maximum of 174 $\mu g \cdot l^{-1}$, 4 h after the lead was placed on the left arm and decreased rapidly to background levels after 6 h. By measuring the area under the curve in Fig. 1, the 24-h average lead in sweat was calculated as 16 $\mu g \cdot l^{-1}$, much less than the average lead in sweat over 24 h determined for Subject A (46 $\mu g \cdot l^{-1}$).

Sweat excretion of lead varied between subjects depending on skin hydration, occlusion, physical activity and atmospheric conditions. When lead was rubbed onto the forearm of subject A, with no covering over the treated area, lead in sweat from the other arm increased from a background of < 1 $\mu g \cdot l^{-1}$ to a maximum of 46 $\mu g \cdot l^{-1}$ after 2.5 h. This maximum was lower than the 71 μg Pb/l found when lead was applied to the arm and covered with Parafilm and polyethylene wrap for 24 h. Consecutive sweat samples taken from the same spot on the forearm showed the same varia-

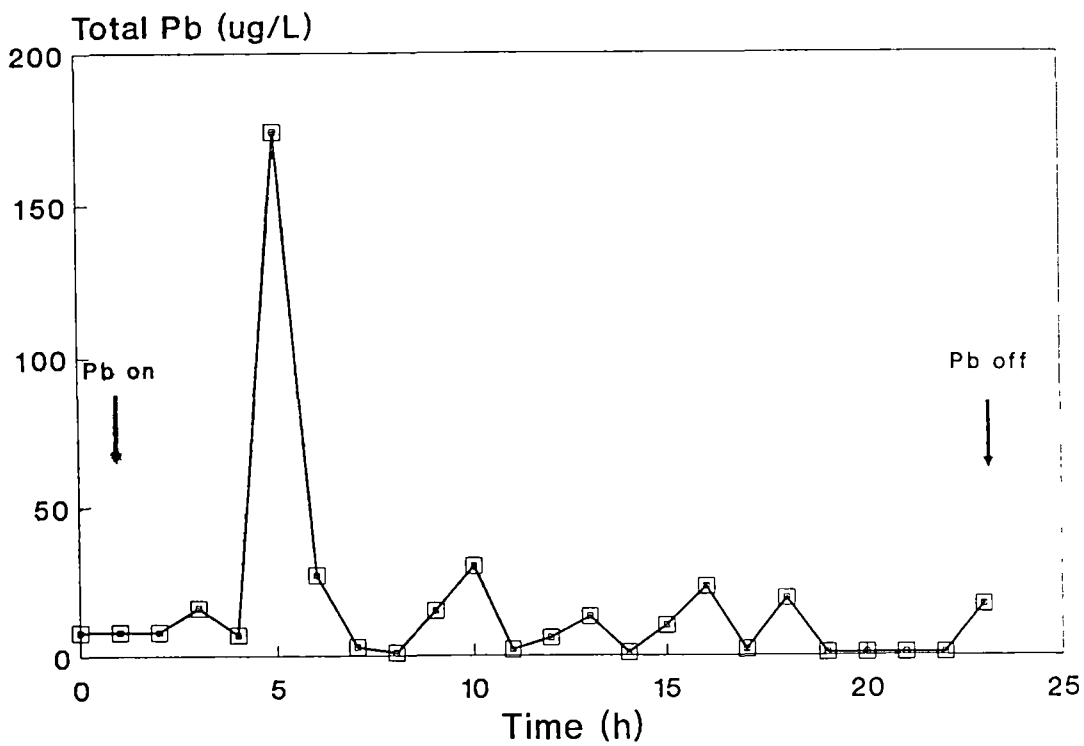

Fig. 1. Total lead in sweat measured hourly by ASV during a 24-h skin lead exposure (Subject C)

tion in lead concentration as samples taken from different spots on the arm.

The quantitative lead absorption experiment showed that, of the 4.4 mg of lead placed on the arm, 3.1 mg was recovered from the filter paper and arm washings, i.e. 29% (1.3 mg) was absorbed into or through the skin. It is possible that some of the absorbed lead was still present in the epidermis and had not yet entered the circulatory system

3.2. ^{204}Pb Experiments

Concentrations of total lead and ^{204}Pb in sweat (Experiment 1) measured by ICPMS are shown in Fig. 2. Total lead in sweat reached a maximum of 70 $\mu g \cdot l^{-1}$ after 1.5 h, compared with background levels before exposure of 6 ± 5 $\mu g \cdot l^{-1}$. ^{204}Pb increased in parallel with total lead in sweat, reaching a maximum of 2.3 μg $^{204}Pb \cdot l^{-1}$. ^{204}Pb abundance (at. %) reached 3.95% after 5 h, compared with the natural abundance of 1.44%.

In Experiment 2, total lead and ^{204}Pb in blood during skin lead exposure to $Pb(NO_3)_2$, were determined using TIMS. To confirm lead was absorbed in this subject, total lead in sweat was also monitored by ASV. Lead in sweat was below detection limits (0.5 $\mu g \cdot l^{-1}$) before ^{204}Pb was placed on the arm Lead in sweat increased significantly over the 48 h lead exposure, with a maximum of 61 μg Pb $\cdot l^{-1}$ after just 1 h exposure Lead in sweat returned to background levels after exposure ceased

Concentrations of total lead and ^{204}Pb in blood, together with ^{204}Pb abundance, are shown in Table 1. There was no significant change in total lead in blood over the 48-h skin exposure, however, there was a small increase in ^{204}Pb (0.6 $\mu g \cdot l^{-1}$) More importantly, there was a significant increase in ^{204}Pb abundance, first observable after 6.5 h and with a maximum abundance of 1.824 at. % at 47 h. This increase in ^{204}Pb in blood confirmed that ^{204}Pb placed on the skin was absorbed through the skin and entered the circulatory sys-

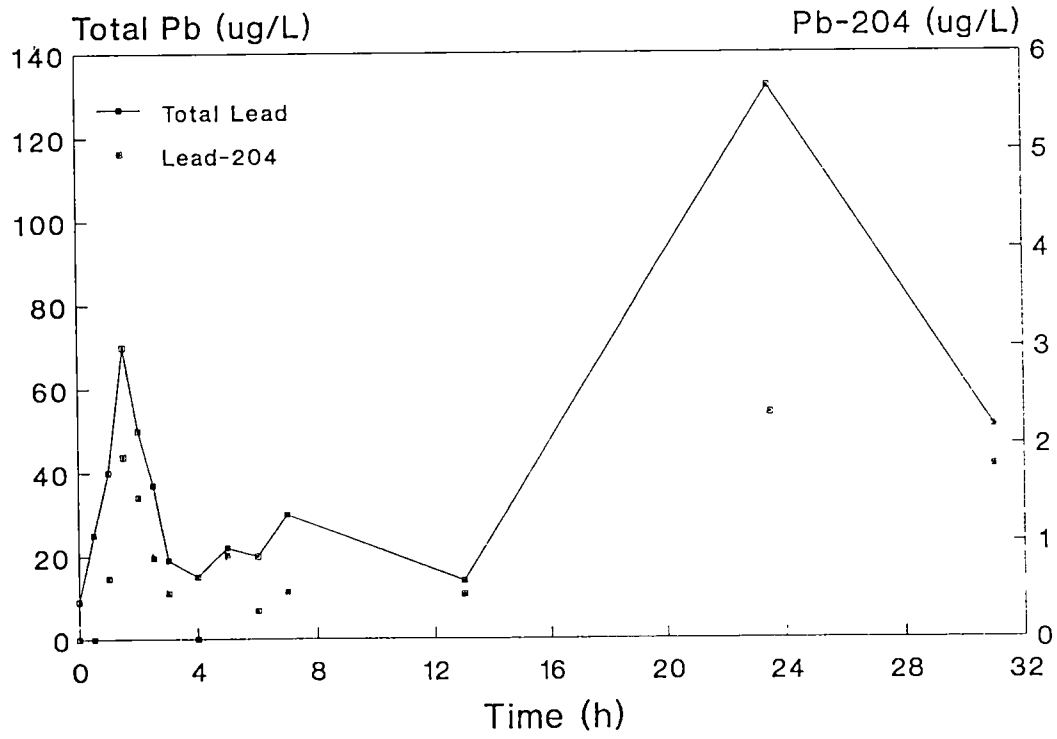

Fig. 2 Concentrations of total lead and ^{204}Pb in sweat measured by ICPMS after application of $Pb(NO_3)_2$, enriched in ^{204}Pb, to the skin for 24 h (Experiment 1)

Table 1
Concentrations of total Pb and ^{204}Pb, and ^{204}Pb abundance in whole blood, measured by TIMS (Experiment 2)

Time (h) after lead placed on the arm	Total Pb ($\mu g \cdot l^{-1}$)	^{204}Pb ($\mu g \cdot l^{-1}$)	^{204}Pb (at percent)
Before	148	2.13	1 439[a]
2	138	1 98	1 440
6 5	144	2 13	1 479
24	146	2 47	1 692
31	144	2.53	1 757
47	148	2.70	1 824

[a]Relative standard deviation was 0 001 at. %

tem to be transported throughout the body and excreted in sweat on the opposite arm.

The ^{204}Pb experiment was repeated (Experiment 3) using a different subject (B), and $Pb(CH_3COO)_2$ enriched in ^{204}Pb in place of $Pb(NO_3)_2$, and 24 h skin exposure. Iontophoresis sweat, urine, blood and serum samples were collected before, during and after exposure and analysed by TIMS.

Concentrations of total Pb, ^{204}Pb and ^{204}Pb abundance in blood are shown in Table 2. Results with Subject B confirmed results obtained with Subject A in Experiment 2. While there was no increase in total Pb in blood, there was a significant increase of 0.5 μg $^{204}Pb \cdot l^{-1}$ over 47 h. There was also a significant increase in ^{204}Pb abundance, which was detectable 6 h after the lead was placed on the arm and remained elevated at least 24 h after the lead was removed (Fig. 3).

Table 2
Concentrations of total Pb and ^{204}Pb, together with ^{204}Pb abundance (at percent) in whole blood measured by TIMS (Experiment 3)

Time after lead placed on the arm	Total Pb ($\mu g \cdot l^{-1}$)	^{204}Pb ($\mu g \cdot l^{-1}$)	^{204}Pb (at percent)
Before	52	0 736	1 415[a]
Before	56	0.792	1 414
10 min	58	0.822	1 417
20 min	55	0 777	1 413
30 min	54	0 764	1 415
45 min	53	0.750	1 415
60 min	55	0 778	1 415
2 h	54	0 765	1 417
6 h	53	0 765	1 443
24 h	50	1 07	2 142
31 h	53	1.19	2.236
47.5 h	52	1.23	2.360

[a]Relative standard deviation was 0.001 at. %.

Total lead in serum ranged from 0.17–0.53 $\mu g \cdot l^{-1}$, with no significant increase over 48 h (Table 3). A significant increase in ^{204}Pb abundance in serum was observed after 24 h and remained high over 48 h, similar to ^{204}Pb in whole blood.

Lead in urine ranged from 2.2 to 3.5 $\mu g \cdot g^{-1}$ creatinine (3.6–8.1 $\mu g \cdot l^{-1}$), with a mean of 3.0 ± 0.5 μg Pb $\cdot g^{-1}$ creatinine over 16 days (Table 4). No increase in total Pb in urine was observed. However, both the concentration of ^{204}Pb and ^{204}Pb abundance increased significantly 6 h after Pb was placed on the arm (Fig. 4). By integrating the ^{204}Pb v time curve, it was calculated that approximately 6% of the increased ^{204}Pb in blood over 24 h was excreted in urine. ^{204}Pb abundance in urine reached a maximum after 24 h and was still higher than background after 16 days.

In contrast to Subject A, no increase in total lead in sweat, analysed by TIMS (or by ASV in a separate experiment), was observed during the skin lead exposure. A significant increase in both concentration and abundance of ^{204}Pb was detected after 24 h (Table 5). A small increase in ^{204}Pb abundance was found 2 h after the lead was placed on the arm, but this decreased to background levels before a large increase after 24 h.

In Experiment 4, sauna sweat samples were obtained to provide larger volumes of sweat for analysis, together with urine samples. Concentrations of total Pb and ^{204}Pb in urine and sweat samples, analysed by both ICPMS and TIMS, are shown in Tables 6 and 7. Good agreement between ICPMS and TIMS was obtained for the sweat samples and most of the urine samples. ^{204}Hg interfered with ^{204}Pb determination by ICPMS, leading to erroneous results for the urine sample taken at 15.25 h. No significant increase in total Pb or ^{204}Pb concentration in urine was found after 47 h. For sweat there was a significant breakthrough of ^{204}Pb after 25 h. Even when concentrations of Pb in sweat and urine were normalised to chloride

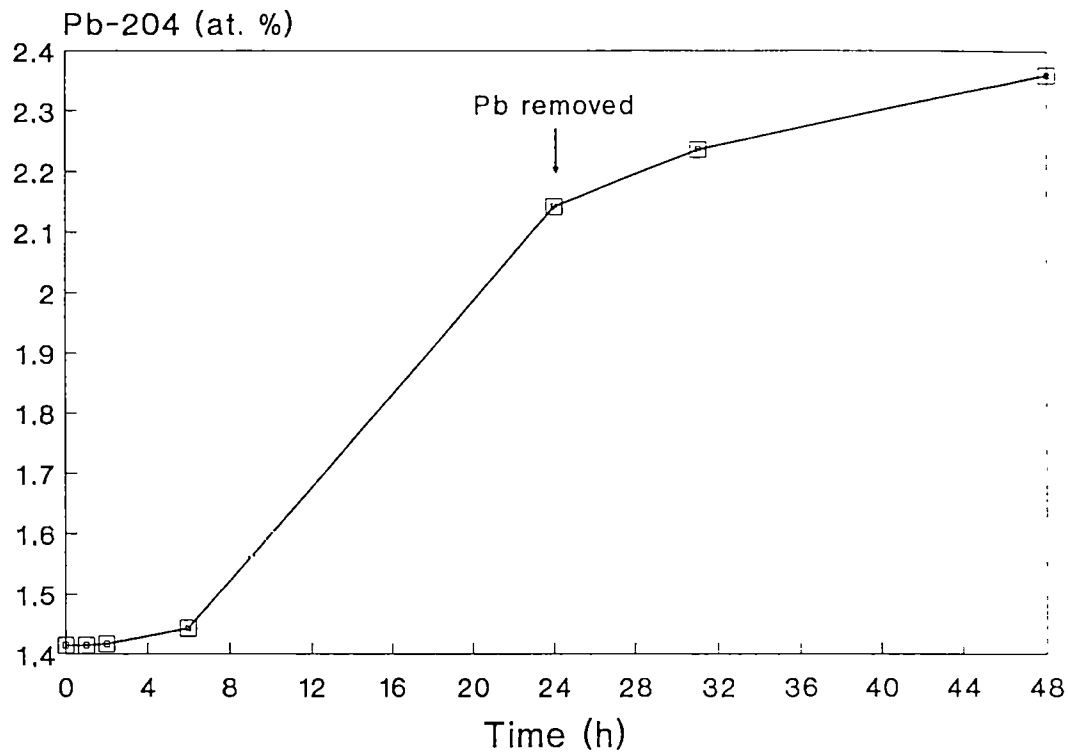

Fig 3 ^{204}Pb abundance in blood determined by TIMS after application of $Pb(CH_3COO)_2$, enriched in ^{204}Pb, to the skin for 24 h (Experiment 3)

Table 3
Concentrations of total Pb and ^{204}Pb, together with ^{204}Pb abundance in serum, determined by TIMS (Experiment 3)

Time after lead placed on the arm	Total Pb (ng · l^{-1})	^{204}Pb (ng · l^{-1})	^{204}Pb (at percent)
Before	200	2 89	1 445[a]
10 min	410	5 88	1 434
20 min	220	3 11	1 414
30 min	530	7 36	1 389
45 min	490	7 01	1 431
60 min	290	4 07	1 403
2 h	300	4.19	1 397
6 h	310	4 44	1 432
24 h	170	3.04	1.788
31 h	370	6.15	1 662
47.5 h	240	4 19	1.746

[a]Relative standard deviation was 0 024 at. %

Table 4
Concentrations of total Pb and ^{204}Pb, together with ^{204}Pb abundance in urine, determined by TIMS (Experiment 3)

Time after lead placed on the arm	Total Pb (μg/g creatinine)	^{204}Pb (μg/g creatinine)	^{204}Pb (at percent)
Before	3 0	0 042	1 400[a]
Before	3 3	0.047	1 424
14 min	3 1	0.044	1.419
51 min	3.6	0.052	1 444
76 min	3 3	0.046	1 394
131 min	2 2	0 032	1 455
4 h	2 5	0.036	1 440
6 h	3 4	0.069	2 029
24 h	2 9	0.091	3 176
31 h	3 2	0.096	3.001
47.5 h	3 5	0.078	2 229
15 days	2 2	0 043	1 955
16 days	2.4	0 048	2 000

[a]Relative standard deviation was 0 023 at. %

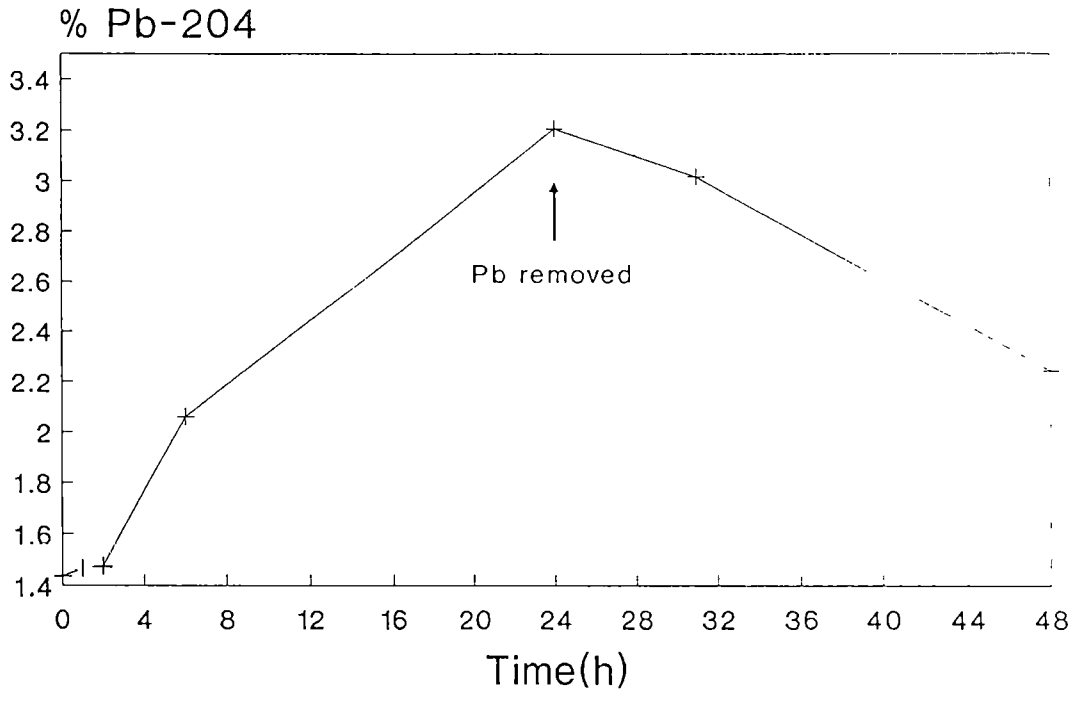

Fig 4 ^{204}Pb abundance in urine determined by TIMS after application of $Pb(CH_3COO)_2$, enriched in ^{204}Pb, to the skin for 24 h (Experiment 3).

and creatinine, respectively, no significant increase in total Pb was detected.

In contrast, ^{204}Pb isotope abundance results (Table 8, Figs. 5 and 6) show that there was a significant increase in % ^{204}Pb in sweat and urine after 1.5 h, followed by a gradual increase in sweat to 25 h.

Table 5
Concentrations of total Pb and ^{204}Pb, together with ^{204}Pb abundance in iontophoresis sweat, determined by TIMS (Experiment 3)

Time (h) after lead placed on the arm	Total Pb ($\mu g \cdot l^{-1}$)	^{204}Pb ($\mu g \cdot l^{-1}$)	^{204}Pb (at percent)
Before	12.6	0 181	1 437[a]
Before	3.7	0.053	1 431
1	4.6	0 067	1.460
2	2.4	0.039	1 626
3	1.8	0.026	1 462
4	3.7	0 053	1 444
5	4.3	0 063	1.473
6	8.2	0 120	1 468
24	10 8	1 094	10 127

[a]Relative standard deviation was 0 04 at %.

3.3. *In vitro experiments*

Ultrafiltration of sauna sweat. Sixty-one percent of lead in sauna sweat was associated with high molecular weight particles (> 30 000 MW). The remaining 39% of lead in sweat passed through the 30 000 MW microconcentrator, with 29% also passing through a 10 000 MW cutoff However, despite UV irradiation, ASV signals were severely depressed in sweat samples which had been passed through the microconcentrators, presumably from wetting agents used in the ultrafiltration membranes. Despite this, results were similar to those obtained previously (Stauber and Florence, 1988) in which 30% of lead in sweat was associated with < 10 000 MW particles. Large differences between individuals were found: lead in sauna sweat of Subject E was almost all associated with low molecular weight particles

Table 6
Concentrations of total Pb and ^{204}Pb in urine, determined by ICPMS and TIMS (Experiment 4)

Time (h) after Pb placed on the arm	ICPMS		TIMS	
	Total Pb (μg/g creatinine)	^{204}Pb (μg/g creatinine)	Total Pb (μg/g creatinine)	^{204}Pb (μg/g creatinine)
Before	4 6[a]	0.097[b]	3 1[c]	0 045[d]
1 5	2.2	0 090	2 5	0.082
3 25	2.3	0 046	1 6	0 029
6	2 5	0.047	2 1	0 033
15.25	2.3	—[e]	3.1	0 050
25	2.7	0 061	2 6	0.040
47	3 4	0 083	2 1	0 031

[a]RSD was ~7%.
[b]RSD was ~6%
[c]RSD was ~1%
[d]RSD was <1%
[d]^{204}Hg interference

Lead dissolution in sweat Only small amounts of Pb metal powder and PbO could be dissolved in sweat after shaking for 16 h (Table 9). More lead dissolved in Milli-Q water and synthetic sweat than in sauna sweat. Very little Pb dissolved in lactic acid or NaCl at concentrations found in synthetic sweat.

Uptake of ^{210}Pb into erythrocytes. The uptake of ^{210}Pb into erythrocytes from saline, synthetic sweat and sauna sweat is shown in Fig. 7. Lead uptake into erythrocytes at 37°C was rapid. After 5 min, approximately half the lead was associated with the erythrocytes (48, 41 and 57% for saline, synthetic sweat and sauna sweat, respectively) After 15 min, 75% of ^{210}Pb was associated with the red blood cells, with only a small further increase over 120 min. There was no significant difference in uptake of lead from saline, synthetic sweat or sauna sweat.

The pooled blood contained 43 μg Pb · l^{-1}. The lead in the Seronorm standards was always within 10% of the reference value.

Table 7
Concentrations of total Pb and ^{204}Pb in sauna sweat, determined by ICPMS and TIMS (Experiment 4)

Time (h) after Pb placed on the arm	ICPMS		TIMS	
	Total Pb (μg l^{-1})	^{204}Pb (μg · l^{-1})	Total Pb (μg l^{-1})	^{204}Pb (μg l^{-1})
Before	31[a]	0 46[b]	44[c]	0 63[d]
1 5	9 9	0 17	9 5	0 17
3	5 5	0 61	5 4	0 70
6	9 3	0 51	8 3	0 60
25	24	6 7	27	9 1

[a]RSD was 5%.
[b]RSD was 2%.
[c]RSD was 1–4%
[d]RSD was 1–4%

Table 8
^{204}Pb abundance in sweat and urine determined by ICPMS and TIMS (Experiment 4)

Time (h) after Pb placed on the arm	Sample	^{204}Pb (at percent)	
		ICPMS (± 2σ)	TIMS (± 2σ)
0	Sweat	1 481 ± 0 027	1 4473 ± 0 0022
1.5	Sweat	1 754 ± 0 0054	1.7674 ± 0.0027
3	Sweat	11 06 ± 0 31	12 850 ± 0 019
6	Sweat	5 15 ± 0 39	7 249 ± 0 011
25	Sweat	27 66 ± 0.066	33 738 ± 0.051
0	Urine	1.55 ± 0 16	1.457 ± 0.002
1.5	Urine	2.94 ± 0.20	3.279 ± 0 005
3.25	Urine	1.46 ± 0.08	1 775 ± 0.003
6	Urine	1.40 ± 0 14	1.606 ± 0 002
15	Urine	1 41 ± 0 07	1 587 ± 0 002
25	Urine	1.63 ± 0 13	1 541 ± 0.002
47	Urine	1.79 ± 0 07	1 527 ± 0 002

Table 9
Lead dissolved from Pb metal powder or lead oxide (PbO) after shaking for 16 h in various solutions

Lead	Solution	Dissolved Lead (ppm)
Pb grain (<200 μm)	Milli-Q	57
	Synthetic sweat	56
	Sauna Sweat	6 0
	NaCl	3.7
	Lactic Acid	0 01
PbO	Milli-Q	220
	Synthetic Sweat	40
	Sauna Sweat	20

Experiments to determine the rate of leaching of ^{210}Pb from erythrocytes into saline and synthetic sweat showed that after 48 h, only 4% and 7% of the ^{210}Pb was leached from the erythrocytes in saline and sweat, respectively. Most of the ^{210}Pb taken up over 30 min remained in the erythrocytes for over 2 days.

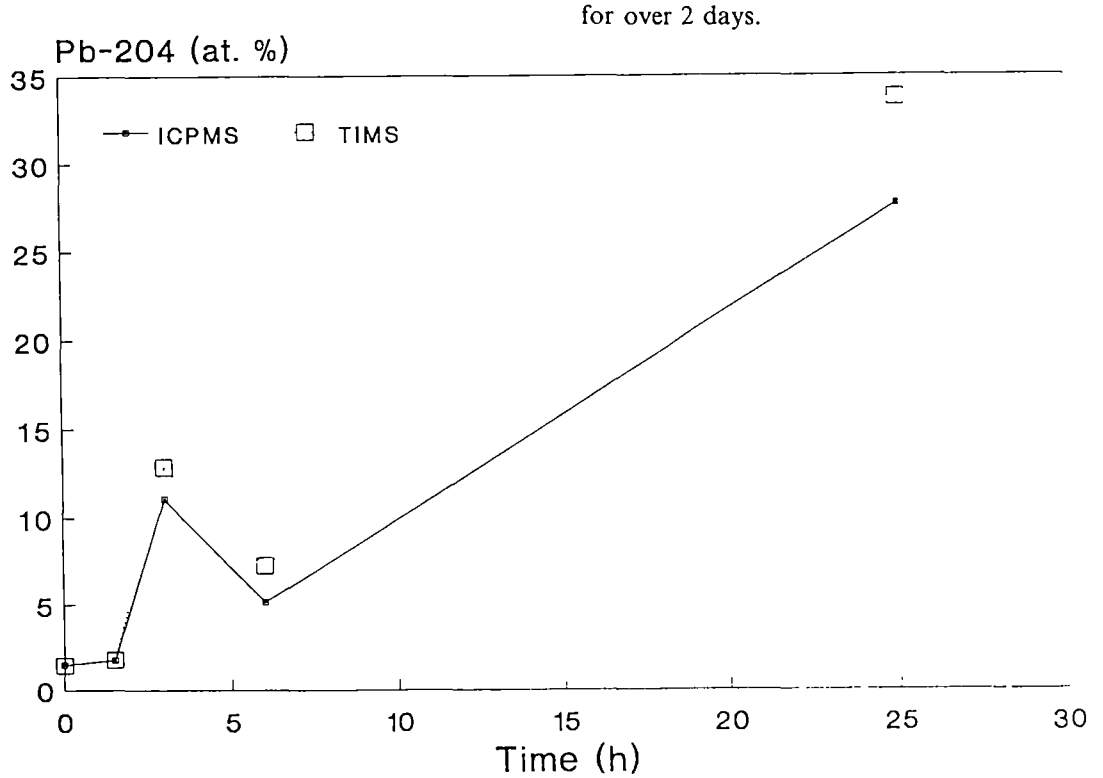

Fig 5 ^{204}Pb abundance in sweat determined by TIMS and ICPMS after application of $Pb(CH_3COO)_2$, enriched in ^{204}Pb, to the skin for 24 h (Experiment 4)

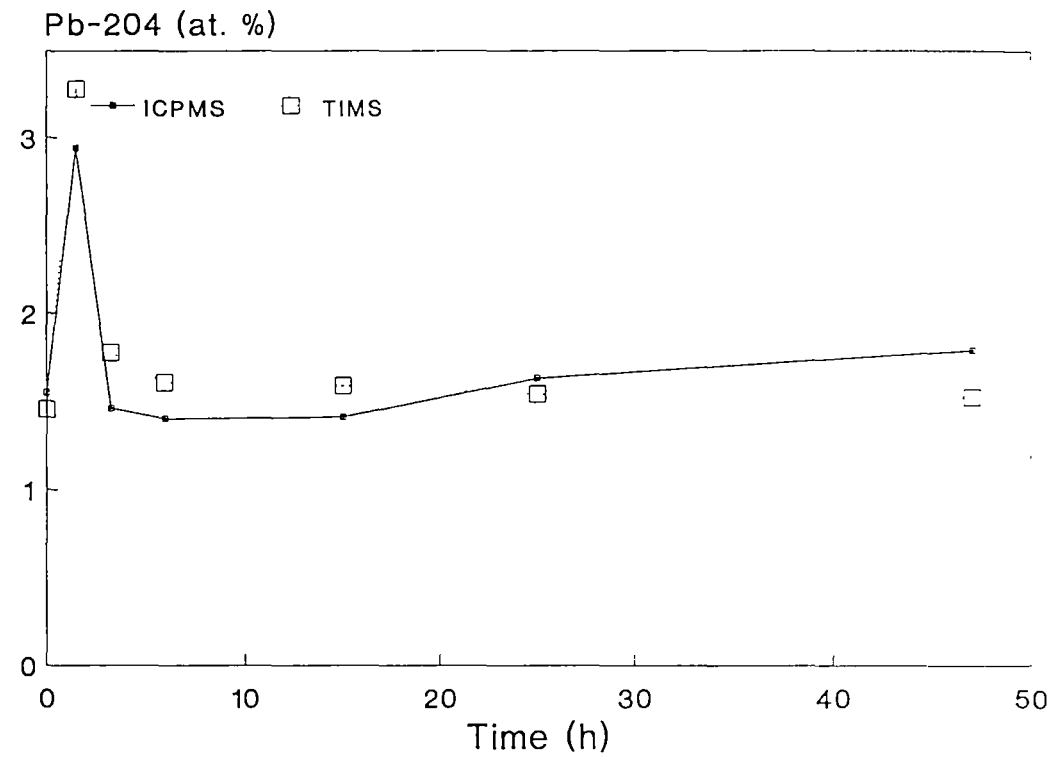

Fig 6 ^{204}Pb abundance in urine determined by TIMS and ICPMS after application of $Pb(CH_3COO)_2$, enriched in ^{204}Pb, to the skin for 24 h (Experiment 4).

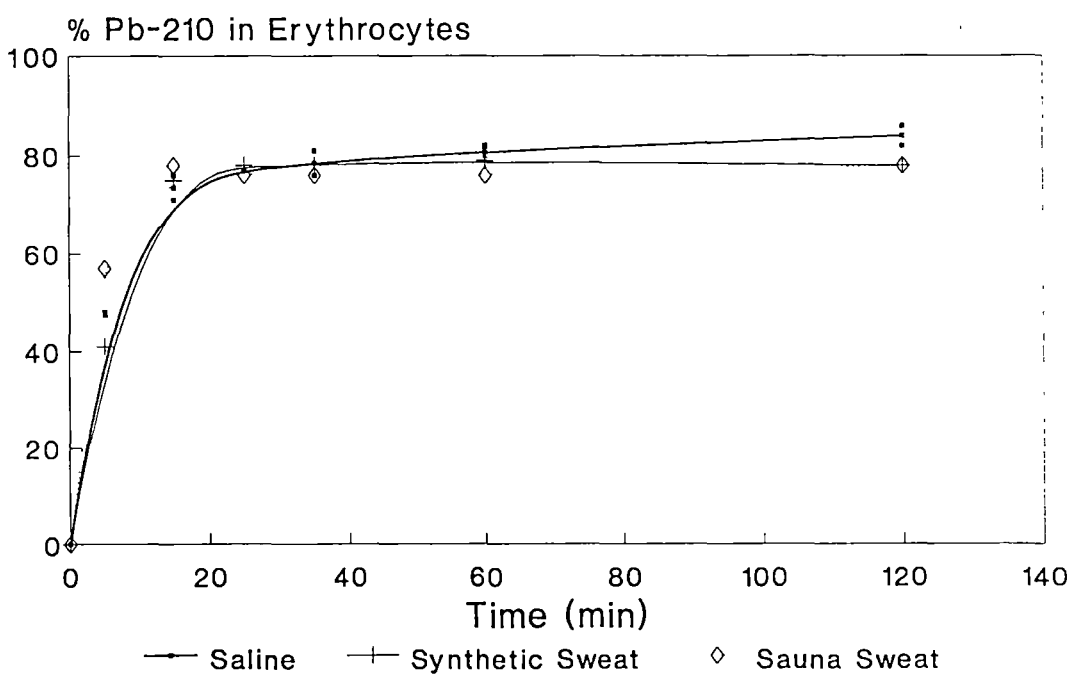

Fig 7 Uptake of ^{210}Pb into erythrocytes (in vitro) from saline, synthetic sweat and sauna sweat

4. Discussion

Three different analytical techniques, TIMS, ICPMS and ASV, confirmed that lead, as Pb $(NO_3)_2$ or $Pb(CH_3COO)_2$, was rapidly absorbed through the skin and detectable in sweat, blood and urine within 6 h of skin application. The use of the stable lead isotope, ^{204}Pb and the technique of TIMS, which measured ^{204}Pb abundance with a precision of 0.1%, enabled detection of small increases in lead in these body fluids, which was not previously detectable by ASV (Lilley et al., 1988).

For the first time, increases in ^{204}Pb in blood, serum and urine were found after application of ^{204}Pb to the forearm. While small increases in ^{204}Pb in blood (0.5–0.6 $\mu g \cdot l^{-1}$) and ^{204}Pb abundance in blood and urine were found in two different subjects, no increase in total lead in blood or urine was observed. This is in agreement with previous experiments in this laboratory (Lilley et al., 1988), in which no increase in total lead in blood or urine could be detected by ASV. Moore et al. (1980) also showed that lead acetate may be absorbed through the skin in small quantities, with no detectable change in total lead in blood, ALA dehydratase, blood porphyrin, urinary ALA or coproporphyrin.

Of the 5 mg of lead applied to the skin in one experiment, 1.3 mg was absorbed into or through the skin after 24 h. Initial rapid uptake was probably through sweat glands and hair follicles, which can act as diffusion shunts for rapid absorption, particularly for polar molecules (Scheuplein and Blank, 1971). This may be followed by slower diffusion via the transepidermal pathway (across the stratum corneum either intercellularly or intracellularly) and finally into the microcirculation (Fig. 8). Even after 24 h, much of the lead absorbed may have been retained in the epidermis, and not yet absorbed into blood capillaries. High levels of ^{204}Pb in blood and urine some 2 and 16 days, respectively, after exposure may reflect this slower route of absorption or alternatively, the slow elimination rate of lead from erythrocytes, in agreement with lead ingestion studies.

Integration of the sweat lead-time curve (Fig. 1) over 24 h, showed that the 24-h average total lead in sweat over this period was 16 $\mu g \cdot l^{-1}$ for Subject C and 46 $\mu g \cdot l^{-1}$ for Subject A. Daily sweat

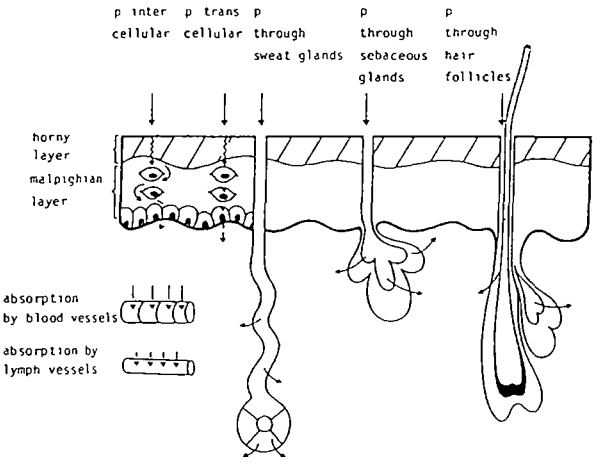

Fig 8 Pathways of percutaneous penetration (p = permeability) — reproduced from Bayer A G Training Centre Health Care Sector.

excretion varies from 0.05–4.0 l, depending on temperature, humidity, exercise and acclimatization (Vander et al., 1986). Assuming that a total of 500 ml of sweat was produced in 24 h, a total of 23 μg Pb $\cdot l^{-1}$ and 8 μg Pb $\cdot l^{-1}$ were excreted in sweat in Subjects A and C, respectively. This represents 1.5% and 0.6% of the total lead that was skin-absorbed. Campbell et al. (1984) and Rabinowitz et al. (1976), in pharmacokinetic studies of lead absorption following intravenous injection of radioactive ^{203}Pb or intake by ingestion, found approximately 2% and 0.7%, respectively, of the lead dose was excreted in sweat. Thus similar amounts of lead, whether injected, ingested or skin-absorbed, are eliminated in sweat.

Sweat excretion of lead varied between subjects depending on skin hydration, occlusion, physical activity and atmospheric conditions. In one subject (B), only 0.007% of the applied ^{204}Pb was excreted in sweat over 24 h and no increase in total lead in sweat was observed. If it is assumed that similar amounts of lead were absorbed as in the quantitative absorption experiment using Subject A, 0.03% of the absorbed ^{204}Pb was excreted in sweat over 24 h.

No significant increase in total lead in blood over 24 h was observed, however, increases in ^{204}Pb concentration and abundance were detected. Integration of the blood ^{204}Pb-time

curve over 24 h (Experiment 3), shows that the average increase in ^{204}Pb concentration in blood was 0.12 $\mu g \cdot l^{-1}$ Assuming a total blood volume of 6 l, 0.72 μg ^{204}Pb entered the blood in the first 24 h, equivalent to 0.2% of the skin-absorbed ^{204}Pb. This is negligible compared with the 58% of intravenous injected lead present in erythrocytes after 20 h (Chamberlain, 1985; Campbell et al, 1984).

In the same experiment no increase in total lead in urine was detected, which was expected, as any such increase would have had to originate from plasma. However, an increase in ^{204}Pb concentration and abundance in urine was observed 6 h after application of the lead. Integration of the urinary ^{204}Pb-time curve shows that the average increase in the concentration of ^{204}Pb in urine was 0.025 $\mu g \cdot g^{-1}$ creatinine. Assuming an average creatinine excretion of 1.7 g/day, only 0.006% of the absorbed ^{204}Pb was excreted in urine in the first 24 h. This was equivalent to 6% of the ^{204}Pb increase in blood. Similar results have been reported for excretion of intravenously administered lead, with 1–3% of the absorbed dose excreted in 24 h. Renal clearance rate (urinary Pb μg per day/blood Pb $\mu g \cdot kg^{-1}$) was 0.1 kg/day, the same as that found for clearance of intravenously injected lead by Chamberlain (1985). Renal clearance of lead with respect to serum was ~11 ml/min, which was within the range of 15 ± 6 ml/min determined by Manton and Cook (1984).

Since the initial circulation of skin-absorbed lead around the body must involve blood, it may be expected that the fate of lead introduced into the blood stream by percutaneous absorption and by direct intravenous injection would be similar. Chamberlain (1985) and Campbell et al. (1984) found that injected ^{203}Pb partitioned rapidly ($t_{0.5}$ = 0.5–1 min) from the plasma into the extracellular fluid (e.c.f.) compartment. At the same time, ^{203}Pb attached strongly to erythrocytes, depleting the plasma and reversing the gradient between plasma and e.c.f., thus causing ^{203}Pb to return to the blood. After 1 h, only 2–3% of the injected lead remained in the plasma and 45–50% was in the erythrocytes.

Skin-absorbed lead obviously behaves very differently to intravenously injected lead. The lead still transfers very rapidly from plasma to the e c.f and can therefore be detected in sweat 1–2 h after lead application. Lead in saliva also increased 3 h after lead was placed on the arm (Lilley et al, 1988), in agreement with Rabinowitz et al (1976) who postulated that sweat and salivary lead originated from the same pool. Apparently skin-absorbed lead does not return to blood from the e.c.f. as no increase in ^{204}Pb in serum was detectable for the first 24 h and increases in ^{204}Pb in blood, detected after 6 h, were small. Small quantities of ^{204}Pb were measured in urine after 6 h, and continued to be excreted for at least 16 days.

It is possible that skin-absorbed lead is in a physicochemical form that partitions strongly into e.c.f., but has very low affinity for erythrocytes, in which case there would be no driving force to bring the lead back from the e.c.f compartment. Ultrafiltration experiments showed that up to 70% of lead in sweat was associated with high molecular weight (>30 000) particles. One may speculate that, on passage through the skin, lead forms a stable protein complex that is not absorbed by erythrocytes.

To test this, the uptake of lead (as ^{210}Pb) into erythrocytes from sweat was investigated. There was no significant difference in uptake of lead into erythrocytes from saline, synthetic sweat and sauna sweat in vitro at 37°C. It appears that any difference in speciation of lead in sweat solutions compared with saline is not reflected in different rates of uptake into the erythrocytes. There was about ten times more lead present in the sweat solutions as ^{210}Pb, than lead naturally present in sweat (10 $\mu g \cdot l^{-1}$). It is therefore possible that 24 h was not long enough for equilibration of ^{210}Pb and lead present in the sweat. To confirm this, the ^{210}Pb sauna sweat solution was passed through a Chelex 100 (100–200 mesh) column and the effluent counted in a gamma well counter. All the radioactivity was removed by the Chelex resin, suggesting that lead was present in ionic form and had not bound to larger molecular weight proteins in sweat after 24 h. This may account for the similar rates of uptake of ^{210}Pb into erythrocytes from both saline and sweat solutions. Longer equilibration times may be required to further investigate erythrocyte lead uptake and speciation of skin-absorbed lead.

Considering that lead in these experiments was applied to a small surface area (<5 cm^2) and that the average body surface area is 18 000 cm^2, the potential exists for substantial uptake of lead through the skin. Duggan and Inskip (1985) reported 5–142 μg Pb from dust on the hands of children in urban and rural environments. If lead in dust is present as a skin-absorbable species, percutaneous absorption could contribute significantly to lead body burden. Occupational exposure to lead in dust in battery factories and lead mining operations may be even more significant. Although precautions are taken in the lead industry to protect workers from ingestion and inhalation of lead, little attempt is made to avoid skin contact.

It is interesting to note that the compartmental model for prediction of lead body burden (Bert et al., 1989) only fits the observed data when an allowance is made for an 'extraneous' source of lead. The magnitude of this unaccounted lead source ranged from 22–38% of the highest lead intake or 18–39 μg/day. Considering that 9 μg Pb/day is absorbed from diet and 1–50 μg Pb/day is absorbed from air, lead intake from this unaccounted source is significant. It is quite possible that this unmeasured source of exposure required to fit the model comes from percutaneous absorption of lead.

It is still not known whether skin-absorbed lead is retained in soft tissue and bone or largely excreted in sweat and urine. Sweat has been shown to be a significant route of excretion for workers exposed to lead (Lilley et al., 1988; Omokhodian and Howard, 1991). Of particular concern is that skin-absorbed lead is not detectable in blood by the usual techniques of electrothermal AAS or ASV. Since blood lead is the main criterion by which the industry determines exposure, skin absorption of lead would remain undetected. The possibility remains that a significant proportion of the lead body burden of workers results from skin absorption.

5. Acknowledgements

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Toxicity of nickel ores to marine organisms

T.M. Florence[a], J.L. Stauber*[b], M. Ahsanullah[c]

[a]*Centre for Environmental and Health Science, 112 Georges River Crescent, Oyster Bay, NSW 2225, Australia*
[b]*CSIRO Centre for Advanced Analytical Chemistry, Division of Coal and Energy Technology, PMB 7, Menai, NSW 2234, Australia*
[c]*Australian Nuclear Science and Technology Organization, PMB 1, Menai, NSW 2234, Australia*

Abstract

Queensland Nickel proposes to import New Caledonian (Ballande) and Indonesian (Gebe) nickel ores, one option being ship-to-barge transfer in Halifax Bay, North Queensland. Because small amounts of ore may be spilt during the unloading and transfer operations, it was important to investigate the potential impact of the spilt ore on the ecological health of the Bay. Long-term leaching of the ores with seawater showed that only nickel and chromium(VI) were released from the ores in sufficient concentrations to cause toxicity to a range of marine organisms. The soluble fractions of nickel and chromium(VI) were released from the ores within a few days. Nickel, chromium(VI) and the ore leachates showed similar toxicity to the juvenile banana prawn *Penaeus merguiensis*, the amphipod *Allorchestes compressa* and both temperate (22°C) and tropical (27°C) strains of the unicellular marine alga *Nitzschia closterium*. In a series of 30-day sub-chronic microcosm experiments, juvenile leader prawns *Penaeus monodon*, polychaete worms *Galeolaria caespitosa* and the tropical gastropod *Nerita chamaeleon* were all very resistant to the nickel ores, with mortality unaffected by 700 g ore per 50 l seawater. The growth rate of the leader prawns was, however, lower than that of the controls. From these data, a conservative maximum safe concentration of the nickel ores in seawater is 0.1 g l^{-1}. The nickel ore was not highly toxic and if spilt in the quantities predicted, would not have a significant impact on the ecological health of the Bay.

Key words: Nickel; Chromium; Toxicity; Algae, Ore; Invertebrates

1. Introduction

Queensland Nickel plan to import New Caledonian (Ballande) and Indonesian (Gebe) lateritic nickel ores to supplement the Australian Greenvale ore at present being processed in the Company's refinery at Yabulu, 30 km north of Townsville, Queensland, Australia. One option considered was to ship the ore directly to the refinery by ship-to-barge transfer in Halifax Bay. It was proposed to unload ore from cargo vessels at a single point mooring in the Great Barrier Reef Marine Park onto open-hold shuttles by means of a gantry crane-fully enclosed conveyer belt system. Some dredging of the Bay would be necessary to enable the shuttles to approach the unloading facility. Because small amounts of ore (a maximum of 16 tonnes per year according to Lassing and Dibben, 1991) may be spilt during the unloading and transfer operations, it was important to in-

* Corresponding author.

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vestigate the potential impact of the spilt ore on the ecological health of the Bay. The Great Barrier Reef Marine Park is an environmentally sensitive area, while Halifax Bay is an important recreational and commercial fishery, particularly for prawns and crabs.

Iron, aluminium and manganese are the major metals present in the ores with nickel, chromium, magnesium and cobalt present in concentrations greater than 0.1% (w/w). The ores also contain significant amounts of zinc and copper at concentrations less than 0.1% (w/w) (Table 1).

Extensive baseline studies of the marine environment of Halifax Bay were conducted prior to the opening of the Nickel refinery in 1973 (Water and Trade Wastes Consultants, 1973). In Halifax Bay, chromium concentrations of 0.6 $\mu g\ l^{-1}$ and nickel concentrations ranging from $<0.1-10.7\ \mu g\ l^{-1}$ have been reported (Carey et al., 1982; Queensland Nickel Pty Ltd., 1984). Bioconcentration factors (wet wt.) for nickel from seawater range from 370 for oysters and mussels to 1000 for macroalgae (Water Research Centre, 1984a,b), with bioconcentration factors for chromium about one third of those for nickel. Lethal toxicities of nickel and chromium to marine organisms have been reported to range from 0.1 to 1000 mg l^{-1}, with invertebrates possibly more sensitive than vertebrates (Connell and Miller, 1989) (Table 2).

The US EPA has set chronic toxicity limits for the protection of marine life for nickel and chromium in seawater of 8 $\mu g\ l^{-1}$ and 50 $\mu g\ l^{-1}$, respectively (Table 3). However, it is possible that nickel and other metals leached from the ores in seawater may be particularly toxic to benthic organisms (Connell and Miller, 1989). Judell (1989) reported on the toxicity of nickel ores to benthic organisms in bays in New Caledonia which had been impacted by the ores over 100 years. Although considerable benthic activity was observed, no quantitative measurements were made. Bivalves inhabiting locations where sediments contained substantial quantities of metals showed no unusual accumulations of metals in their tissues. Over the past few years, we have developed sub-lethal, chronic bioassays using a local isolate of the unicellular marine diatom *Nitzschia closterium* (Mann and Florence, 1987; Stauber and Florence, 1987,1989,1990). This particular species was chosen because it is widely distributed in Australian waters and the availability of both tropical and temperate strains makes it particularly suitable for site-specific toxicity testing. Because cells divide 1.4 times per day, a number of generations of cells are exposed to the toxicant over the 3-day bioassay. *Nitzschia*

Table 1
Composition of the nickel ores[a]

Element	Concentration (%)
Iron	36 4–47.6
Silica	2 0–15.0
Nickel	1 5–1 7
Aluminium	1.2–2.8
Chromium	1 0–1 7
Magnesium	1 0–6.0
Manganese	0 5–2.0
Cobalt	0 10–0 24
Calcium	0.01–1 50
Zinc	0.01–0.10
Copper	0 01–0.05

[a]From Queensland Nickel Pty Ltd. (1989).

Table 2
The acute toxicity of nickel and chromium(VI) to marine organisms (96-h exposure)[a]

Organism	LC_{50} (mg l^{-1})	
	Nickel	Chromium
Fish		
Fundulus heteroclitus	350	91
Menidia menidia	8 0	13
Molluscs		
Nassarius obsoletus	72	105
Mya arenaria	320	57
Crustaceans		
Acartia clausi	2 1	6 6
Pagurus longicarpus	47	10
Tigriopus japonicus	6 4	17
Annelids		
Ctenodrilus serratus	17	4 3
Nereis virens	25	2.0
Echinoderms		
Asterias forbesi	150	32

[a]From WRC (1984a,b)

Table 3
Criteria for the toxicity of nickel and chromium(VI) in seawater

Type of criterion	Limit (mg l^{-1})		Reference
	Nickel	Chromium	
Protection of marine life	0 008	0.050	US EPA (1987)
Protection of marine life	0.030	0.015	WRC (1984a,b)
Protection of marine life	0.020	0 020	Klapow and Lewis (1979)
Protection of marine life	0.025	0 010	Hart (1982)
Protection of marine life	0.008	0 050	SPCC (1990)
Protection of marine life	0.008	0.050	ANZEC (1990)
Not to be exceeded	0.140	0.170	US EPA (1987)
Not to be exceeded	0.450	0.200	Klapow and Lewis (1979)

closterium is particularly sensitive to a wide range of metals. Table 4 shows the concentrations of a number of metals which give a 50% reduction in cell division rate of *Nitzschia* over 72 h (72-h EC_{50}).

In this study, toxic metals in seawater leachates of the ores were identified and the leachates tested for acute and chronic toxicity to a range of marine organisms.

Table 4
Toxicity of some metals to *Nitzschia closterium*[a]

Metal	EC_{50}($\mu g\ l^{-1}$)[b]
Arsenic(III)	7
Arsenic(V)	>2000
Cadmium	350
Chromium(III)	>5000
Chromium(VI)	3500
Cobalt	>500
Copper	10
Iron(III)	>500
Lead	>500
Manganese(II)	>500
Mercury(II)	9
Nickel	250
Selenium(IV)	1000
Selenium(VI)	>2000
Silver	150
Zinc	70

[a]Florence and Stauber (1991).
[b]Concentration for 50% reduction in algal cell division rate.

2. Experimental

2.1. Analytical procedures

Trace element determination

Seven trace elements (Ni, Cr, Fe, Cu, Zn, Cd and Mn) were determined quantitatively in the ore leachates by inductively-coupled plasma emission spectrometry (ICP-AES), and 55 elements semi-quantitatively by inductively-coupled plasma mass spectrometry (ICP-MS). To concentrate heavy metals, especially arsenic, for the ICP-MS determinations, the leachates (10 g of 'as received' ore shaken with 200 ml of seawater for 4 h) were treated with 3 mg of iron(III) (as $FeCl_3$), adjusted to pH 6–7, allowed to stand for 24 h, filtered through a Whatman No. 1 filter paper and washed with Milli-Q water. The hydrated iron oxide precipitate was dissolved by adding 2 × 5 ml of hot 2 M HNO_3 and the acid solution analysed by ICP-MS.

'Total' chromium(VI) in the ores was determined by shaking the ore in 50 ml of 0.2 M NaOH for 22 h. Chromium in the leachates was determined by a sensitive, highly selective spectrophotometric method using diphenylcarbazide in phosphoric acid medium (Anon, 1976)

2.2. Properties of the ore

Ore settling experiments

Each ore (3 g) was shaken with 100 ml of

seawater for 24 h, aliquots transferred immediately to a 1-cm spectrophotometer cell and percent transmission followed at 600 nm for 24 h

Ore leaching experiments

Shake extraction. Ore leachates in seawater were prepared by shaking various weights of ore (as received) with 100 ml of seawater in a wrist-action shaker. The leachates were then filtered through a 0.45-μm membrane filter. The effect of shaking time on the composition of the leachate was also investigated, using shaking times of between 4 and 65 h.

Standard elutriation. Air was bubbled vigorously through a slurry of 5 g of dry ore equivalent in 100 ml of seawater for 1 h, the ore allowed to settle for 30 min and the elutriate filtered through a 0.45-μm membrane filter (Lee et al., 1976)

Column leaching Two glass columns (7 cm × 2 cm), with medium porosity glass discs, were prepared for long-term ore leaching experiments. Pieces of glass fibre filter were boiled with water, and the slurry added to the columns, which were fitted with adaptors to allow seawater to flow through at a rate of 0.25 ml min^{-1}. In the first experiment, approximately 0.5 g of Ballande or Gebe ore (as received) was weighed into each column, while in a second experiment, 2 g of each ore were used. The leaching was carried out for 15 days until the concentrations of chromium and nickel in the eluants were below 5 μg l^{-1} and 3 μg l^{-1} for Ni and Cr, respectively. The eluants were analysed for Ni, Cr, Co, Fe, Mn, Cu, Pb, Cd, Zn and Cr(VI).

Ore lump leaching. To simulate the leaching of ore spilt in the Bay by gentle currents, a 'lump' of Gebe ore (24.5 g wet wt.) was lightly moulded into a ball, placed in a 2-l beaker and covered carefully with 1.8 l of seawater. A 0.5-cm glass tube was positioned 1.5 cm into the seawater and air bubbled slowly throughout, to provide gentle stirring. Samples were taken daily from the top of the beaker and analysed for nickel and chromium.

Ore metal bioavailability experiments The fraction of metal available to filter feeding animals as a result of digestion-absorption was estimated by leaching ore particles with hydrochloric acid Ballande and Gebe ores (1 g) were shaken in a wrist-action shaker for 6 h with 100 ml of 1 M HCl, and the solutions were filtered and analysed by ICP-AES.

2.3 Properties of the sediment

Halifax Bay sediments from three locations were collected by a grab sampler, sealed in air-tight plastic-lined drums and stored at 4°C until required. Pore waters were separated from 60- to 80-g sediment samples, before and after use in the bioassays, by centrifugation in 50-ml centrifuge tubes. The pore water was filtered through a 0.45-μm membrane filter before analysis by ICP-AES. A seawater blank was prepared in the same way.

To simulate trace metal and nutrient release during dredging, the standard elutriation test (used for the ores) was also carried out on the sediment samples. Nitrate plus nitrite (NO_x) in the elutriate was determined by the cadmium reduction method no. 4500-NO_3-F (APHA, 1989). Free reactive phosphorus (*o*-phosphorus) was determined using the automated ascorbic acid reduction method no. 4500-P-F (APHA, 1989).

Bioassay procedures

Toxicity tests with the marine diatom Nitzschia closterium The toxicity of Ni, Cr(VI), Ni + Cr(VI) and leachates of the Ballande and Gebe ores to both temperate and tropical isolates of the unicellular marine alga, *Nitzschia closterium* (Ehrenberg) W. Smith, was investigated.

The temperate strain (CS 5) was originally isolated from Port Hacking, NSW and the tropical strain (CS 114) from the Coral Sea, near the Great Barrier Reef. The diatoms were cultured in f medium (Guillard and Ryther, 1962), with the iron and trace element concentrations halved. The batch cultures were maintained on a 12-h light, 12-h dark cycle (Philips TL 40 W fluorescent daylight, 4500 lux) at 21°C (temperate strain) and 27°C (tropical strain) (Lumsden and Florence, 1983).

Cells in log phase growth (<7 days old) were centrifuged at 2500 rev./min on a Jouan CR4.11 centrifuge with E4 swingout rotor. The nutrient medium was discarded and the cell pellet resuspended in ~30 ml of 0.45-μm filtered seawater. The centrifugation and washing proce-

dure was repeated three times to remove culture medium which would otherwise ameliorate toxicity (Stauber and Florence, 1989).

Seawater controls, together with five ore leachate concentrations, each in duplicate, were prepared in 200-ml erlenmeyer flasks precoated with Coatasil (Ajax) to prevent absorption of metals onto the glass. To each flask, containing 50 ml seawater, 0.5 ml of 26 mM sodium nitrate and 0.5 ml of 1.3 mM potassium dihydrogen phosphate were added. Each flask was inoculated with 3–5 × 10^4 cells ml^{-1} of a prewashed *Nitzschia* suspension, and incubated at 21°C or 27°C on a 12:12 h light/dark cycle at 14 000 lux. Numbers of live cells in each flask were determined daily for 3 days by counting in a hemocytometer using phase contrast microscopy. Aliquots of cells from each flask were homogenized in a 15-ml hand-held tissue grinder (Wheaton) with Teflon pestle, to break cell clumps.

A regression line was fitted to a plot of $\log_{10}$ cell density versus time (h) for each flask and the cell division rate (μ) determined from the slope. Cell division rates per day (3.32 μ × 24) were calculated and compared to cell division rates of the controls. Probit Analysis software was used to calculate the EC_{50} i.e., the effective concentration of leachate which gave a 50% reduction in cell division rate compared to the controls. LOEC (the lowest concentration of ore leachate which significantly decreased cell division rate) and NOEC (the highest concentration of leachate which had no significant effect on cell division rate) were estimated using US EPA statistics protocols (Weber et al., 1989).

Acute toxicity tests with the juvenile banana prawn Penaeus merguiensis. Juveniles (approximately 2 weeks old) of the banana prawn *Penaeus merguiensis* were air-freighted to Sydney from the Gold Coast Marine Hatchery, Beenleigh, Queensland. They were fed cockles and fish pellets, and acclimated to laboratory conditions for 10 days prior to toxicity testing

Toxicity tests were carried out with 32‰ seawater in a laboratory controlled at 20°C and a photoperiod of 14 h light and 10 h dark. Four dilutions of leachate, together with controls, were prepared in acid-washed plastic cups. Twenty juveniles were exposed individually (one per cup), without feeding, at each dilution to avoid problems of cannibalism. The experimental solution in each cup was replaced after 48 h. Mortality after 96 h was determined

Acute toxicity tests with the amphipod Allorchestes compressa. Static toxicity tests, using the marine amphipod *Allorchestes compressa*, originally obtained from Queenscliff, Victoria, were carried out under the same conditions as the banana prawn assays. Healthy adults (1–1.5 cm) were exposed to four leachate dilutions plus controls (10 animals per 800 ml) for 96 h without feeding. Observations for mortality were made twice daily. The criterion for determining death was the absence of movement when animals were gently prodded with a glass rod. Dead animals were removed after each observation (Ahsanullah, 1982).

Microcosm toxicity studies. Thirty-day microcosm toxicity studies were carried out using the leader prawn *Penaeus monodon* and the gastropod *Nerita chamaeleon*.

Microcosms were established in 70-l glass tanks (74 × 36 × 30 cm) with continuously flowing seawater (25 ml min^{-1}) at 27 ± 0.5°C. The covered tanks were continuously aerated, and monitored for salinity, pH, temperature and dissolved oxygen.

Sediment from three points in Halifax Bay was sent by air freight in plastic-lined drums and kept at 4°C for 2 days before use. The three sediments were thoroughly mixed, and approximately 15 kg weighed into all tanks The system was allowed to settle for 2 days, before *Zostera* (15 plants ex Sydney University) and *Cymodocea* (five plants ex Magnetic Island) were planted in each tank. In the first experiment, 50 juvenile leader prawns (*Penaeus monodon*) were added to each tank. The prawns (1.5–2 cm total length) were obtained from a commercial farm in Queensland and were acclimatised at 27°C in a flowing, aerated seawater tank for 10 days before use. The prawns were fed during the experiment on cockles and a commercial food from the supplier, containing 38% protein, 5% fat, 3% fibre, 12% moisture and 14% ash. Steady feeding of the prawns was essential to limit predation.

A sample of Ballande nickel ore was ground and sieved through a #50 sieve, the <#50 mesh fraction being used in the assays. The Gebe ore was crumbled by hand. Two tanks were kept as controls, and to the others weighed amounts of ore (25–700 g per 50 l) were added; the ore was sprinkled over the surface of the tank to simulate an ore spillage.

The experiment was conducted for 30 days, during which time samples of the tank water and sediment were taken for analysis. At the end of the experiment the surviving prawns were counted, measured and weighed. A polychaete (*Galeolaria caespitosa*) worm count was also made. Although polycheates were not added deliberately, they were present in the seawater and sediment and because the same amount of mixed sediment was added to each tank, the initial worm population would have been very similar.

In a second experiment, each tank was partitioned into 20 separate compartments using 3-mm nylon mesh (oyster mesh), and one juvenile prawn (8–10 mm) was placed in each compartment. Prawns were weighed as a group at the beginning and the end (28 days) of the experiment. Control tanks and tanks containing different ore concentrations were each repeated in triplicate.

The microcosm experiments were repeated with the tropical gastropod (snail) *Nerita chamaeleon*, collected from Cairns in Northern Queensland. A total of 50 snails were added to each tank and the recoveries counted after 30 days.

A variety of statistical tests, including the *t*-test, the Mann-Whitney *U*-test (parametric tests) and the Wilcoxon rank sum test (non-parametric test), were used to analyse the microcosm data.

Table 5
Trace elements released from the ores during the standard elutriation test[a]

Metal[b]	Ore (mg l^{-1})	
	Gebe	Ballande
Ni	0.31	0 011
Cr	2.90	5.15
Cr(VI)	3.00	5 18
Fe	0.009	<0.003

[a]Ore (5 g, dry weight) in 100 ml seawater.
[b]Detection limits were 0.005, 0 002 and 0 002 mg l^{-1} for Ni, Cr and Fe, respectively.

Table 6
Trace elements in the ore leachates[a]

Element	Concentration ($\mu g\ l^{-1}$)	
	Gebe	Ballande
Ag	0 003	0 003
As(III)	<0 002	<0 002
Bi	0 009	0 010
Cd	<0 001	<0.002
Co	0.80	0.69
Cu	2 1	1.0
Ga	0.10	0 069
Ge	0 10	0 10
Hg	<0 002	<0.002
Zn	<0.002	<0 002

[a]Ore (9.3 g, normalised to 30% moisture) shaken in 200ml seawater for 4 h.

3. Results and discussion

The moisture contents of the Gebe and Ballande ores were 36.2% and 22.9%, respectively, for the first ore samples, and 32.1% and 22.6%, respectively for the second batch. All tests were carried out on the 'as received' ores, and the results normalised to a moisture content of 30% (Florence and Stauber, 1989; Florence et al., 1991).

3.1. Composition of the ore leachates

The concentrations of Ni, Cr, Cr(VI) and Fe released from the ores during the standard elutriation test, which were similar to those obtained in the shaken leachates, are shown in Table 5. The concentrations of a range of other elements in the ore leachates are given in Table 6. The leachates contained less than 0.1 $\mu g\ l^{-1}$ of Ag, As, Bi, Cd, Hg, Mn, Pb, Sb, Se, Tl and Zn. In addition to Ni and Cr, only Co, Cu and Fe were present at concentrations above 0.1 $\mu g\ l^{-1}$.

The effect of shaking time on the concentrations of chromium(VI) and nickel in the leachates is shown in Figs. 1 and 2. Chromium(VI) dissolved rapidly from both ores with more chromium

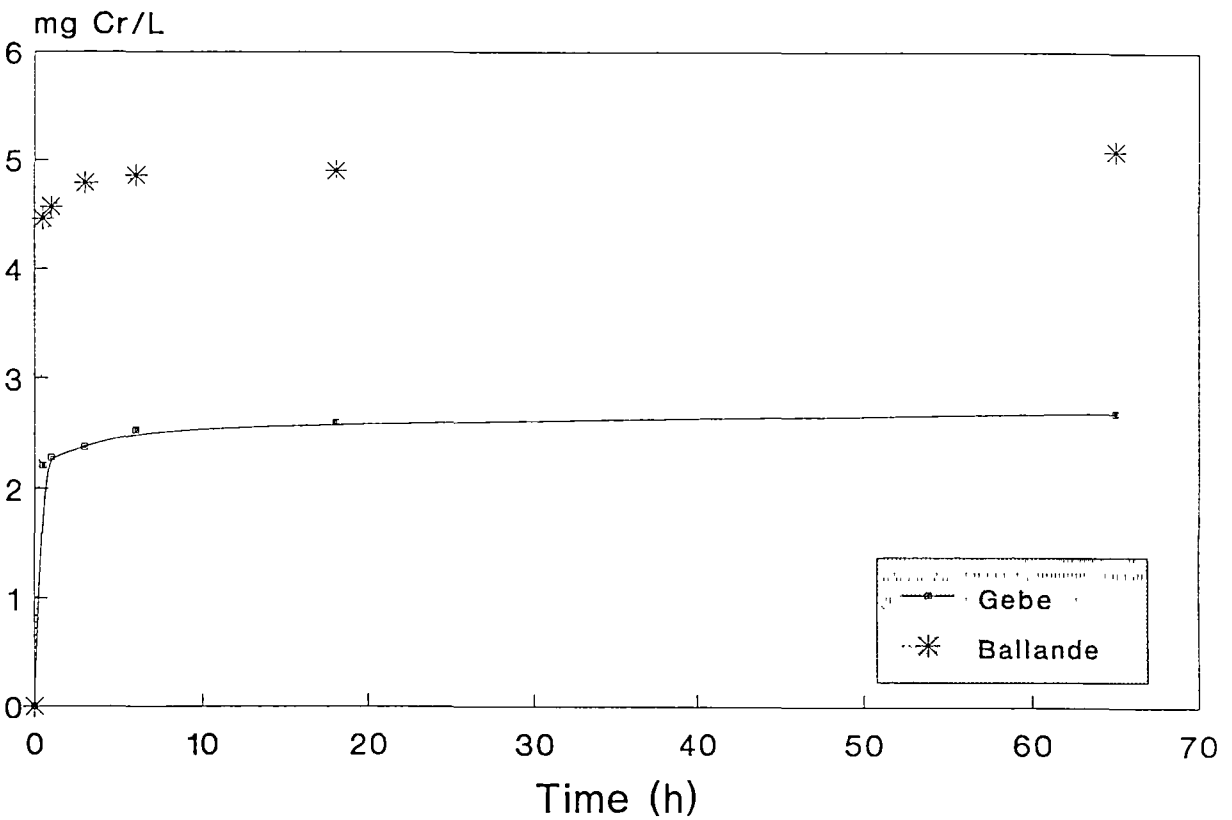

Fig. 1. The effect of shaking time on the chromium concentration in the ore leachate (1 9 g ore shaken with 100 ml seawater)

released from Ballande ore than from Gebe ore. All the chromium released from the ores was present as Cr(VI). Nickel dissolved at a slower rate with more nickel released from the Gebe ore than the Ballande ore.

The effect of ore:seawater ratio on the concentrations of chromium(VI) and nickel in the leachates is shown in Figs. 3 and 4. The concentration of chromate formed in the seawater leachates was not proportional to ore concentration, but approached a limiting value. A similar situation was observed with nickel, although nickel in the Gebe leachates continued to increase slowly with ore concentration.

'Total' Cr(VI) concentrations in the ores, determined by extraction with 0.2 M NaOH in seawater, was 0.023% and 0.066% for the Gebe and Ballande ores, respectively. In order to test if chromate was present in the ore or formed from Cr(III) during the shaking in aerated seawater, 1 g of Gebe ore was shaken for 2 h in 50 ml of seawater, which had been deaerated with nitrogen. The filtrate contained 2.9 mg Cr(VI) l^{-1}, compared with 3.1 mg l^{-1} in aerated seawater. In addition, when 10 mg l^{-1} of Cr(III) chloride was shaken in seawater in the absence and presence of ore for 24 h, no additional Cr(VI) (<0.05 mg l^{-1}) was produced. Clearly the chromate was present in the ore, and was not formed during shaking with seawater.

Probably all of the chromate in the ore leachate was produced as a result of hydrolysis of a metal chromate, as the ores had very little chromate present as a soluble metal salt. Cr(VI) in the ore leachates increased with increasing pH, and was much higher in seawater (pH 8.2) than in

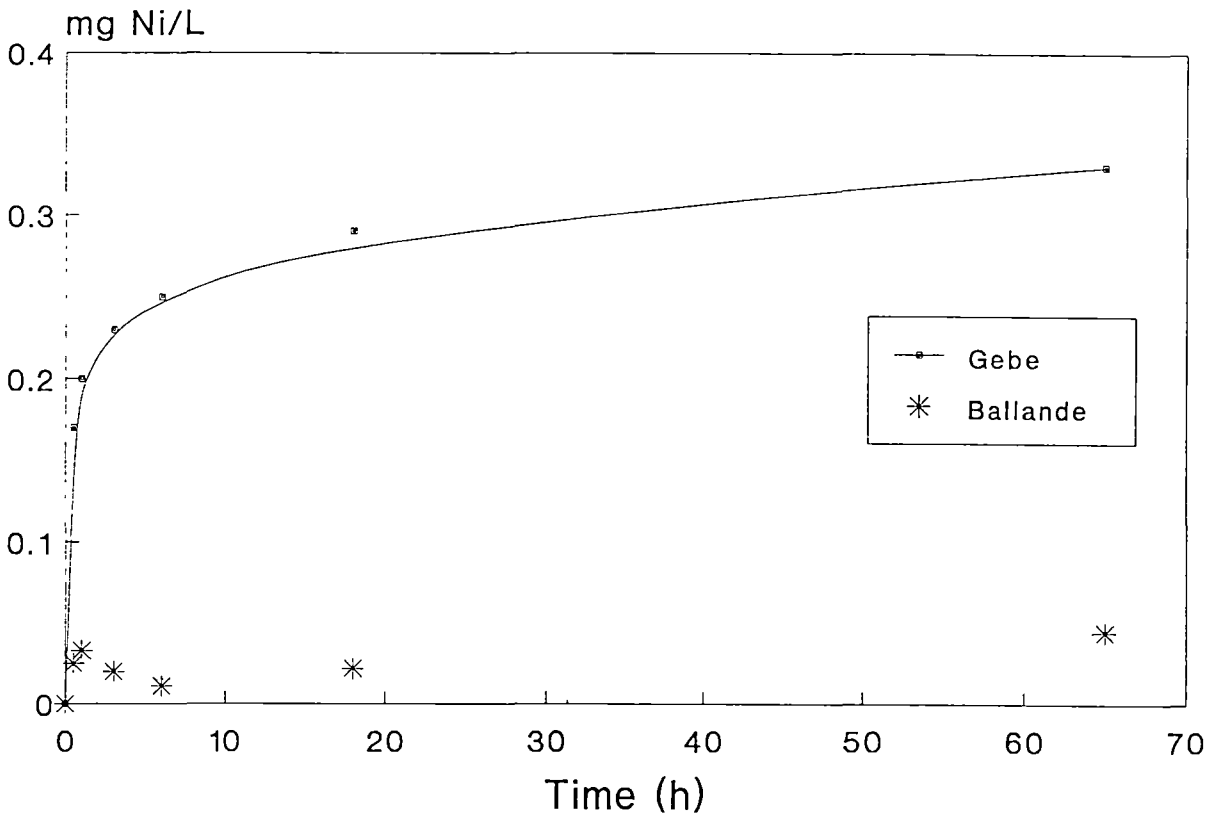

Fig 2. The effect of shaking time on the nickel concentration in the ore leachate (1.9 g ore shaken with 100 ml seawater).

demineralized water (pH 6.5). During leaching, the pH of the seawater dropped from 8.2 to 7.5, as its alkalinity was consumed in the hydrolysis reaction. The iron contents of the ores were high and plots of [Cr(VI)] vs. [alkali consumed] were linear, with slopes in the range 0.4 to 0.29. This suggests (Mellor, 1952) that the hydrolysis reaction may be:

$$Fe_2(CrO_4)_3 + 6NaOH \rightarrow 2Fe(OH)_3 + 3Na_2CrO_4$$

Below pH 3, where Fe(III) hydrolysis does not occur, no chromate was liberated by leaching.

3.2. Column leaching experiments

Column leaching experiments showed that both nickel and chromium were released rapidly from the ores. In the 0.5 g Ballande ore leaching experiment, the eluant contained <2 $\mu g\ l^{-1}$ of Fe, Mn, Cu and Zn, and <20 $\mu g\ l^{-1}$ Pb after the passage of 662 ml seawater. Spectrophotometric analysis of the seawater eluants showed that all the chromium found by ICP-AES was present as Cr(VI). A similar pattern was observed in the 0.5 g Gebe column leaching experiment.

In the 2-g experiments, over 80% of all the chromium was eluted in the first three days, with 50% eluted in the first 7 h. Eighty percent of the nickel was released in the first 4 days. The concentrations of nickel and chromium in the eluants were close to background seawater levels after only 13–15 days.

The rapid release of the soluble metal fraction has important environmental implications. If small

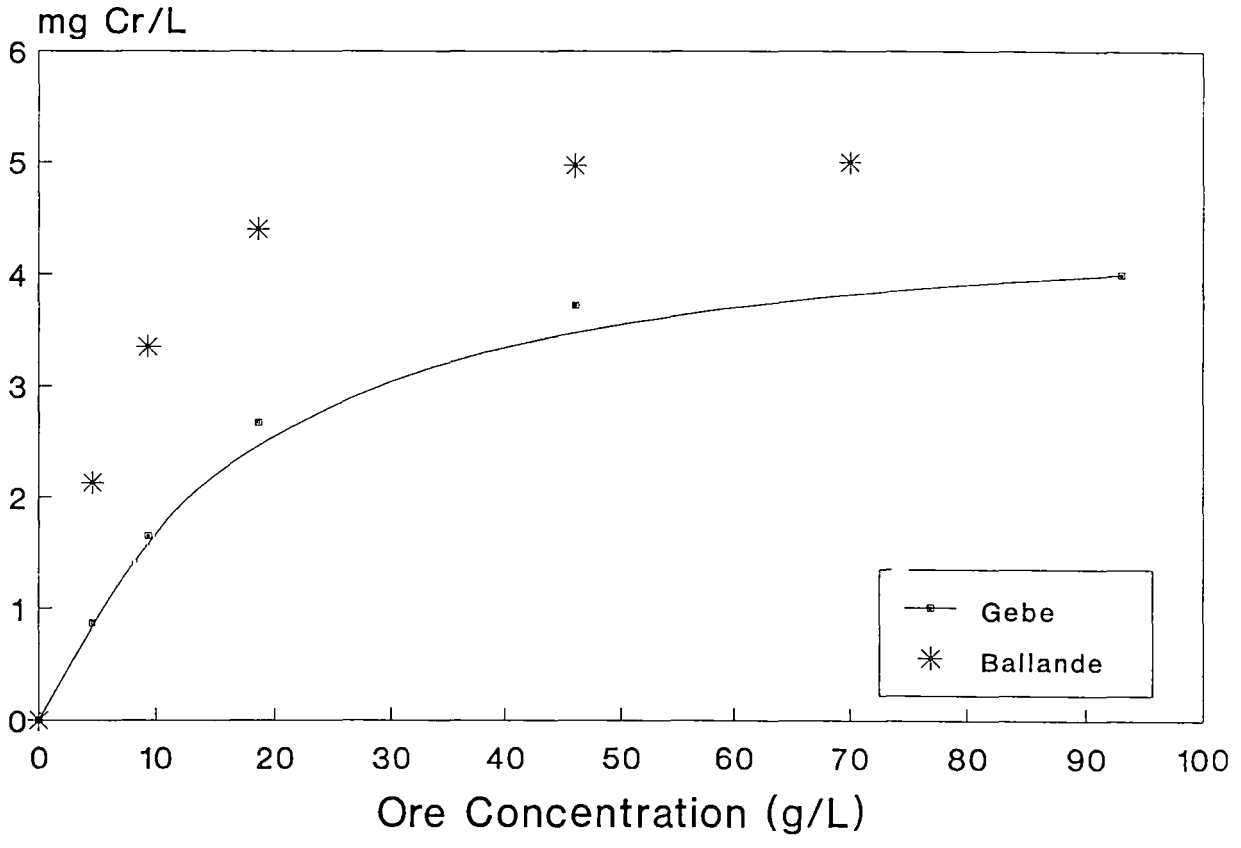

Fig. 3. The effect of ore.seawater on the chromium concentration in the ore leachate (ore weights shaken for 5 h in seawater)

amounts of ore were spilt over a relatively long period, the soluble metals would be unlikely to be present at toxic concentrations. However, large spills would lead to high, localised concentrations of nickel and chromium, whose toxic effects would depend on dilution by currents and tidal action.

3.3. Ore lump leaching experiments

The maximum nickel concentration leached from the lump of ore was 0.165 mg l^{-1} after 4 days, whereas chromium took nearly 10 days to reach its maximum concentration of 2.03 mg l^{-1}. This was in contrast to batch and column leaching experiments, in which chromium leached much faster than nickel. Apparently, under conditions of gentle agitation, where the rate of transport of seawater to the ore surface was limited, the supply of the alkalinity necessary for hydrolysis of the metal chromate was the rate determining step. Release of chromium from a pile of spilt ore may therefore be slower than that predicted from the column leaching experiments (Florence, 1991)

3.4. Ore settling experiments

The results of the ore settling tests are shown in Fig. 5. The Gebe ore settled at a constant rate, while the Ballande ore settled rapidly at first, followed by a slower rate over 0.5–3 h. After 24 h, 77% and 60% of the Gebe and Ballande ores, respectively had settled. A significant fraction of

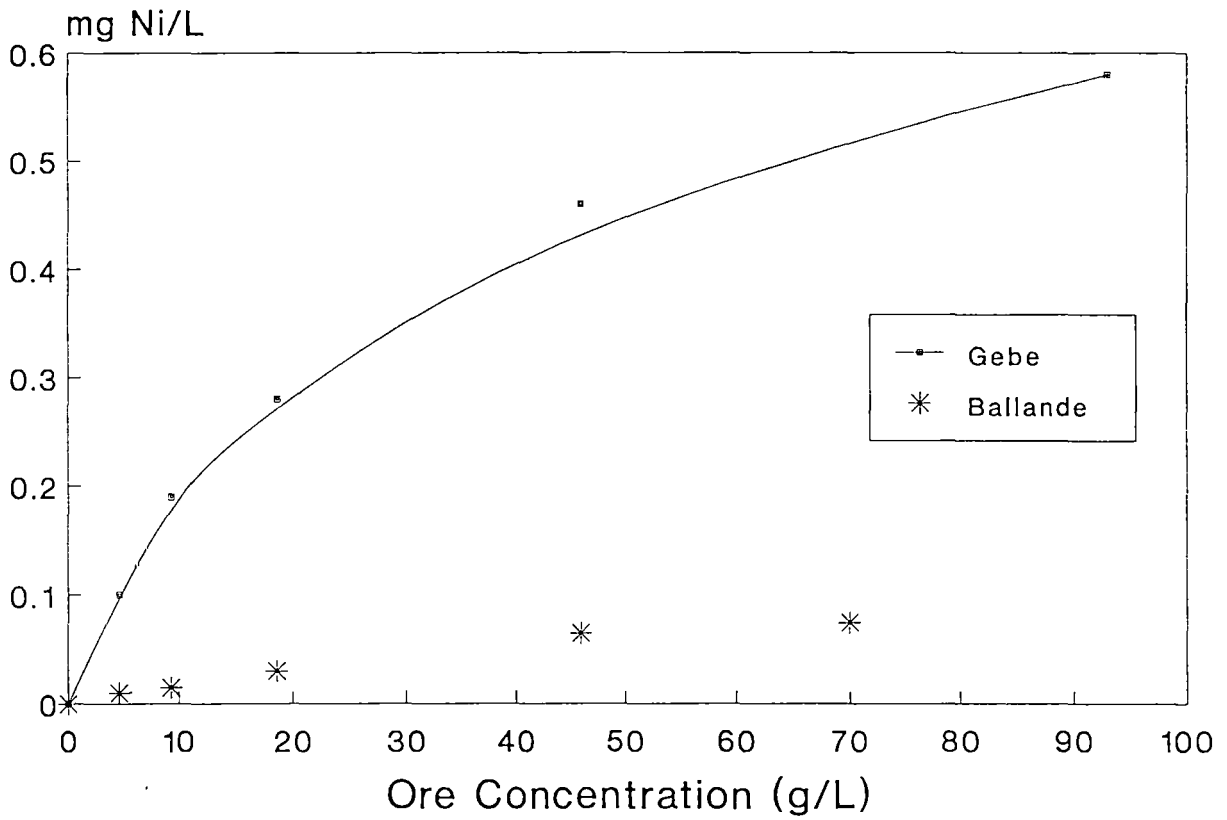

Fig 4 The effect of ore seawater ratio on the nickel concentration in the ore leachate (ore weights shaken for 5 h in seawater)

both ores was very fine and stayed in suspension for a few days. This is in agreement with Connell and Miller, 1989, who reported that the ores contained a high proportion of fine clays, with particle sizes ranging from <45 μm to 200 mm.

3.5. Ore metal bioavailability experiments

The results of the 1 M HCl leach of the ores, expressed as mg extractable metal per kg of ore (dry weight) are shown in Table 7. In most cases the fraction of metal in the ore available to a filter feeder was very small. HCl (1 M) was used to simulate the worst case situation. It appears, benthic organisms would be unlikely to accumulate significant amounts of metal as a result of digestion of ore particles.

3.6. Properties of the sediment

The elutriation tests on Halifax Bay sediments, which attempt to simulate the release of nutrients and metals from sediments during dredging, showed that only small amounts of these substances were released (Table 8). The nitrate + nitrite concentration increased significantly; however, inputs from rivers during storm events would be expected to have a greater impact on the Bay, whose waters are usually low in nitrate (5–23 μg N l^{-1}) (Water and Trade Wastes Consultants, 1973).

Pore waters from the Halifax Bay sediments were low in heavy metals, with the exception of manganese in two of the samples (Table 9). These high manganese concentrations were surprising because neither the overlying seawater nor the

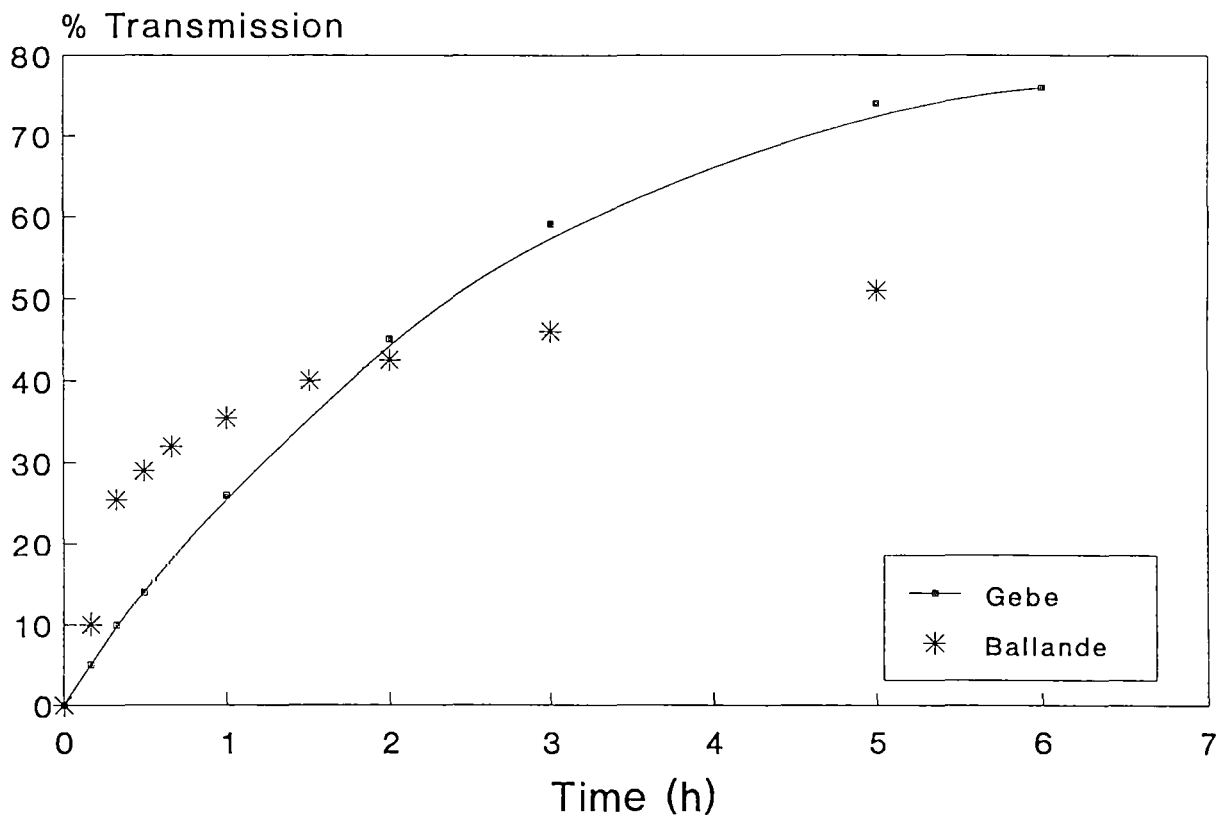

Fig 5. Gebe and Ballande ore settling rates

Table 7
Bioavailable metal in the ores[a]

Metal	Extractable metal (mg/kg ore)		Extractable metal (% of total metal)	
	Gebe	Ballande	Gebe	Ballande
Ni	476	130	2.9	0 92
Cr	302	704	2.1	3 3
Fe	810	494	0.20	0.11
Mn	51	77	0 81	0.53
Cu	6	11	6 0	15.9
Pb	5	10	—	—
Cd	0 2	0 2	—	—
Zn	17	20	4.3	4 5

[a]Metals extracted in 1 M HCl

elutriate seawater contained significant amounts of manganese (<1 μg l^{-1}). Manganese values in pore water as high as 30 mg l^{-1} have been reported (Brannon et al., 1980), as a result of the high mobility of manganese in sediments. Because much of the manganese in sediments is present as colloidal manganese dioxide, which has been shown to ameliorate metal toxicity (Stauber and Florence, 1987), manganese at these concentrations is unlikely to be toxic to marine organisms

3 7. Toxicity to the marine diatom Nitzschia closterium

The analysis of the ore leachates used in the

Table 8
Elutriation test of Halifax Bay sediments

Elutriate constituent[a]	Sediment samples			Blank[b]
	1	2	3	
Moisture (%)	23	28	32	—
Nitrate + nitrite (mg/l)	0 30	1 10	0 60	0 03
o-Phosphorus (μg/l)	16	27	26	10
Mercury	0·3	0 3	0 2	0 1
Lead	<20	<20	<20	<20
Cobalt	<3	<3	<3	<3
Copper	4	4	7	<3
Chromium	2	3	8	<2
Iron	3	4	5	<2
Manganese	<1	<1	<1	<1
Nickel	9	9	7	<5
Cadmium	8	<5	7	<2
Zinc	<3	<3	<3	<3

[a]All metal concentrations are μg l^{-1}
[b]Blank seawater used for elutriation tests

algal assays is shown in Table 10. For equivalent weights of ore, four times more nickel was leached from Gebe ore than Ballande, whereas Ballande ore leached twice as much chromium. The response of the tropical and temperate strains of the marine alga, *Nitzschia closterium* is shown in Tables 11 and 12. Cell division rates of controls were 1.4 ± 0.2 doublings per day for the temperate strain and 0.8 ± 0.07 doublings per day for the tropical strain.

The cell division rate of the tropical diatom was reduced by 50% by concentrations of 7.1 g l^{-1} of Ballande ore and >24 g l^{-1} of Gebe ore (ore weights normalised to 30% moisture). No toxicity was observed at concentrations of Ballande ore in seawater below 1.4 g l^{-1} and below 7.1 g l^{-1} for Gebe ore. The toxicity of both ore leachates to tropical *Nitzschia* was due entirely to chromium(VI) leached from the ores by seawater. More chromium was leached from Ballande ore (Table 10), accounting for its greater toxicity to this strain than Gebe ore. Nickel, at concentrations up to 0.5 mg l^{-1}, had no effect on the cell division rate of tropical *Nitzschia* (Table 11).

The chromium(VI) EC_{50} to *Nitzschia* was 3.5 mg l^{-1}, with a maximum allowable toxicant concentration (the mean of the NOEC and LOEC) of 1 mg l^{-1}. This is in agreement with Missimer et al., 1989, who reported a MATC of 1.2 mg Cr l^{-1} for the marine diatom *Skeletonema costatum*. Chromium(VI) toxicity to marine algae has been shown to depend on the salinity and the sulfate concentration in seawater, as chromate and sulfate compete for active transport across the cell membrane (Reidel, 1984). No synergistic effect of nickel and chromium was observed.

The temperate strain of *Nitzschia* was more sensitive to Gebe ore than the tropical strain, with

Table 9
Analysis of Halifax Bay pore waters

Metal (μg l^{-1})	Sediment samples		
	1	2	3
Pb	<20	<20	<20
Co	<5	<5	<5
Cu	6	4	<3
Cr	<2	<2	<2
Fe	3	5	20
Mn	<1	460	2000
Ni	<5	<5	<5
Cd	4	<2	<2
Zn	8	<3	<3

Table 10
Analysis of ore leachates for algal toxicity assays

Ore	Wt of Ore (g)[a]	Metal in leachate (mg l^{-1})		Metal in leachate (mg g^{-1} ore)	
		Nickel	Chromium	Nickel	Chromium
Gebe	0 71	0 084	1 0	0.012	0 14
	1 43	0 162	1 8	0 011	0 13
	2.86	0 313	2 8	0.011	0 10
Ballande	0.14	0 005	0 75	0 004	0 54
	0 57	0 009	2 5	0 002	0 44
	1 00	0 016	3.5	0 002	0 35
	1 43	0 040	4 1	0 003	0 29

[a]Ore weights normalised to 30% moisture. Ore shaken for 4 h in 100 ml seawater, prior to filtering

EC_{50} values of 11 g l^{-1} of Gebe ore and 6 4 g l^{-1} of Ballande ore. No toxicity was observed at concentrations below 4.7 g l^{-1} and 1.9 g l^{-1} for Gebe and Ballande ores, respectively. By comparing values in Tables 10, 11 and 12, it is apparent that the observed toxicity was due to a combination of nickel and chromate released from the ores in seawater. Nickel was much more toxic to the temperate strain of the alga, as was Gebe ore (which leached more nickel) and chromium and nickel combined. The EC_{50} for nickel was 0.25 mg l^{-1}, similar to toxicities reported for other marine diatoms (Tadros et al., 1990). There was evidence of possible synergistic toxicity between nickel and chromate: a mixture of 0.1 mg Ni l^{-1} and 2 mg Cr(VI) l^{-1} reduced the cell division rate by 67%, even though the separate metals at these concentrations had no effect on growth rate.

Table 11
Toxicity of ore leachates to tropical and temperate strains of *Nitzschia closterium*

Ore[a]/Metal	72-h EC_{50} (mg l^{-1})	
	Tropical *Nitzschia*	Temperate *Nitzschia*
Gebe	>24 000	11 000
Ballande	7100	6400
Nickel	>0 5	0.25
Chromium	3.5	3 5

[a]Ore weights normalised to 30% moisture

3.8 Acute toxicity to invertebrates

Toxicity of the ore leachates to both the juvenile banana prawn *Penaeus merguiensis* and the amphipod *Allorchestes compressa* are shown in Table 13. Control survival rates after 96 h were >85%. The Gebe and Ballande ore leachates showed similar toxicity to both banana prawns and the amphipod. The marine alga *Nitzschia closterium*, however, was more sensitive to Ballande ore leachates.

The 96-h LC_{50} values for chromium and nickel to *Allorchestes compressa* were 5.6 mg l^{-1} and 34 mg l^{-1}, respectively. This amphipod was more sensitive to both chromium and nickel than other invertebrate species such as juvenile *Callinectes sapidus* (Frank and Robertson, 1979) and *Pagurus longicarpus* (Eisler and Hennekey, 1977).

Denton and Burdon-Jones (1982) found the 96-h LC_{50} for nickel to juvenile banana prawns to

Table 12
No observable effect concentrations (NOEC) of ore leachates to tropical and temperate strains of *Nitzschia closterium*

Ore[a]	NOEC(g l^{-1})	
	Tropical *Nitzschia*	Temperate *Nitzschia*
Gebe	7 1	4 7
Ballande	1 4	1 9

[a]Ore weights normalised to 30% moisture

Table 13
Acute toxicity of nickel ores to marine organisms

Organism	96-h LC_{50} (g l^{-1})	
	Gebe	Ballande
Banana Prawns	15	20
Amphipod (*Allorchestes compressa*)	12	15
Diatom (*Nitzschia closterium*)[a]	12	7

[a]72-h EC_{50}, temperate strain

Table 15
Maximum safe concentrations of nickel ores in seawater

Criterion	Maximum safe ore concentration (g l^{-1})	
	Gebe	Ballande
Prawns (LC_{50} × 0.01)	0 15	0 20
Amphipod (LC_{50} × 0 01)	0 12	0 15
Diatom (LOEC × 0 1)	0 50	0.30
US EPA, 50 μg Cr(VI) l^{-1}	0 20	0 10
US EPA, 8 μg Ni l^{-1}	0 23	4 1

be 3.55 mg l^{-1} at 30°C, with toxicity increasing in longer term experiments (Denton and Campbell, in press).

Table 14 compares the sensitivity of *Nitzschia closterium* to several metals with other representative marine organisms, including polychaete worms, gastropods, crabs and fish. While this compares a sublethal effect (reduction in cell division rate) for *Nitzschia* with lethality (organism death) in other marine organisms, it is useful to note that *Nitzschia* is at least 25 times more sensitive than other organisms to nickel, copper, zinc and cadmium.

3.9. Microcosm studies

Leader prawn experiments

Throughout the 30-day experiments, oxygen was 6.0 ± 0.3 ppm, salinity 32.0 ± 0.1 and temperature 26.5 ± 0.2°C in the tanks. Control tanks were more turbid than the tanks containing ore, and turbidity decreased with increasing ore concentration. Light microscopic examination of water samples showed that none of the tanks contained significant numbers of bacteria, and tanks containing ore had less suspended sediment. Concentrations of Ni, Cr (all present as Cr(VI)), Mn, Fe and Cu in the tank waters were below the limit of detection in less than 24 days. Neither Cu nor Zn were significantly higher than the control tanks, and Co, Cd, Sn, Pb and As were not detectable.

Concentrations of Ni, Cr, Co, Fe and Mn in sediment pore waters from the bioassay tanks were determined by ICP-AES. Chromium concentrations were <5 μg l^{-1}, even in tanks containing 700 g ore per 50 l, presumably because of the high solubility and anionic nature of the chromate ion. Nickel concentrations ranged from <5 μg l^{-1} in controls to 34 μg l^{-1} in tanks containing the highest concentration of ore. Manganese in the pore waters (3.4–6.9 mg l^{-1}) was considerably higher than values obtained for the same sediments before use in the bioassay tanks, prob-

Table 14
Sensitivity of some marine organisms to heavy metals

Organism	96-h LC_{50}[d] or 72-h EC_{50} (μg l^{-1})			
	Nickel	Copper	Zinc	Cadmium
Nitzschia closterium	250	10	70	350
Polychaete worms	17 000	300	5000	9000
Gastropods	80 000	1000	80 000	25 000
Crabs	10 000	3000	5000	2000
Fish	10 000	1000	10 000	30 000

[d]Bryan (1976)

ably due to mobilisation of manganese from biological activity over the 30 days. Manganese values as high as 30 mg l^{-1} have been reported in pore waters (Brannon et al., 1980), with most of the manganese present as colloidal manganese dioxide, which can ameliorate metal toxicity (Stauber and Florence, 1987).

The *Cymodocea* (seagrass) used in the microcosms showed no difference in growth or appearance after 30 days between control tanks and tanks containing ore. The temperate *Zostera* did not grow very well in either control or assay tanks. Both Gebe and Ballande ores, even at the highest concentration tested (700 g per 50 l), had no significant effect on either the survival or growth of the juvenile leader prawns. Similarly, the polychaete worm density, which ranged from 35–72 worms per tank, was unaffected by any amount of ore.

Prawn recovery varied from 54–88%, with no significant difference between controls and tanks containing ore. The low recoveries in the controls (56–76%) were thought to be due to cannibalism, so the experiment was repeated with each prawn in a separate compartment. Despite the use of mesh to separate the prawns, the recovery of prawns in the control tanks (45–80%) was no better than previously. The US EPA allows minimum control recoveries of 70–90% for chronic bioassays of 7–9 days' duration. An average recovery of 70% for controls in a 30-day experiment, however, is acceptable, as the longer the duration of the experiment, the more likely there will be losses in controls. The two series of experiments on leader prawns were in excellent agreement, and gave no indication of any lethal (survival) effects of the highest amounts of ore tested. There was, however, a significant difference in the growth rate of the prawns in the tanks containing ore compared to controls in the second experiment. For the Ballande ore, a correlation coefficient of −0.991 was calculated for weight increase vs. amount of ore, with a regression equation of:

$$\text{weight increase} = 27.0 - 0.0286 \times \text{weight of ore}$$

Using this equation, 94 g of Ballande ore (1.9 g l^{-1}) would cause a decrease in growth of 10% over 28 days. Gebe ore had a slightly smaller effect on growth. The effect of the ores on the growth rate of the prawns probably occurred in the first week, since most of the nickel and chromium was leached from the ores over this period.

Gastropod experiments

Recovery of snails was 100% in all tanks, assays and controls after 30 days. Gebe and Ballande ore (25–700 g per 50 l) was not toxic to *Nerita chamaeleon* at any concentration of ore tested.

The microcosm results suggest that the ore particles per se did not cause toxicity; rather, the ore leachates, containing nickel and chromium, were the toxic agent.

3.10. Maximum safe concentrations of ores

The acute toxicity data from Table 13 can be used to calculate acceptable, long-term-no-effect concentrations of Ballande and Gebe ores in seawater. Application factors of 0.01 × LC_{50} for invertebrates and 0.1 × the lowest observable effect concentrations for algae were used. These, together with acceptable concentrations using US EPA criteria, are shown in Table 15. A conservative maximum ore concentration of 0.1 g l^{-1} in seawater suggests no known impact on marine life would occur at this ore concentration.

The no-effect-concentrations observed in the microcosm experiments range from 1–14 g ore l^{-1} for both Gebe and Ballande ore. This is much higher than the limit of 0.1 g l^{-1} calculated from the acute toxicity tests, because the ore leachates were continually removed by the seawater as would be the case in a real spill situation

The effect of the Yabulu Nickel Refinery effluent on the coastal waters of Halifax Bay over an 8-year period was reported by Carey et al., 1982 Connell and Miller, 1989, estimated that up to 10 000 kg of nickel would have been discharged into the Bay during this time, assuming maximum permissible nickel concentrations in the effluent of 1 mg l^{-1}. No adverse marine ecological effects were detected. Small changes in benthic invertebrate communities and zooplankton biomass were found to be unrelated to effluent discharge.

In comparison, the proposed new unloading facility would add about 1150 kg per year from ore

spillage, with a smaller contribution from dust fallout (21 kg). Small infrequent spills may lead to nickel and chromium concentrations of 10–100 $\mu g\ l^{-1}$ within a mixing zone, depending on leaching rates and redox conditions in the sediment. Metal enrichments to the sediments suggest benthic species may be more at risk than pelagic species Much of the nickel, however, is unlikely to be biologically available (Connell and Miller, 1989). Observations in New Caledonia, where some bays have been impacted by nickel ores for over 100 years, and in Foundry Cove, Hudson River, New York, which has received industrial effluents containing nickel for over 25 years, showed that there had been no change in either the concentration or diversity of benthic organisms. In the case of Foundry Cove, the macrobenthos adapted to the heavy metals and showed greater tolerance to them than control organisms (Klerks and Levinton, 1989).

4. Conclusions

(i) The toxicity of the ore leachates was due entirely to chromium(VI) and nickel, which dissolved rapidly from the ores in seawater. No other metal ion of those determined was present in the leachates in sufficient quantities to cause toxicity.

(ii) Reduction in the cell division rate of the marine diatom *Nitzschia closterium* was a more sensitive indicator of toxicity than mortality to banana prawns and amphipods over 96 h.

(iii) The temperate strain of *Nitzschia* was more sensitive to nickel and Gebe ore leachates than the tropical strain, with nickel and chromium exerting synergistic toxicity.

(iv) Thirty-day microcosm studies showed that seagrass, juvenile leader prawns, polychaete worms and a tropical gastropod were all very resistant to the nickel ores. The growth rate of the leader prawns was, however, lower than the controls, with 1.9 $g\ l^{-1}$ of Ballande ore decreasing growth by 10% over 28 days.

(v) Elutriation tests on Halifax Bay sediments showed that there would be no deleterious releases of nutrients or heavy metals during dredging of the Bay.

(vi) A conservative maximum ore concentration of 0.1 $g\ l^{-1}$ in seawater suggests that no known impact on marine life in Halifax Bay would occur at this ore concentration.

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Toxicity Testing Using Marine and Freshwater Unicellular Algae

J. L. Stauber

Centre for Advanced Analytical Chemistry CSIRO Division of Coal and Energy Technology

Private Mail Bag 7, Menai, NSW 2234, Australia

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ABSTRACT:

Unicellular algae, at the base of most aquatic food chains, are particularly sensitive to a wide range of pollutants and are therefore an important part of a battery of toxicity tests for hazard assessment and aquatic environment protection. The use of bioassays for toxicity assessment, based on growth inhibition in algae, is described and compared to alternative measures of toxic stress in algae, such as ^{14}C uptake, respiration, ATP and enzyme activity. Factors affecting toxicity, including the test medium, inoculum pretreatment, incubation conditions and test duration are reviewed. Selection of sensitive and ecologically-relevant test species for both freshwater and marine toxicity testing is essential. Bioassays using the freshwater green alga *Chlorella protothecoides* and a local species of the marine diatom *Nitzschia closterium* were developed and their sensitivity to metals, chlorate and chlorophenolic compounds were compared to standard overseas test species. The bioassay using *Chlorella protothecoides* was applied to assessing the bioavailability and toxicity of copper in the Fly River System, Papua New Guinea. The marine algal assay with *Nitzschia closterium* was used to test the toxicity of a complex pulp mill effluent and its high molecular weight fraction, and compared to bioassays with bacteria, seaweed and fish larvae.

KEY WORDS:

algae, toxicity, bioassays, marine, freshwater.

Introduction

Toxicity tests with unicellular algae were originally developed to assess the eutrophication potential in freshwaters receiving effluents (USEPA, 1971). Over the last two decades, algal bioassays have been more widely used to assess biostimulatory and inhibitory effects of natural waters, wastewaters, specific chemicals and sediment leachates (Van Coillie *et al.*, 1983; Sonnassy *et al.*, 1977; Florence *et al.*, 1994).

Single-celled algae are the foundation of most aquatic food chains and account for much of the productivity of aquatic ecosystems. Any adverse impact on algae may directly or indirectly affect organisms at higher trophic levels. Marine and freshwater algae have been shown to be particularly sensitive to a wide range of organic and inorganic pollutants (Florence and Stauber, 1991; Stauber *et al.*, 1994a). Because toxicity tests using algae are chronic tests, covering several generations of algal cells in a few days, and determine sublethal effects, rather than mortality, algae have often been shown to be more sensitive than other test species (Joubert, 1981; Walsh and Merrill, 1984). In addition, clonal cultures of unicellular algae can be easily established in the laboratory, providing genetically uniform cultures for toxicity testing. Toxicity tests with algae, in common with other microbial tests, are highly reproducible (Stauber *et al.*, 1994a) and are, therefore, an essential part of a battery of toxicity tests for hazard assessment and aquatic environmental protection.

Growth Inhibition Bioassays

Most algal bioassays are growth inhibition tests, which measure the decrease in growth rate (cell division rate) or final cell biomass after a few days in the presence of toxicant, compared to controls in dilution water. Standard protocols for algal toxicity testing have been published by Weber *et al.* (1989), OECD (1984), ASTM (1992) and Environment Canada (1992). Healthy exponentially-growing cells are added to a range of test concentrations (at least 5 dilutions in a geometric series) and incubated under controlled light and temperature conditions. The original standard algal bottle test (USEPA, 1971) was carried out over 14 days, however, this has been reduced to 96 h and more recently 48-72 h tests (Walsh *et al.*, 1988; Stauber *et al.*, 1994a) to prevent loss of toxicants by degradation or evaporation.

Algal growth is usually estimated daily by counting cells microscopically in a haemocytometer or in an automatic particle counter, or by measuring other biomass parameters such as cell fluorescence, optical density, turbidity, dry weight or chlorophyll *a*. Good correlation between the different methods for estimating population density is generally

found (Walsh *et al.*, 1988). However, toxicants may cause a change in cell chlorophyll and therefore cell fluorescence, without causing a change in cell numbers, so these techniques may be unreliable. Dry weight and optical density were not found to be sensitive enough to measure changes in cell density for routine algal assays (Stauber *et al.*, 1994a). Turbidity was sensitive, however, because turbidity was a complex function of cell volume, size and pigment content, it was highly variable between toxicity tests and required constant recalibration with cell counts. Neither turbidity nor optical density as a measure of cell density is suitable for testing complex, highly-coloured effluents.

At the end of the test, growth rates or final cell biomass at each treatment concentration are determined and compared to controls. Toxicity is expressed as an EC50 i.e. the effective concentration to decrease growth by 50%. Various statistical protocols are used to estimate NOEC (the highest test concentration to cause no significant effect on algal growth) and LOEC (the lowest test concentration to cause a significant effect on growth compared with controls) (Weber *et al.*, 1989).

Alternative Effect Parameters

Other algae effects parameters, apart from growth inhibition, have also been used, including carbon assimilation in photosynthesis (Van Coillie *et al.*,1983; Kusk and Nyholm, 1991), respiration (Stauber and Florence,1987), ATP (Kwan, 1989; Hickey *et al.*, 1991) and enzyme activity (Stauber and Florence, 1990).

Chiaudani and Vighi (1978) compared the sensitivity of growth, photosynthesis, ATP and fluorescence to copper after 1 and 7 days using the freshwater green alga *Selenastrum capricornutum* Printz. They found that photosynthesis (measured as uptake of ^{14}C) was the most sensitive endpoint, followed by ATP, fluorescence and growth. For the same test species, however, Hickey *et al.*, (1991) found that growth inhibition over 96 h was ten times more sensitive than ATP determinations after 4 h exposures to nine metals and four organic compounds. Clearly, the toxicity response is dependant on the chemical contaminant, the species of algae and the test endpoint.

Effect Parameter	% of Control	
	Copper (200µgL^{-1})	Zinc (200µgL^{-1})
Growth (Cell Division)	0	0
Photosynthesis	116±16[a]	114±11
Respiration	108±13	117[b]±21
ATP	108±12	144±28
ETC Activity[c]	119±19	238±23
Thiols	59±14	310[b]±99

[a] One standard deviation
[b] 500 µg Zn L^{-1}
[c] Electron transport chain (dehydrogenase) activity

Table 1. *The effect of copper and zinc on various bioassay endpoints for Nitzschia closterium.*

The sensitivity of various effects parameters to copper and zinc in the marine alga, *Nitzschia closterium* (Ehrenberg) W. Smith is shown in Table 1 and Figure 1. In the presence of 200 µg Cu L^{-1}, only cell division rate (growth rate) over 72 h and cellular thiol content (-SH) after 24 h were significantly inhibited compared to controls (100%). No significant effects on photosynthesis (^{14}C uptake), respiration, ATP or electron transport chain (dehydrogenase) activity after 24-h exposure to copper were observed (Stauber and Florence, 1987). In the presence of zinc (200 and 500 µg L^{-1}), the only endpoint sensitive enough to detect toxicity was inhibition of cell division rate. No significant effect of zinc on photosynthesis or respiration was found (Stauber and Florence, 1990). There was significant enhancement of ATP, dehydrogenase activity and cellular thiol content compared with controls and these parameters may be useful biomarkers for zinc toxicity in this species. However, in general, inhibition of growth rate was the most sensitive and reproducible indicator of toxicity to metals for this marine alga, and it is this parameter on which a standard algal toxicity test has been based (Stauber *et al*, 1994a).

Factors Affecting Toxicity

The Test Medium

Most algal toxicity test protocols recommend that the tests be carried out in the same medium used to culture the algae in the laboratory, with the omission of EDTA which would otherwise complex metals and reduce toxicity (Weber *et al.*, 1989; Chiaudani and Vighi, 1978). However, Stauber and Florence (1989) showed that algal culture medium containing chelators such as citrate, and adsorbents such as iron and silicate, can ameliorate the toxicity of some compounds, particularly metals. They found that the concentration of copper giving 50% reduction in cell division rate in the freshwater green alga *Chlorella pyrenoidosa* (now *C. protothecoides*) increased from 16 µg L^{-1} in synthetic softwater to greater than 200 µg L^{-1} in high nutrient MBL medium. Similarly, marine medium f reduced copper and zinc toxicity to *Nitzschia closterium* by 10 to 20 fold compared to seawater. For this reason, recent protocols for toxicity testing with marine and freshwater algae (Stauber *et al.*, 1994a) recommend the use of natural filtered seawater or synthetic water enriched only in nitrate and phosphate, together with Hepes buffer for freshwater testing. This simple dilution water is adequate to maintain exponential growth rates for up to 96 h and maximises the sensitivity of the toxicity test.

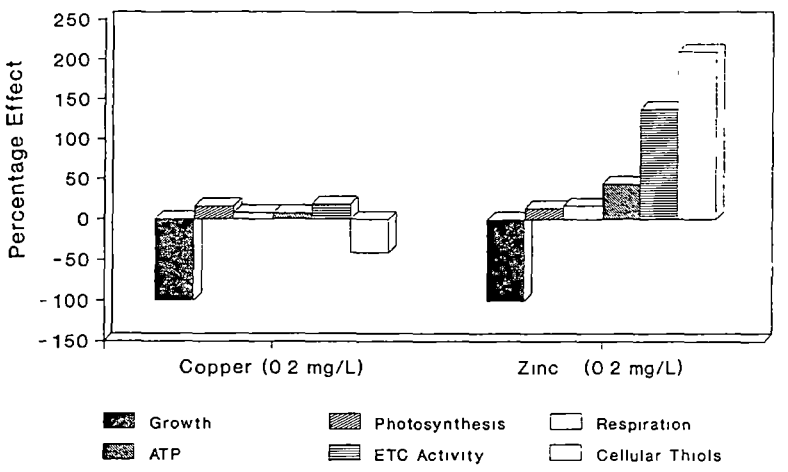

Figure 1. *The sensitivity of different bioassay endpoints (effect parameters) to copper and zinc in Nitzschia closterium. The percentage effect on the y axis is the difference between the measured parameter and the control (as a percentage). An effect above the 0 line indicates enhancement, while an effect below the zero line indicates inhibition.*

Toxicity tests should imitate, as closely as possible, the composition of the natural medium. Apart from synthetic waters, natural water samples including riverwater, lakewater or seawater samples, collected from the area of interest, filtered and supplemented with minimal nutrients, have also been used in routine toxicity tests (Homstrom, 1990).

Test Inoculum Size, Age and Pretreatment

Healthy exponentially-growing cells, usually less than 7 days old, are used as the toxicity test inoculum to prevent a lag in growth in short-term bioassays (Stauber *et al.*, 1994a).

One factor not usually considered in standard algal toxicity tests is the pretreatment of algal cells prior to use as the assay inoculum. Stauber and Florence (1985a) showed that the toxicity of copper to the marine alga Nitzschia closterium, depended on the concentration of iron in the culture medium used to grow the cells before their inoculation into the assay flasks. Cells cultured under the iron levels in normal f medium (790 $\mu g\ L^{-1}$) were more tolerant to copper than cells cultured in iron-deficient medium. Similarly, manganese, cobalt, aluminium and chromium (III) protected against copper toxicity, by forming around the algal cells metal (III) hydroxides, which adsorbed copper and reduced copper penetration into the cells (Stauber and Florence, 1985b; Stauber and Florence, 1987). It is important to standardise trace metal concentrations in culture media used to grow cells for test inocula and to wash the cells by centrifugation/washing procedures prior to use in the algal toxicity tests.

The toxicity of effluents and specific chemicals has been shown to depend on the number of cells added initially to the test flasks (Florence and Stauber, 1986). In general, the higher the initial cell density, the less sensitive the test to toxic compounds, due to increased binding of toxicants with increasing number of cells (Homstrom, 1990). The initial cell density is thus a compromise between maximising sensitivity of the assay, and having enough cells for the determination of cell density each day over 48-96 h. Most protocols recommend an inoculum size of 1 to 4 x 10^4 cells mL^{-1}.

Incubation Conditions

The importance of light and temperature for algal growth in toxicity tests is well recognised and has been reviewed by Bonin *et al.* (1986) and Walsh (1988). The intensity and quality of light and day/night cycles will strongly influence the growth and physiological state of algal populations. Light intensity may affect the release of photosynthetic products from algal cells, which in turn may alter the uptake of a test substance and therefore its toxicity. In addition, light intensity and quality can alter the rates of photodegradation of test compounds. For example, the standard high light intensities (14 k lux) required when testing the toxicity of highly-coloured pulp mill effluents, also contributed to the photodecomposition within 24-h of chlorocatechols, often present in the effluents (Stauber *et al*, 1994b), making the interpretation of toxicity indices difficult.

Each algal test species has specific optimal temperatures for maximum growth rate. In general, toxicity test protocols with freshwater green algae recommend 24° C, while tests with temperate marine diatoms should be carried out at 20 ± 2°C. Toxicity tests with effluents and natural waters should be carried out at temperatures appropriate to the receiving water e.g. 29°C for tropical studies (Florence *et al.*, 1994).

Test Duration

Current test protocols now recommend toxicity tests of short duration, usually 48-72 h (OECD, 1984; Walsh *et al.*, 1988) to avoid changes in pH that occur over the longer-term as a result of the utilisation of carbon dioxide and

bicarbonate and the uptake of nitrogen by algal cells. Such changes may interfere in algal metabolism, and may alter the chemical behaviour and mode of action of the toxicant (Swanson *et al.*, 1991). In addition, photodegradation of organic compounds over time, will be minimised in short-term tests. Longer exposure times may result in decreasing toxicity of the test substance (Walsh *et al.*, 1982).

It is essential to use rapidly-growing algae in such short-term tests. Most standard tests require final cell densities in the controls to be greater than 2 x 10^5 cells mL^{-1} (Weber *et al.*, 1989) or 10^6 cells mL^{-1} (Greene *et al.*, 1988), with variation between the replicate controls of less than 20%. The OECD protocol requires that the cell numbers in the controls should have increased by a factor of 16 within 3 days.

Selection of Sensitive Test Species

Freshwater Algae

The most commonly used algal species for toxicity testing is the green alga *Selenastrum capricornutum*, which is widely distributed in eutrophic lakes in North America. The choice of this as a standard test species was largely dependent on its ability to grow rapidly under laboratory conditions and the ease with which it could be counted, compared to other filamentous and clumping algae. Standard protocols for toxicity tests with this species have been published by Weber *et al.*, 1989, OECD (1984), ASTM (1992) and Environment Canada (1992).

The standard *Selenastrum* test has recently been modified by the use of microplates in place of flasks (Blaise, 1986; Environment Canada, 1992). This has enabled greater replication and increased cost effectiveness and has eliminated the potential for cross-contamination between tests. An interlaboratory calibration exercise with the modified test found an overall precision of 8.7% for control growth and coefficients of variation of 24% for cadmium and 35% for phenol toxicities (Thellen *et al.*, 1989).

Despite its wide distribution in eutrophic waters in North America, *S. capricornutum* has not been positively identified in Australian freshwaters (D. Cannon, pers comm.) and its ecological relevance in Australian aquatic ecosystems is uncertain. Moreover, *S. capricornutum* is only a moderately sensitive test species and certainly less sensitive to metals than other species of freshwater algae (Stauber *et al.*, 1994a).

A standard toxicity test protocol using the freshwater green alga, *Chlorella protothecoides* Kruger in Australia has recently been published (Stauber *et al.*,1994a). *C protothecoides* is widely distributed in Australian lakes and rivers and other *Chlorella* species are present in soils (Megharaj *et al.*, 1992). In addition, *C protothecoides* is particularly sensitive to a range of toxicants, especially metals.

Toxicant	72H EC50 (µM) *Chlorella protothecoides*	*Selenastrum capricornutum*
Copper	0.21 (0.17-0.27)[a]	0.61 (0.52-0.71)
Chromium (VI)	2 (0.5-4)	9.6 (0.6-23)
Chlorate	1200	730 (470-1000)
4-Chlorophenol	350 (310-390)	400 (340-510)

[a] *95% confidence limits*

Table 2. *Toxicity of chemicals to freshwater algae.*

Table 2 compares the toxicity of copper, chromium(VI), chlorate and 4-chlorophenol to *Chlorella protothecoides* and *Selenastrum capricornutum* under identical assay conditions. *C. protothecoides* was more sensitive to both metals than *S. capricornutum*, and showed similar sensitivity to 4-chlorophenol. *S. capricornutum* was slightly more sensitive to chlorate, one component of bleached kraft pulp mill effluents. The toxicity of chlorate has been shown to be dependant on the nitrate concentration in the assay medium. At high nitrate concentrations (>1 mgL^{-1}) chlorate is less toxic, due to competitive uptake between nitrate and chlorate (Stauber *et al.*, 1994a). Other species of green algae have also been either recommended as standard test organisms or widely used for aquatic toxicity testing. These include *Scenedesmus subspiatus* and *Chlorella vulgaris* (OECD,1984), and *Chlamydomonas reinhardii*, *Chlamydomonas variabilis*, *Chlorella pyrenoidosa* (now *protothecoides*) and *Scenedesmus quadricauda* (Van Coillie *et al.*, 1983; Swanson *et al.*, 1991). Swanson *et al.* (1991) noted that algae from other taxonomic groups including two blue-green algae, *Anabaena flos-aquae* and *Microcystis aeruginosa*, and one diatom, *Navicula* sp. had been used frequently in pesticide toxicity testing. Hornstrom (1990) found that, of 21 species of natural phytoplankton, diatoms were the most sensitive taxonomic group to four metals and three organic compounds tested, whereas chrysophytes were the most tolerant.

Because of the uncertainty regarding sensitivity among algal species, and the difficulty in identifying any particular taxonomic group as most sensitive, the OECD (1989) recommended that the algal growth inhibition test be changed from a single species to a battery of tests with a number of species, although particular species to be used were not identified. Swanson *et al.* (1991) and Maestrini *et al* (1984) suggested that a species battery should include a

representative of each of the major algal classes, while Hornstrom (1990) suggested that a test battery containing up to five species, if correctly selected for sensitivity and widespread distribution, would be sufficient.

Marine and Estuarine Algae

While marine algae have been used extensively in aquatic toxicity testing, standardised test protocols recommending particular test species have rarely been published. One of the first standard protocols to assess the impact of dredging and ocean disposal of sediments (USEPA, 1976) proposed that a minimum of three species from different taxonomic groups be used for marine toxicity tests. Two standard test organisms were recommended comprising the diatom *Skeletonema costatum* (Greville) Cleve which is widely distributed and ecologically important, and *Thalassiosira pseudonana* (Hust.) Hasle and Heimdal, of lesser significance ecologically, but which is sensitive to heavy metals and has short generation times (about 8 h). The protocol used long-term tests of at least 169 h or until the stationary phase of growth was achieved. The use of indigenous algae, in addition to standard test organisms, was also recommended.

While *Skeletonema costatum* is generally sensitive to pollutants, its current use as a standard test species is limited because it is a chain-forming diatom which is difficult to count using automatic particle counting techniques. Endpoints, other than growth, have therefore been proposed for this species (Kusk and Nyholm, 1991).

The marine green algae, *Dunaliella tertiolecta* Butcher was also proposed as a standard test organism (USEPA, 1974; Maestrini *et al.*, 1984) but this species has subsequently been shown to be particularly insensitive to metals and pesticides (Swanson *et al.*, 1991; Stauber *et al.*, 1994a).

Recently, the diatom *Minutocellus polymorphus* Hasle, von Stosch and Syvertsen, has been used in toxicity tests because of its general sensitivity. It is widely distributed in estuaries and the open ocean, and its unicellular growth habit enables easy counting (Walsh, 1988; Walsh *et al.*, 1988). Its rapid growth allows a test duration of only 48 h and the bioassay has been scaled down to microplates, as has been done with the freshwater *Selenastrum* test (C. Hickey, pers comm.).

The marine diatom *Nitzschia closterium* may be more appropriate as a standard test organism for Australian ecosystems. This alga is both a benthic and planktonic species and is widely distributed in Australian coastal waters. It was originally isolated from NSW coastal waters, but tropical clones from the North West Shelf and the Coral Sea have also been established, making this species particularly suited to site specific toxicity testing. This species has been shown to be particularly sensitive to a wide range of organic and inorganic compounds (Florence and Stauber 1991; Stauber *et al.*, 1994a) compared to other organisms.

Species	72H EC50 (µM) Cu	Zn	Cr(VI)	Cd	Pb	ClO_3
Nitzschia closterium	0 15	0 97 (0 90-1 04)[a]	47 (40-54)	3 1	>2 4	>3 0
Skeletonema costatum	>3 9	2 0[b]	26[d] (18-35)	1 3[b]	0 1[b]	–
Minutocellus polymorphus	–	0 3[c]	–	0 6[c]	0 3[c]	
Dunaliella tertiolecta	–	–	327 (154-721)	–	–	108

[a] *95% confidence limits* [b] *From Walsh et al. (1988)*
[c] *From Walsh et al. (1988) -48-h EC50* [d] *From Kusk and Nyholm (1991) - 6h EC50*

Table 3. *Toxicity of inorganic compounds to marine algae.*

Table 3 compares the sensitivity of *Nitzschia closterium* with other standard test algae, to chlorate and metals. *N. closterium* is particularly sensitive to copper, zinc and chromium(VI), but less sensitive to cadmium and lead than the two diatoms *Skeletonema costatum* and *Minutocellus polymorphus*.

Nitzschia closterium is also sensitive to organic compounds. The toxicity of a range of chlorophenolic compounds found in bleached pulp mill effluents to *N. closterium* and the marine bacterium *Photobacterium phosphoreum* (Microtox®) is shown in Table 4. In general, *N. closterium* is more sensitive to chloroguaiacols, chlorovanillin, trichlorosyringol and chlorosyringaldehydes, while *P. phosphoreum* is more sensitive to chlorophenols and chlorocatechols. In particular, *N. closterium* is about one order of magnitude more sensitive than *P. phosphoreum* to 2-chlorosyringaldehyde, the major chlorophenolic compound in some modern bleached-eucalypt kraft pulp mill effluents when chlorine dioxide is used in the bleaching sequence (Wallis *et al.*, 1993). In general, bacterial bioassays are more sensitive than algal tests to organic compounds, while algae are much more sensitive than bacteria to metals (Hickey *et al.*, 1991).

Nitzschia closterium also compares favourably in sensitivity with higher organisms. A suite of toxicity tests, using local species, has recently been developed for the National Pulp Mills Research Program (Stauber *et al.*, 1994b). Two reference toxicants, chromium(VI) and 4-chlorophenol, were recently tested using Microtox (*Photobacterium phosphoreum*), the *N. closterium* 72-h growth-inhibition assay, a 3-h fertilisation-inhibition bioassay with the macroalga *Hormosira banksii* (Stauber *et al.*, 1994) and a 96-h acute lethality test with larvae of the tidepool fish,

Chlorophenol	EC50 (X10^{-5}M) *Nitzschia closterium* (72h)	*Microtox* (15min)
Phenol	57 (52-61)[a]	21 (17-28)
4-Chlorophenol	6 3 (5 3-7.3)	0 64 (0.47-0 91)
2,4-Dichlorophenol	5 6 (5 3-5.8)	2.2
2,4,6-Trichlorophenol	2.5 (1.2-4 0)	3 6
4,5-Dichlorocatechol	3.3 (2 8-3 9)	1 1[b] (0 77-1.7)
3,4,5-Trichlorocatechol	0.2	0.34 (0 009-9)
Tetrachlorocatechol	0 67 (0 60-0.74)	0.06 (0.01-0 30)
4,5-Dichloroguaiacol	2 0 (1.9-2.1)	5 5 (2.6-11)
4,5,6-Trichloroguaiacol	1 8 (1.7-2.0)	2.4 (1.6-3.4)
Tetrachloroguaiacol	0.70 (0 65-0.75)	1.1 (0.53-2.1)
5,6-Dichlorovanillin	10.6 (9.8-11.5)	27 (26-29)
2-Chlorosyringaldehyde	37 (26-52)	320 (310-330)
2,6-Dichlorosyringaldhyde	77 (6.1-9.9)	25 (14-47)
3,4,5 Trichlorosyringol	1.1 (0.45-2.7)	17 (13-22)

[a] *95% confidence limits* [b] *5-min EC50*

Table 4. *Toxicity of chlorophenolic compounds to Nitzschia closterium and Photobacterium phosphoreum.*

Parablennius tasmanianus (Tasmanian blenny) (Munday and Deavin, 1992). The sensitivities of these species are compared in Figure 2. Toxicity is expressed as arbitrary toxic units (100/ μ M EC50). *N. closterium* and Tasmanian blenny larvae were the most sensitive to chromium(VI), whereas Microtox was the most sensitive to 4-chlorophenol. Coefficients of variation for the *N. closterium* bioassay to chromium(VI) and 4-chlorophenol were 7% and 8% respectively.

Specific Applications of Algal Bioassays

Growth-inhibition bioassays using the freshwater green alga *Chlorella protothecoides* and the marine diatom *Nitzschia closterium* have been used extensively for assessing the toxicity of complex effluents, natural waters, specific chemicals and ore elutriates (Florence and Stauber, 1994; Stauber *et al.*, 1994a,b). Two specific applications are the use of *C. protothecoides* for investigating the bioavailability of copper from the Ok Tedi copper mine in Papua New Guinea, and the use of *N. closterium* for determining the toxicity of pulp mill effluents.

Bioavailability of Copper to *C. protothecoides*

Ok Tedi Mining Limited is currently investigating the aquatic behaviour and fate of mine-derived sediments in the Ok Tedi/Fly River Systems. The ultimate aim is to determine the bioavailability of copper released from the sediments. *C. protothecoides* is particularly sensitive to copper, with a 72-h EC50 value of 13 μg Cu L^{-1} (95% confidence limits of 11 to 17 μg L^{-1}) in synthetic medium-hardwater (80 mg $CaCO_3$ L^{-1}). Significant inhibition of growth rate occurs at concentrations as low as 3 μg Cu L^{-1}.

Eight riverwater samples from the Ok Tedi, Ok Mani and Fly Rivers, together with samples from two off-river water bodies, were collected and immediately filtered through 0.45 μm membrane filters. Dissolved copper, nitrogen (NOx), orthophosphate, pH and salinity were determined on each sample. All samples were tested in duplicate for toxicity to *C. protothecoides* at pH 7.9, and matched to the same hardness, nitrate and phosphate concentrations as the controls in synthetic water. Cell densities were determined daily for 3 days and growth rates calculated from the slope of the regression line of log (cell density) versus time. The tests were acceptable if control growth rates were 1.3±0.3 doublings per day.

Dissolved copper concentrations in the riverwater samples ranged from 0.5 to 12.6 μg Cu L^{-1}, and decreased with increasing distance from the mine. Mean algal cell division rates in each water sample, as a function of copper concentration, are shown in Figure 3. Control cell di-

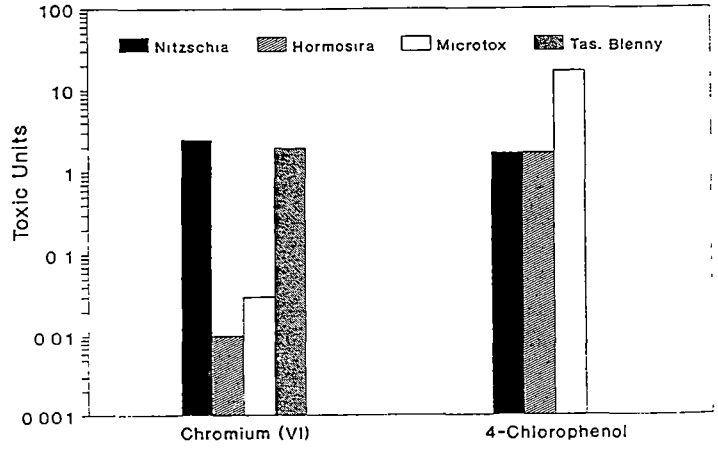

Figure 2. *Toxicity of two reference toxicants, chromium (VI) and 4-chlorophenol, to P photobacterium (Mictrotox), N. closterium, H. banksii and P. tasmanianus*

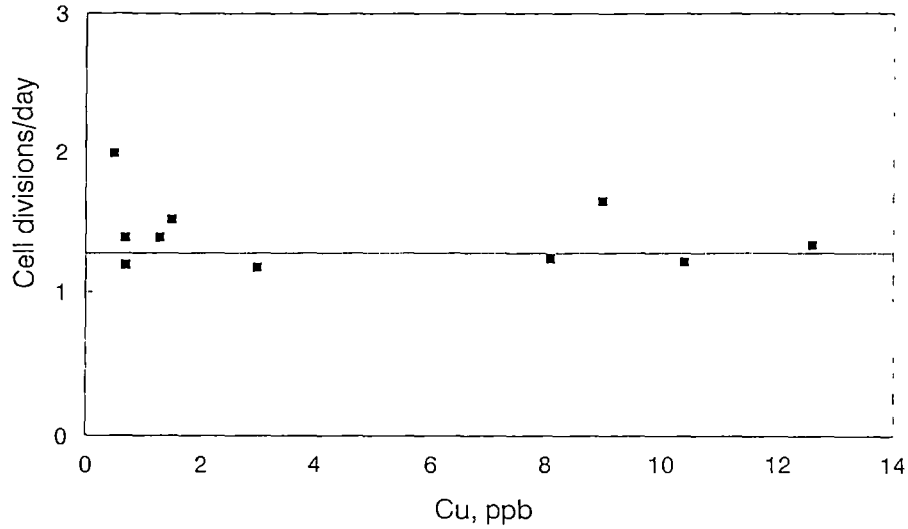

Figure 3. *Growth (cell division rate) of C protothecoides versus total dissolved copper concentration in riverwater samples from the Fly River System, Papua New Guinea. Growth rate of the controls (±95% confidence limits) is shown as the horizontal lines.*

vision rates, with 95% confidence limits, are shown as horizontal lines in the figure. None of the samples were found to be toxic to *C. protothecoides*, with algal growth rates the same as the synthetic water controls. The growth of *C. prototheccoides* was significantly enhanced in one sample, possibly due to additional organic nutrients such as humic/fulvic acids.

The lack of toxicity of any of the riverwater samples to the alga suggested that less than 3 µg Cu L^{-1} was bioavailable, otherwise significant decreases in algal growth would have been detected. Even the sample which contained 12.6 µg Cu L^{-1}, was not toxic, suggesting that copper in these samples was complexed or bound to colloidal material and unavailable for uptake into the algal cells.

Toxicity of Pulp Mill Effluents to N. closterium

The *Nitzschia closterium* bioassay was one of a suite of bioassays used to determine the toxicity of bleached eucalypt kraft pulp mill effluents (BEKME). The toxicity of BEKME is considered to be due mainly to chlorate, chlorophenolic compounds, resin acids (softwoods only) and high molecular weight lignin derivatives. Mature eucalypt kraft-oxygen pulp was bleached using a four stage sequence with 100% chlorine dioxide substitution. Effluents from each stage were obtained by hand-squeezing the bleached pulps and washing with Milli-Q water. The combined bleach effluent was divided into three subsamples. For toxicity testing of the untreated effluent, the first sample was adjusted to 31 ppt salinity using artificial sea salts. To provide a high molecular weight fraction for toxicity testing, the second sample was ultrafiltered three times through a 90 mm cellulose acetate membrane having a nominal molecular weight cutoff of 1000. To provide a secondary-treated effluent, the third subsample was inoculated with 100 mL of fresh anaerobic sewage sludge (mixed domestic/industrial sludge from Dutson Downs) and incubated anaerobically for three weeks, followed by an 18 day aerobic treatment. The composition of the effluents is shown in Table 5. All three effluents, after salinity and pH adjustment to simulate a marine discharge, were tested for toxicity to Microtox,

	mg L^{-1}		
	Untreated	Untreated High Molecular Weight Fraction	Treated
AOX[a]	8.6	2.8	3.2
TOC[b]	184	109	85
BOD_5[c]	140	89	55
COD[d]	480	269	346
Apparent Colour (Hazen Units)	65	46	70
Suspended Solids	8	3	19
Chlorate	77	<0.2	37[e]
2-Chlorosyringaldehade	0 089	<0.0005	0.03
Ammonia (mg N L^{-1})	0.41	<0.1	0.4
Oxidisable Nitrogen	0.066	0 02	0.02
Ortho-Phosphorus	<0.05	<0 01	1.2
pH	9	4.9	7.9-8.0

[a] *Adsorbable organic halide* [b] *Total organic carbon*
[c] *Biochemical oxygen demand* [d] *Chemical oxygen demand*
[e] *After storage for 37 days*

Table 5. *Composition of bleached eucalypt kraft mill effluents.*

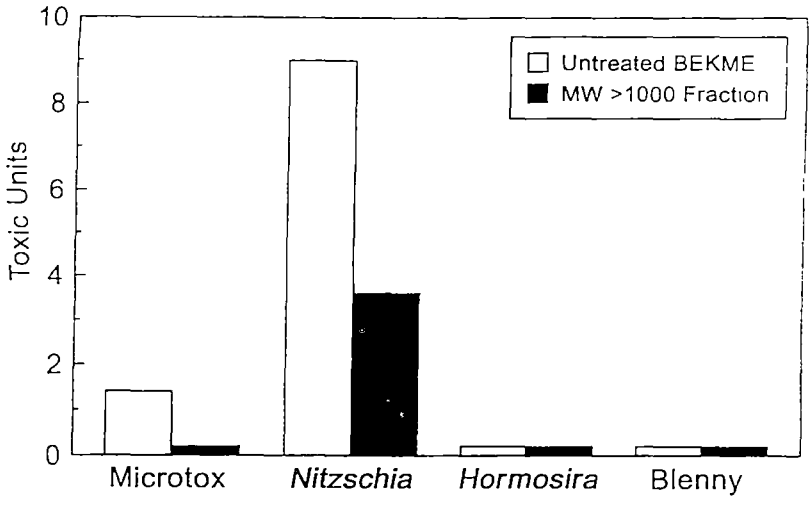

Figure 4. *Toxicity of untreated bleached-eucalypt kraft pulp mill effluent and its high molecular weight fraction (>1000) to the marine test species. Toxic units are <1 for Hormosira banksii and Tasmanian blenny larvae.*

Nitzschia closterium, *Hormosira banksii* and Tasmanian blenny larvae (Stauber *et al.*, 1994b).

The toxicity of the untreated effluent and its high molecular weight fraction to each of the test species is shown in Figure 4. The untreated effluent was toxic to both Microtox and *Nitzschia closterium*, with EC50 values of 72% (95% confidence limits of 50-100%) and 11% (2-25%) respectively. Approximately 40% of the toxicity to *Nitzschia* was associated with the high molecular weight fraction, whereas toxicity to Microtox was due to low molecular weight compounds. The high molecular weight fraction (>1000) contained polysaccharides and lignin breakdown products, both chlorinated and non-chlorinated, which have been shown to be toxic to algae and the early life stages of marine animals and plants (Stockner and Costella, 1976; Higashi *et al.*, 1992). In contrast, toxicity studies with freshwater species, found that most of the toxicity of bleached-softwood kraft effluents was associated with low molecular weight compounds, such as resin acids, fatty acids and chlorinated aromatic compounds (Rogers, 1973). The untreated effluent was not toxic to *Hormosira* or the Tasmanian blenny larvae, with EC50 values >100%. The treated effluent was not toxic to any of the test species, with EC50 values greater than 100%.

The *Nitzschia closterium* growth inhibition bioassay was the most sensitive bioassay in this suite of tests for assessing the potential toxicity of complex effluents. This bioassay has also been used to monitor changes in effluent toxicity with changing bleaching sequences, as mills move towards totally chlorine-free bleach sequences to reduce the concentrations of organochlorines in the effluents.

Conclusions

Single-celled algae are just one component of a battery of tests with organisms from all trophic levels, for use in environmental risk assessments. Their sensitivity and reproducibility makes them convenient and cost-effective tools for monitoring the potential toxicity of environmental samples, complex effluents and specific chemicals. With current restrictions by animal ethics committees on the use of fish in acute lethality tests, short term chronic microbial tests such as algal bioassays will play an increasingly important role in the protection of the aquatic environment in the future.

Acknowledgments

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Environmental Contamination and Toxicology

New Algal Enzyme Bioassay for the Rapid Assessment of Aquatic Toxicity

S M Peterson, J. L Stauber

CSIRO Division of Coal and Energy Technology, Centre for Advanced Analytical Chemistry, Private Mail Bag 7, Menai, NSW, 2234, Australia

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Bioassays with single-celled algae have been used extensively over the past 10 years to determine the toxicity of complex effluents, leachates, natural waters and specific chemicals. The standard bioassay measures the inhibition of algal growth rate over 72 hr exposure to a toxicant. Although such growth inhibition tests are sensitive and use an ecologically relevant endpoint, cost-effective assessment of toxicity is limited. There has been an increasing demand for tests that are rapid and inexpensive. Bioassays that detect sub-acute endpoints such as inhibition of enzyme activity (Gilbert *et al.* 1992; Jung *et al.* 1995; Manseal *et al.* 1995) show promise in meeting these requirements.

The enzyme β-D-galactosidase has recently been used in a rapid toxicity test with the invertebrate *Daphnia magna* (Janssen and Persoone 1993). Juvenile *D magna* were exposed to a series of toxicant dilutions for one hour before the addition of a fluorogenic substrate, 4-methylumbelliferyl β-D-galactoside Fifteen minutes later, the number of fluorescent test animals was compared to controls.

The galactosidase enzyme has also been found in other organisms such as sediment bacteria and algae (Casal *et al.* 1985; Stemmer *et al.* 1990, Davies *et al* 1994). This paper describes the development of a rapid toxicity test based on galactosidase activity in the green alga *Dunaliella tertiolecta.* This 3-hr bioassay is compared to standard 72-hr algal growth inhibition tests.

MATERIALS AND METHODS

The fluorogenic substrate was prepared by dissolving 0.0625 g of 4-methylumbelliferyl β-D-galactoside (MU-gal) in 10 mL of dimethylformamide with sonication and then diluting with 10 mL of Milli-Q water. To ensure a low background fluorescence, the reagent was cleaned according to the method of Apte and Batley (1994). The purified reagent was diluted to 250 mL with Milli-Q water and filter sterilized before use in the assay.

A base solution, required at the end of the assay to enhance the fluorescent signal, was prepared by dissolving 2.8 g sodium carbonate, 1.2 g sodium bicarbonate and 10 g sodium citrate in 100 mL of Milli-Q water. All reagents were AR grade

Correspondence to S M Peterson

Dunaliella tertiolecta Butcher was chosen as the test species as it had a suitably high galactosidase activity (Davies *et al.* 1994) and was available in axenic culture (CSIRO Culture Collection Strain CS-175, Hobart, Tasmania, Australia). *Dunaliella* was cultured in a modified half-strength f medium (Guillard and Ryther 1962). The culture was maintained on a 12:12 hr light·dark cycle (Philips TL 40W fluorescent white, 4000 lux) at 21°C.

Exponentially growing cells of *Dunaliella tertiolecta* were washed three times in seawater to remove culture medium and counted using a Coulter Multisizer II with 70 µm aperture. For the enzyme assay, algal inoculum was added to each test flask containing the toxicant and 50 mL of filter-sterilized seawater, to produce a final cell density of 2 x 10^5cells/mL. The test flasks were incubated in the light at 21°C for 2 or 24 hr, and then 5 mL aliquots of algal solution were asceptically transferred to sterile glass culture tubes (16x100 mm) containing 4 mL of MU-gal solution and 1 mL of 0.5 M PIPES buffer. After mixing, the tubes were incubated in a water bath Incubation temperatures of 21, 30 and 44.5°C were used and the incubation period was varied from 30 to 120 min. At the end of the incubation, the tubes were cooled under running water for 5 min and 400 µL of base solution was added to convert all the liberated MU to its anionic (and most fluorescent) form.

Dunaliella was exposed to five different metals (Hg^{+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Cr^{6+}), which were prepared as aqueous solutions in dilute acid, and five different organic compounds (sodium dodecyl sulfate (SDS), phenol, 4-chlorophenol, diuron, atrazine). All organic compounds were dissolved in Milli-Q water, except diuron and atrazine which were dissolved in AR grade methanol. For each toxicity assay, five replicate tubes of five different concentrations of toxicant were assayed along with five replicates of the control (no toxicant). Methanol controls were also run during the diuron and atrazine experiments. As the substrate underwent a limited amount of chemical hydrolysis during the incubation period, it was necessary to run blank solutions of MU-gal with no algae, to account for this contribution to the fluorescent signal. In addition, a blank containing algae plus toxicant (no MU-gal) was included to correct for any background algal fluorescence.

The fluorescence was measured using a Perkin-Elmer LS-5 Luminescence Spectrometer with the following settings: excitation wavelength 375 nm (slit width 10 nm); emission wavelength 465 nm (slit width 20 nm). The instrument response was calibrated daily using standard 4-methylumbelliferone (MU) solutions (20 nM, 50 nM and 80 nM). All results were reported as concentrations of MU.

Toxicity was determined by the reduction in fluorescence in the presence of toxicant compared to the controls. EC50 values (the effective concentration of toxicants to reduce enzyme activity by 50%) were calculated using trimmed Spearman Karber analysis, probit analysis or Microtox® (Microbics Corp.) software.

Growth inhibition bioassays with *Dunaliella tertiolecta* were carried out according to the method of Stauber *et al.* (1994), using the same washed inoculum as the enzyme bioassays. Triplicate seawater controls (50 mL) together

with at least 5 concentrations of toxicant (in triplicate) were prepared To each flask, 0.5 mL of 26 mM sodium nitrate and 0 5 mL of 1.3 mM potassium dihydrogen phosphate were added Each flask was inoculated with 2-4 x 10^4 cells/mL of the prewashed *Dunaliella* suspension and incubated at 21°C on a 12·12 hr light:dark cycle at 14000 lux.

Cell density in each flask was determined daily for three days using a Coulter counter. A regression line was fitted to a plot of $\log_{10}$ cell density versus time (hr) for each flask and the cell division rate determined from the slope. EC50 values were determined using the same statistics protocols as the enzyme bioassays. The bioassay was acceptable if the cell division rate in the controls was 1.3±0 3 doublings per day

RESULTS AND DISCUSSION

The effect of incubation temperature on fluorescence response of the controls is shown in Figure 1. The fluorescence response of the assay increased only slightly from 21°C (the usual *Dunaliella* culture temperature) to 30°C and then increased by nearly two orders of magnitude at 44.5°C. Even at 44.5°C, microscopic examination showed that the *Dunaliella* cells were intact and healthy. A separate experiment in which cells were killed with 4% formalin prior to incubation with MU-gal confirmed that the enzyme was only present in live algae.

The fluorescent signal was also found to increase with increasing incubation time from 30 to 120 min (Fig. 2), with a maximum fluorescence soon after 120 min. Background fluorescence from chemical hydrolysis of the substrate also increased with increasing incubation time, with a rapid increase after 90 min. Coefficients of variation also increased with length of incubation. Consequently, a one hour incubation time was chosen as a compromise between maximum fluorescence response and minimum background fluorescence.

The assay was performed with a number of different cell densities to ensure that fluorescence response increased linearly with the number of algal cells. Figure 3 shows that the fluorescence response was proportional to the number of cells over 2 orders of magnitude (10^4-10^6 cells/mL).

Once the optimal bioassay conditions were established, the toxicity of 5 metals and 5 organic compounds to *Dunaliella* was tested (Table 1). The alga was much more sensitive to metals than to organics. Similar insensitivity to organic compounds has been observed in other enzyme tests, including bacteria galactosidase assays (Dutton *et al.* 1988; Barnhart *et al.* 1983; Jung *et al.* 1995). Longer exposure times increased the sensitivity of the alga to organics, whereas metals were less toxic after 24 hr exposure than after 2 hr. It is possible that the metals may affect only enzyme that has already been expressed, but do not inhibit further biosynthesis of enzymes (which may be more resistant to the metals). The organics, on the other hand, may restrict enzyme expression so that the activity appears to decrease with time relative to the controls. This may explain the observations of Dutton *et al.* (1988) who found that galactosidase activity in bacteria was not affected by organic toxicants, whereas galactosidase biosynthesis was.

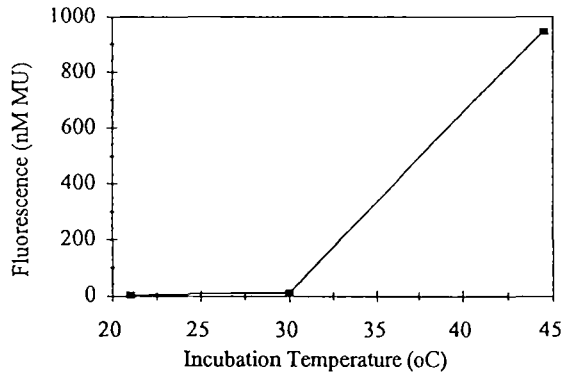

Figure 1. Effect of incubation temperature on utilization of substrate by *Dunaliella tertiolecta.* Incubation time: 60 min.

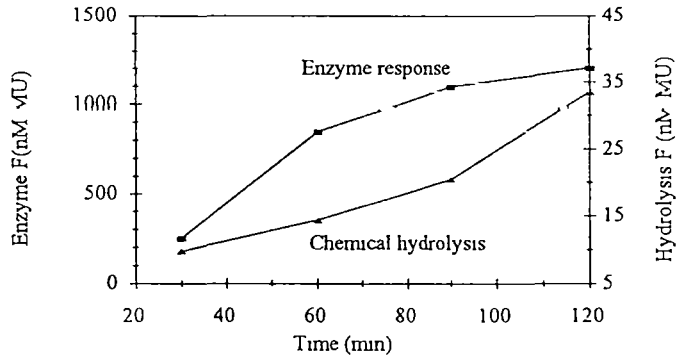

Figure 2. Effect of incubation time on fluorescent signal by *Dunaliella tertiolecta* and on chemical hydrolysis of substrate. (Note. Different scales on Y-axes) Incubation temperature: 44.5°C

Table 1. Inhibition of β-D-galactosidase in *Dunaliella tertiolecta* after exposure to metals and organic compounds for 2 hr (Concentrations in mg/L; Coefficients of variation from 3-5 replicate experiments in parentheses).

Toxicant	2-hr EC50 (CV)	Toxicant	2-hr EC50
Copper	0.035 (10%)	Sodium Dodecyl Sulfate	50
Mercury	0.081 (10%)	4-Chlorophenol	>230
Zinc	0.076 (22%)	Phenol	>470
Cadmium	0.351 (3%)	Diuron	>200
Chromium (VI)	7.0 (5%)	Atrazine	>100

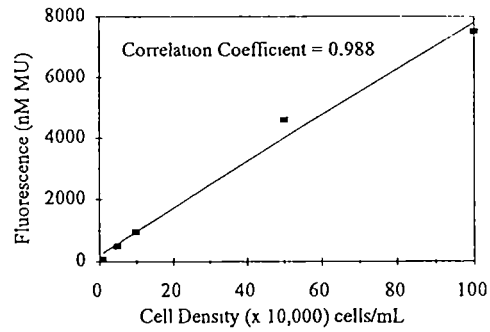

Figure 3. Effect of cell numbers of *Dunaliella* on fluorescent signal. Incubation temperature: 44.5°C; incubation time: 60 min.

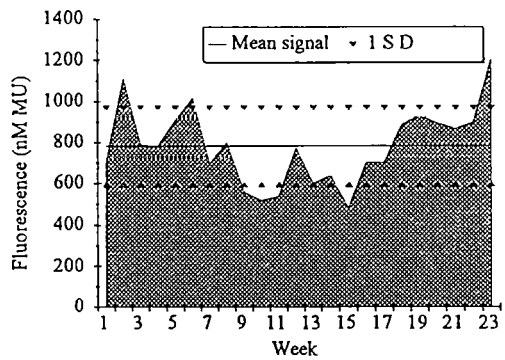

Figure 4. Variation of fluorescence produced by control samples from weekly cultures of *Dunaliella*. Mean signal ± one standard deviation is shown.

Weekly variations in the fluorescence response of the controls is shown in Figure 4. This variability was due to different inoculum sizes in the algal culturing procedure prior to the enzyme assay, rather than variation in the enzyme assay itself. Evidently, toxicity is a relative response compared to healthy controls rather than an absolute response to enzyme activity. Despite this, the new bioassay was highly reproducible with excellent replication within experiments (mean coefficient of variation: 4%), as well as between experiments (coefficients of variation: 3-22%).

Table 2 compares the sensitivity of the new *Dunaliella* galactosidase test with standard 72-hr growth tests with *Dunaliella* and the marine diatom *Nitzschia closterium*. The new enzyme assay was much more sensitive than 72-hr growth tests with *Dunaliella* for all compounds tested, except SDS (Table 2). Because *Dunaliella* growth is not a particularly sensitive endpoint (Abalde *et al.* 1995), the *Dunaliella* enzyme test was also compared to 72-hr growth tests using the sensitive diatom *Nitzschia closterium*. *Nitzschia* was more sensitive than the enzyme assay for some toxicants, while similar toxicity to zinc and cadmium was

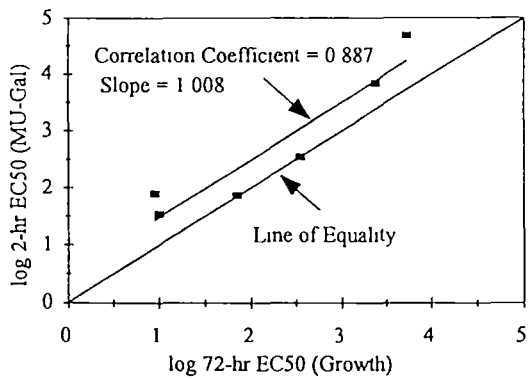

Figure 5. Correlation between 2-hr EC50 from β-D-galactosidase activity in *Dunaliella tertiolecta* compared to 72-hr EC50 from growth of *Nitzschia*

observed for both tests (Table 2). Figure 5 shows the correlation between 2-hr EC50 values for the new enzyme assay, compared to 72-hr EC50 values for *Nitzschia* with six toxicants. Good correlation for the 6 toxicants was obtained (r^2= 0.887; slope = 1.008).

The *Daphnia* test (Janssen and Persoone 1993) was more sensitive than *Dunaliella* to mercury, cadmium and chromium, whereas *Dunaliella* was more sensitive to copper, zinc and SDS (Table 2). However, the coefficients of variation for the *Daphnia* test were higher (15-32%), probably due to the qualitative determination of fluorescence in the water fleas.

Table 2. Comparison of EC50 values obtained from different tests (mg/L) for six different toxicants.

Toxicant	*Dunaliella* Galactosidase test	*Dunaliella* Growth test	*Nitzschia* Growth test[a]	*Daphnia* Galactosidase test[b]
Mercury	0.081	0.300	0 009	0.015
Copper	0.035	0.576	0.010	0.059
Zinc	0.076	>6	0.070	0.978
Cadmium	0.351	>6	0.350	0.210
Chromium (VI)	7.0	17.8	2.4	0.250
SDS	50	15	5.3	74

[a] From Stauber *et al.* 1994; [b] From Janssen and Persoone 1993

The new algal enzyme bioassay was both sensitive and reproducible and correlated well with other environmentally relevant parameters such as growth The major advantage of this test is that toxicity information can be obtained

within 3 hr rather than 72 hr in a standard growth test, making the algal enzyme test a cost-effective way to monitor the toxicity of a range of chemicals.

Further work is underway to reduce the test time to 1 hr and to assess its suitability for monitoring complex effluents. In addition, an enzyme test using freshwater algae is currently being developed for monitoring pollutants in freshwater aquatic systems.

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Skin Absorption of Ionic Lead Compounds

Ref: lead5

T. MARK FLORENCE D.Sc.
Centre for Environmental & Health Science
Oyster Bay, NSW, Australia
JENNY L. STAUBER M.Sc., LES S. DALE B.Sc. and DALE HENDERSON.
CSIRO Centre for Advanced Analytical Chemistry
Lucas Heights, NSW, Australia
BEVERLY E. IZARD and KERYNNE BELBIN
Australian Nuclear Science and Technology Organisation
Lucas Heights, NSW, Australia

Lead, as a solution of lead acetate or nitrate, was applied to the skin of mice. In less than 24 h there were large increases in lead in skin, muscle, pancreas, spleen, kidney, liver, cecum, bone, heart and brain, but not in blood. For i.v.-injected lead there were increases in blood lead and in all organs except skin and muscle. Previous research found that inorganic lead can also be absorbed through the skin of humans. The results show the importance of taking into account the possibility of skin absorption of lead in occupational exposure, especially since blood lead, the prime index of lead status, is not an indicator of skin absorption.

Key words: lead, skin absorption, blood lead, occupational exposure, lead uptake

INTRODUCTION

We showed previously [1-3] with human volunteers that inorganic lead salts such as lead chloride and nitrate are rapidly absorbed through the skin from aqueous solution and enter the extracellular fluid compartment, resulting in high concentrations of lead in sweat and saliva but not in blood. We proposed [3] that the behavior of skin-absorbed lead in the body is quite different from that of i v.-injected or ingested lead, because lead which has passed through the skin is in a physico-chemical form which has a low affinity for erythrocytes, but which partitions strongly into the extracellular fluid compartment. By applying solutions of the stable lead isotope Pb-204 to the skin of volunteers and measuring the increase in Pb-204 in blood, we were able to show that skin-absorbed lead has a very short residence time in blood [3] The ability of lead salts to enter the body through the skin without significantly raising blood lead has important implications for occupational health control, because measurement of lead in blood is the prime technique for monitoring lead exposure Surveys of workers in lead industries such as lead battery manufacture showed that many of them had very high levels of lead in sweat, even those who had retired or who had been away on leave for several weeks [1]. Workers in lead industries often wear masks to avoid inhalation of lead, but rarely have skin protection. Some lead battery workers have their skin covered with a layer of finely powdered lead metal/lead oxide [1]. Both lead metal and lead oxide were found to dissolve in sweat and pass through the skin.[1-3] Failure to appreciate the dangers of skin-absorbed metal salts has had serious consequences. In Japan, "smelter disease", which caused hundreds of poisoning cases and several deaths of workers in sulphuric acid plants, was believed to result from sulfur dioxide exposure However the disease was later shown to be caused by skin absorption of mercuric sulfate, and fully encapsulated suits had to be worn by workers to prevent toxicity [4]

Although we were able to show unequivocally with human experiments that simple inorganic lead salts are readily absorbed through the skin, we were unable to determine accurately the amount of lead absorbed and retained by this route or which organs and tissues, if any, accumulated this source of lead. To provide this information, we have now carried out

experiments with mice using both skin-absorbed and i v.-injected lead, and compared the concentrations of lead in several organs after various exposure times The i v experiments were carried out to compare organ retention of lead when the metal entered the bloodstream directly, and when it arrived there via the skin

EXPERIMENTAL PROCEDURE

Female Balb/c mice (20 g, 4-5 months old) were used for the study During the experiments they were provided with water and their normal diet, and kept in a room controlled at 26 ± 1°C. To study the skin absorption of lead a 0.5 cm^2 area of hair (about 3% of total skin area) was shaved from the back of each mouse, leaving a short stubble to ensure that the skin was not damaged. Using a micropipette, 40 µL of an aqueous solution of lead acetate or nitrate (6.4 mg of lead) was spread over the shaved area, then almost dried using a hair dryer. The patch was covered with a square of Parafilm, which was then covered with an H-shaped saddle of Elastoplast which wrapped around the body. The covering remained intact throughout the experiment. Control mice were prepared in a similar manner, but 40 µL of MilliQ water was substituted for the lead solution. After periods ranging from 2 hours to one week, the mice were sacrificed and the organs and tissues were analysed for lead by inductively-coupled plasma mass spectrometry (ICP-MS) after microwave digestion of the tissue in HNO_3 - H_2O_2 For the i.v.-injected experiments, 10 µL of lead acetate in normal saline (70 µg of lead) was injected into the tail of the mouse.

The solution of lead in normal saline was prepared by adding a slight excess of lead acetate to normal saline, allowing the lead chloride precipitate to settle for three days, then collecting the supernatant solution. The lead concentrations of both this solution and the lead acetate solution used for skin absorption were determined just before use by ICP-AES analysis to ±2-3%. The precision of the ICP-MS analyses of tissue lead from the mice was 3-5% and the accuracy, based on the recovery of lead measured in several standard biological materials using the same analytical procedure as used for the mice tissue samples, was 5-10%. Data were analysed using

the statistical computer package MINITAB Control and test means were compared using the non-parametric Mann-Whitney U test

RESULTS AND DISCUSSION

The skin absorption and i.v.-injected results are shown in Table 1, while Table 2 compares the relative affinity of various organs for skin-absorbed and i v.-injected lead. Total analysis of the organs, feces and urine showed that, of the 6 4 mg of lead applied to the skin, 26 μg (0.4%) was absorbed through the skin and entered the circulatory system in 24 h The recovery of the 70 μg i.v.-injected dose of lead was 57 μg (81%) after 24 h, using the same mass balance analysis. Skin absorbed lead concentrated more strongly than i.v.-injected lead in the skin and muscle (remote from patch), but less strongly in the blood, spleen, kidney, liver and bone, while the affinities of the pancreas, cecum, mid-gut, heart and brain for the two sources of lead were similar (Table 2). After 96 h, blood lead in the i v.-injected mice was still seven times higher than that of the control mice, whereas blood lead for the skin-absorbed mice was only 50% higher.

Skin absorption of lead increased with time and, in general, maximum organ concentration occurred after 24-48 h, although this was organ-dependent to some extent No significant difference was found between the absorption of lead acetate and lead nitrate, and organ concentration of lead increased with applied dose. Similar distribution of lead in the organs was found when the lead solution was first absorbed on a membrane filter before applying it to the skin [1-3], although less lead was absorbed when the membrane filter was used

Application of a solution of a simple ionic lead salt to about 3% of the skin surface of a mouse led to increases of two- to 100-fold in the lead concentration of its organs (Table 2). Workers in some lead industries have been observed with much more than 3% of their skin area covered with lead dust [1]. It is quite possible then, that in some circumstances dermal absorption can significantly increase the body burden of lead. Laug and Kunze [5] previously found that application of a solution of lead acetate to the skin of rats caused an increase in lead in their kidneys. However, standard texts on lead toxicity [6], and even recent reviews on the subject

[7], continue to state that dermal absorption of lead is unimportant except for non-polar organo-complexes such as tetraethyllead An insidious aspect of the dermal absorption of lead is that blood lead, which is the prime occupational index of exposure, does not show a measurable increase even in the presence of considerable intake via the skin (Table 1) Clothing that fully covers the skin may be required for workers in some lead industries. Future studies will examine the usefulness of monitoring lead in saliva as a means of detecting skin-absorbed lead in humans

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Table 1. Lead Concentrations in Organs of Mice after Skin Absorption and i.v. Injection of Lead Acetate Solution.

Results show the mean organ concentration of lead in μg/g wet weight and standard deviation, 24 hours after application of 6.4 mg of lead to skin or 70 μg of lead injected

Organ	Control n=25	Skin applied n=4	p*	i.v. injected n=4	p*
Skin**	0.059±0.020	2.42±1.52	0.001	0.28±0 10	0.001
Muscle**	0.055±0.020	0.93±0.78	0.001	0.11±0 08	NS
Patch muscle	0.055±0.020	18.8±4.9	0.001		
Blood	0.035±0.020	0.044±0.027	NS	0 72±0.12	0 001
Pancreas	0.032±0.020	0.38±0.29	0.001	1.24±0.25	0 001
Spleen	0.033±0 020	0.19±0.13	0.001	3 70±0 46	0 001
Kidney	0.041±0.017	4.10±3.81	0.001	22.0±4 7	0.001
Liver	0.027±0 023	0.51±0.33	0.001	6.63±1.54	0.001
Mid-gut	0.108±0.061	1.05±1.17	0.001	2.03±0.43	0.001
Cecum	0.29±0 11	2.72±1.53	0.001	8.75±2.23	0.001
Bone	0 13±0.18	0.93±0.66	0.001	9.55±0.90	0.001
Brain#	0.009±0 013	0.087±0.11	0.01	0 16±0.04	0.005
Heart#	0.039±0.063	0.077±0.056	NS	0.23±0 04	0.005

NS = not significant at p = 0.05

* Mann-Whitney U test comparison of means of controls and tests

** Remote from lead patch

n = 12 for controls

Table 2. Test/Control Ratios of Concentrations of Lead in Mouse Organs after 24 Hours.

Results shown are for skin-applied (6.4 mg Pb) and i v.-injected (70 μg Pb) lead acetate. Last column is the ratio of the two previous columns, corrected for the difference in the measured dose of skin absorbed lead (26 μg) and i.v -injected lead (57 μg)

Organ	Test/Control, Skin Absorbed	Test/Control, i.v. Injected	Columns 3/2, Corrected
Skin*	40.9	4.78	0 053
Muscle*	17.0	2.07	0 055
Blood	1.25	20.3	7 4
Pancreas	11.7	38.4	1.5
Spleen	5 71	112	8 9
Kidney	100	538	2 4
Liver	19.3	248	5 8
Cecum	9.31	30.0	1.5
Mid-gut	9.79	18.9	0 88
Bone	6.94	71.1	4.6
Heart	1.97	5.75	1.3
Brain	9.63	17.6	0.83

* Remote from patch