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The Effect Of Adsorptive Copper Losses On Toxicity Endpoints In Bioassays Using Micromarine Algae.

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The Effect Of Adsorptive Copper Losses On Toxicity Endpoints In Bioassays Using Micromarine Algae.

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Abstract

The purpose of this study is to investigate the effect that adsorptive losses of copper over a bioassay period have on algal bioassay response and ultimately the test outcome. A new tank bioassay is designed to keep copper constant in test solutions over 72 hours. Meanwhile a mathematical model is developed to predict the effect of copper losses on algal growth over a bioassay period. It is shown that standard bioassay, where large contaminant losses occur are likely to underestimate the potential effect of copper. The study also indicates that the error in the estimated toxicity will depend upon exposure duration.

Key words: algal bioassay, copper, ICP-AES, losses, *Phaeodactylum tricornutum*, tank, toxicity endpoints.

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CHAPTER 1. INTRODUCTION

Algal toxicity tests (bioassays) have been developed to quantify the adverse biological effect that contaminants have in aquatic systems. They measure the response of algae after exposure under defined conditions to different concentrations of contaminants. A test system typically consists of test samples containing specific contaminant concentrations and controls which contain no contaminant but duplicate the test conditions. To assess the toxicity of the samples, the responses in the test samples are compared to the responses in the controls.

As a complementary tool to chemical analysis, toxicity tests have found widespread application in the environmental monitoring of metals. Yet in standard toxicity tests, significant adsorptive losses of metals (e.g. copper) from test solutions to test containers (e.g. glass flasks) or to algal cell surfaces have been observed over the test period (e.g. 48-72 h). To overcome this problem, alternative test container materials, shortened test duration, static renewal or low initial cell density have been suggested, however none of these options have completely eliminated the adsorptive loss problems.

In toxicity tests it is important that either contaminant concentrations stay constant during the test duration, or fluctuations adequately mimic the concentration changes occurring in the field. The impact that decreasing contaminant concentrations (e.g. adsorptive losses) has on toxicity responses and test outcomes has not been thoroughly investigated. In this study, a new (tank) bioassay was designed to keep the contaminant (copper) concentrations constant over a test period. The results from these toxicity tests were compared with those of standard test procedures where large contaminant losses occur. The comparison of results from these two test procedures, combined with modeling exercises aimed at predicting effects, allowed the effect of contaminant loss over time on toxicity test endpoints to be more accurately described.

CHAPTER 2. LITERATURE REVIEW

2.1 Copper in the environment

2.1.1 Occurrence of copper

Copper occurs at low background concentrations throughout the earth's crust and lithosphere. Naturally occurring copper in aquatic systems originates mostly from atmospheric precipitation and weathering processes of soils and bedrocks. Copper concentrations in the aquatic environment may approach toxic levels as a result of natural sources such as volcanic ashes or copper mineral deposits, as well as anthropogenic sources (Cimino and Ziino, 1992). Human activities have altered the natural distribution and fluxes of copper, resulting in increasing copper concentrations in the aquatic environment. Anthropogenic point and diffuse sources include industrial (mining, power generation), urban (domestic waste, buildings materials) and agricultural activities (pesticides, herbicides, fertilizers).

Typical concentrations of total dissolved copper in surface open seawater range from 0.03 to 0.15 µg/L (Batley, 1995) and are 0.09-0.3 µg/l in coastal waters (Apte et al., 1998; Batley, 1995). Copper concentrations are expected to be higher in estuarine waters than in coastal waters, with concentrations of up to 16 µg/L reported in estuaries around the world (Mills and Quinn, 1984). Copper concentrations in contaminated areas may be one order of magnitude higher. For example, in Macquarie Harbour, Tasmania, which is affected by particulate and dissolved copper discharges from mines and smelters, copper concentrations in the range 10-42 µg/L were recorded (Stauber et al., 2000). In Chile, concentrations of dissolved copper of up to 40 µg/L were reported close to the disposal of untreated tailings, in comparison with concentrations of 0.5-6.5 µg Cu/L recorded in unpolluted areas along the Chilean coast (Castilla et al., 1997).

2.1.2 Copper speciation and bioavailability

The fate of copper in aquatic systems depends on a complex set of reactions. As a result copper will be present in aquatic systems in a variety of chemical forms, which is known as its speciation. Copper occurs primarily as the hydrated free cupric ion (Cu $(H_2O)_6^{2+}$), as inorganic or organic complexes, as colloids or attached to particulate matter (Table 1). Equilibrium models predict the inorganic carbonato complex ($CuCO_3$), the hydroxy complexes ($CuOH^+$, Cu $(OH)_2$) and the free cupric ion to be the major species of copper in seawater accounting respectively for 82, 7 and 3 % (Stauber, 1996).

Table 1. Examples of copper species in natural water (Modified from xxx and Florence and Batley,1980).

Free metal ion	$Cu(H_2O)_6^{2+}$
Inorganic complexes	$CuCO_3$, $Cu(CO_3)^{2-}$, $Cu(OH)_2$, $Cu(OH)^+$
Organic complexes	
Weak complexes	Cu-fulvic acid
Lipid soluble complexes	Cu-oxinate
Attached to colloids	$Cu\text{-}Fe(OH)_3^-$
Attached to particles	Cu adsorbed onto or contained into clay particles

It is now well established that metal speciation is a critical factor controlling metal bioavailability in natural waters, i.e. the fraction of total metal that an organism can take up or accumulate. Metal speciation depends on a range of parameters, including pH, hardness, salinity, redox potential and dissolved organic matter (Stauber and Davies, 2000). This means that the physico–chemical characteristics of a water body will affect the extent of copper bioavailability and thus the level of toxicity.

In assessing the impact of metals in aquatic systems, it is therefore important to recognize that the total concentration of a metal does not alone control its toxicity. Different physico-chemical forms of copper can cause a wide range of toxic effects.

In general copper toxicity is correlated with the presence of the free metal ion and labile complexes, which are easily dissociable. Studies have shown that free copper ion or inorganic complexes are generally more available than copper in strong complexes or adsorbed to colloidal and particulate matter. Lipid-soluble copper complexes are an exception. Whereas most organic complexes have low toxicity, lipid soluble complexes are highly toxic because they can easily and rapidly penetrate cell membranes.

The physico-chemical characteristics of natural waters influence copper bioavailability. Dissolved organic matter (DOM) appears to ameliorate the toxicity of copper by forming complexes that are either not able to permeate the cell membrane of most organisms or are sufficiently stable that they do not dissociate at the cell membrane (Apte et al., 1993). The occurrence of metals such as iron, aluminum and manganese can also greatly reduce copper bioavailability and toxicity. It has been shown that colloidal manganese and iron hydroxides ameliorate copper toxicity by preventing copper binding at the cell surface (Stauber and Davies, 2000).

2.1.3 Toxicity of copper to algae

Copper is an essential element, which is required to sustain aquatic life. However when copper concentrations exceed the concentration required for optimal growth, copper can become toxic. The free copper ion has been found to be one of the most toxic metal species to algae (Canterford and Canterford, 1980).

Algae are very good indicators of metal toxicity because of their important role in ecosystems. Any adverse effects on algae will have important consequences for the whole ecosystem. All species of algae show a great ability to bioaccumulate copper (Stauber et al., 1986) but the sensitivity of algae to copper can vary greatly (Robinson, 1989; Knauer et al., 1997). The toxicity of copper is mainly associated with the inhibition of cell division, however it has been shown that copper also interferes indirectly with several cellular processes, including enzyme activities, photosynthesis, respiration and electron transport (Stauber and Florence, 1987; Guazon et al., 1994; Peterson and Stauber, 1996; Franklin, 2000; Wong et al., 1994).

Mechanisms of toxicity of copper are largely controlled by the interactions of copper at the cell surface and the uptake of copper into the cell. Lipid bilayer membranes are generally impermeable to charged and polar species. Thus, to enter the cell, copper complexed in solution has to bind with a protein at the cell surface to form a hydrophobic complex that can traverse the membrane (Stauber and Davies, 2000). Copper is then released inside the cell where it may exert adverse effects, i.e.:

Solution: CuL----------protein-membrane: cell

$$CuL + \text{protein} \rightarrow \text{Cu-protein} + L\ (\text{solution})$$

$$\text{Cu-protein} \rightarrow \text{Cu-cell} + \text{protein-membrane}$$

2.1.4 Water quality guidelines

Guidelines for copper concentrations in aquatic ecosystems are typically based on total copper concentrations (Markich et al., 2001). However, these guidelines will generally be overprotective, since only a fraction of the total metal concentration in natural waters will be bioavailable, especially in waterbodies with a high content of particulate and/or DOM. The latest Australian and New Zealand guidelines for fresh and marine water quality recommend a trigger value of 1.3 µg/L for a level of protection of 95% in marine water, i.e. protection of 95% of the species living in the ecosystems (ANZECC/ ARMCANZ, 2000).

Recent research has emphasized the importance of assessing copper toxicity in terms of bioavailable copper, rather than total copper, and has thus reinforced the need for site-specific guidelines.

2.2 Analytical Approaches

It is important to underline the problems associated with the environmental monitoring of trace metals, including copper. Few analytical techniques are capable of quantifying metals at low part per billion concentrations. In addition, because of

these low concentrations, contamination during sample collection, sample storage and preparation for analysis may easily occur, if not done with extreme care. Adsorptive losses of trace metals to container surfaces or suspended solids represents a further source of error.

The environmental monitoring and speciation of copper involves analytical methods such as graphite furnace atomic adsorption spectrometry (GFAAS), anodic stripping voltametry (ASV) or inductively coupled plasma (ICP) associated with atomic adsorption spectrometry (ICP-AES) or with mass spectrometry (ICP-MS). Table 2 compares detection limits for copper by these four methods. Techniques to preconcentrate copper prior to analysis (e.g. solvent extraction of seawater) can both eliminate troublesome matrix effects and greatly lower detection limits (Apte and Gunn, 1987).

Table 2. Detection limits for copper by some analytical techniques (Stauber et al., 1986).

Technique	Freshwater (µg/L)	Seawater (µg/L)
GFAAS	0.5	NA[a]
ICP-AES	1	3
ICP-MS	0.1	NA[a]
ASV	0.2	0.2

[a] Not applicable

2.2.1 Inductively Coupled Plasma- Atomic Emission Spectrometry

Copper measurements can be performed using inductively coupled plasma (ICP) techniques. Usually associated with atomic emission spectroscopy (AES), mass spectroscopy (MS) or even chromatography, ICP is applicable to a wide range of metals and provides low detection limits for many elements. Emission spectroscopy using ICP is a sensitive and rapid method for the determination of trace metals in water samples. Although high spectral interferences (mostly from sodium) represent one of the main disadvantages of this analytical tool, a detection limit of approximately 3 µg/L for copper may be achieved by ICP-AES in seawater.

An ICP source consists of a flowing stream of argon ionized by an applied magnetic field (Figure 1).The argon, previously seeded with free electrons by a tesla discharge, enters the 'torch' through quartz tubes. When the argon reaches the magnetic field, created by surrounding induction coils at the top of the quartz tubes, the free electrons are excited sufficiently to ionize the gas. Further ionization and high thermal energy results in the formation of a plasma at the top of the torch. A sample aerosol is generated in a nebuliser and passes through a spray chamber to ensure only droplets less than 5 μm diameter enter the torch. The appropriate aerosol is then carried into the plasma through an injector tube located within the torch. When injected into the ICP, molecules, which are subjected to temperatures of about 8000 ^{o}K, dissociate and emit light at a characteristic wavelength. The intensity of light is then detected by photomultiplier tubes.

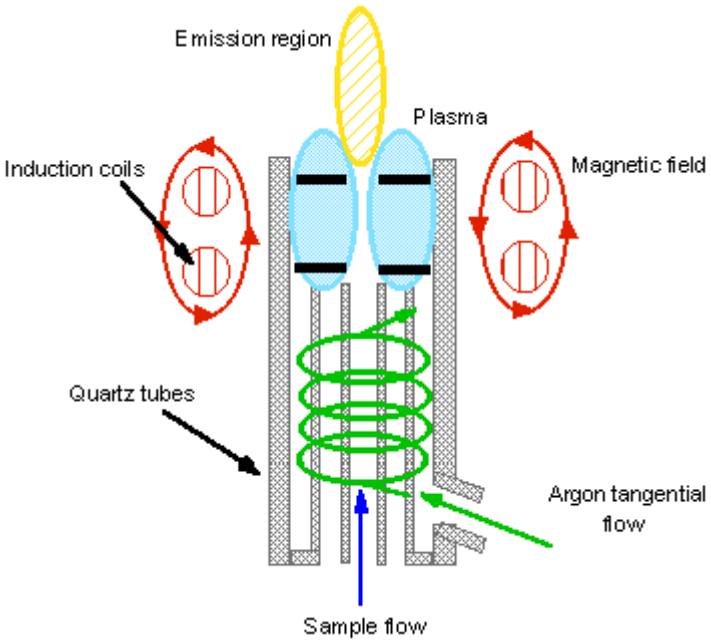

Figure 1. A typical plasma source.

2.2.2 ASV

Anodic stripping voltammetry (ASV) is a very sensitive method for detecting metals at concentrations down to sub part per billion levels. ASV permits simultaneous measurements of several metals and is a species-specific method, making it a powerful tool in trace metal speciation. Although ASV detects only free (or rapidly

labile) metal ions, total metals can be determined after dissociating bound metals and labile forms usually by acidification or UV irradiation.

2.2.2.1 Principles

ASV is an electrochemical method of analysis, which belongs to the voltametry branch of techniques, in which an electrical current at a working electrode is recorded as a function of a potential waveform applied to the electrode. ASV is a two-step technique involving a three-electrode system. The preconcentration step, referred to as deposition, consists of the reduction of metal ions in the sample solution at a negative potential and their concentration onto the electrode. Copper will be reduced at the electrode (Equation 1). Transport of species from the bulk solutions to the surface of the electrode must be controlled by only diffusion and convection phenomenon, where convection may be created by electrode rotation or solution stirring. Electrical migration caused by the electrical field has to be eliminated.

$$Cu^{2+} + 2e^- \rightarrow Cu^0 \qquad \text{(eq 1)}$$

The deposited metals are measured in the second step, referred to as stripping, by applying a positive potential scan. During the scan, each metal is stripped from the electrode when its oxidation potential has been reached. In freshwater samples, copper will be oxidized back into the solution to copper (II) as shown in Equation 2. Yet, in seawater samples, copper will be oxidized to copper (I), as it forms stable complexes with chloride ions (Equation 3).

$$Cu \rightarrow Cu^{2+} + 2e^- \qquad \text{(eq 2)}$$

$$Cu \rightarrow Cu^{+} + e^- \ (+ Cl^-) \rightarrow CuCl^0 \qquad \text{(eq 3)}$$

The oxidation of the metal gives rise to a faradaic current that is measured. The current is proportional to the concentration of the metal on the electrode and thus to the concentration of the metal ion in the sample solution (Equation 4). Between the two steps, a rest period is employed, during which the forced convection is stopped and the deposition current drops to zero. This ensures the concentration of metal in

the mercury drop is uniform and that the stripping step is performed in a quiescent solution.

$ip = kn^{3/2}D^{2/3}rv^{1/2}C_b tm$ (eq 4)

where

ip= peak current, k = constant, n = number of electrons, D = diffusion coefficient, r = radius of the mercury drop, v = scan rate, C_b = concentration of the ion in the bulk solution, t = deposition time, m = mass transport coefficient.

2.2.2.2 Instrumentation

ASV measurements are made in an electrochemical cell (quartz, glass, Teflon) with a three-electrode system, consisting of a working electrode, a reference electrode and an auxiliary electrode. The working electrode is where the electrochemical reaction takes place. The reference electrode, insensitive to the solution, provides a known and stable potential to which the potential of the working electrode is compared. The auxiliary electrode serves to minimize the errors from cell resistance in controlling the potential of the working electrode.

Working electrodes in ASV are either mercury electrodes or inert solid electrodes, the former being frequently used due to its better sensitivity and reproducibility. As the sensitivity of ASV is controlled by the geometry of the electrodes, two different shaped electrodes are used: the hanging mercury drop electrode (HMDE) and the thin mercury film electrode (TMFE). The HMDE dispenses a mercury drop at the tip of a glass capillary. A micrometer controls the drop size. The thin mercury film is formed by added mercuric ions that plate onto the surface of a carbon substrate. The difference is essentially determined by the surface to volume ratio, which is low in the case of the HMDE and high in the case of the TMFE.

2.2.2.3 Oxygen interference

Oxygen can be reduced electrochemically as shown in Equation 5 and 6.

$O_2 + 2H^+ + 2e^- \leftrightarrow H_2O_2$

$H_2O_2 + 2H^+ + 2e^- \leftrightarrow 2H_2O$ (eq 5)

$O_2 + 2H_2O_2 + 2e^- \leftrightarrow H_2O_2 + 2OH^-$

$H_2O_2 + 2e^- \leftrightarrow 2(OH^-)$ (eq 6)

The reduction of oxygen interferes first because the oxygen peak may obscure the peak of interest. In addition, hydrogen peroxide formed during the reduction process can further react with certain metals.

2.3 Use of algal bioassays for assessing copper toxicity in the aquatic environment.

2.3.1 Algal bioassay approaches

Bioassays or toxicity tests are generic tests that use living organisms as indicators of the bioavailability and toxicity of contaminants in aquatic systems. They measure responses of living organisms after exposure to specific concentrations of chemicals, effluents, leachate or receiving waters.

Bioassays are widely used to assess the environmental impact of contaminants, including copper, in aquatic systems. They have the advantage over chemical measurements of giving direct quantitative data on adverse biological effects and are thus of high ecological relevance. Toxicity data derived from bioassays are generally expressed as IC_{50} or IC_{25}, the inhibitory concentration of copper to cause a 50% or 25% effect in comparison to a control. The lowest observable effect concentrations (LOEC) and the no observable effect concentrations (NOEC) are also used to predict toxicity, especially in risk assessments.

Bioassays have traditionally been undertaken on invertebrates and fish, yet microbial bioassays are now increasingly being used. Among the microbial community, algae are particularly suitable for toxicity tests because of their sensitivity and ecological relevance. They are at the bottom of most aquatic food chains. Any adverse impacts

on algae may directly or indirectly affect organisms at higher trophic levels. In addition algae have been shown to be very sensitive to a large range of organic and inorganic contaminants (Stauber and Davies, 2000). Algae divide approximately once per day, therefore toxicity tests using algae are chronic tests, covering several generations of cells over 48 or 72 hours.

2.3.2 Test endpoints

A wide range of algal responses can be used as bioassay assessment endpoints, among them, cell division, cell size, respiration, enzyme activity, and cell fluorescence (Stauber 1995, Franklin, 2000). As there will be great variability in the IC_{50}, depending upon which assessment endpoint is used, the selection of the appropriate endpoint is important (Table 3). Most algal bioassays measure the decrease in growth rate (cell division rate) or the change in cell concentration (cell yield) after a 48-96-h exposure (Stauber, 1995). Growth rate is generally preferred to cell yield because IC estimates from growth rate are more reproducible, more ecologically relevant and less dependent on particular test system parameters (Hornstrom 1990; Nyholm, 1983). Studies have shown that inhibition of the algal cell division rate (growth rate) provides the most sensitive and reproducible chronic endpoint for *Dunaliella tertiolecta*, *Phaeodactylum tricornutum* (Franklin et al., 2000) and *Nitzchia closterium* (Stauber, 1995).

Table 3. The effect of copper on various test endpoints in *Selenastrum capricornutum* and *Phaeodactylum tricornutum* (Franklin et al., 2000).

Test endpoint	Exposure (h)	IC_{50} (µg/L)	
		S.capricornutum	*P.tricornutum*
Cell division rate	72	8±2	10±4
Cell size	72	>70	8±3
Chlorophyll *a* fluorescence	72	13±5	12±2
FDA fluorescence (esterase)	72	40±10	-

2.3.3 Laboratory copper calibration bioassays

Laboratory bioassays using ionic copper spiked into clean freshwater or seawater are used to assess the toxicity of copper in aquatic systems under controlled conditions. Growth inhibition tests (72 h) in minimal nutrient media are typically used. Exponentially growing algal cells, obtained from culture, are exposed to increasing concentrations of copper and cells are counted each day for 3 days. The growth of the algae exposed to the copper is compared to the growth of the algae in a control (no copper). Appropriate statistical tools give the 48-h or 72-h IC_{50}, LOEC and NOEC values, which provide guideline values to develop water quality criteria. Table 4 shows the toxicity of copper to different marine algae derived from a 72-h growth rate inhibition test in filtered seawater with minimal nutrients.

Table 4. Toxicity of copper to marine algae.

Algal species	72-h IC_{50} (µg/L)	Reference
Nitzchia closterium	17	Franklin et al., 2000
Dunliella tertiolecta	576	Peterson and Stauber, 1996
Phaeodactylum tricornutum	10	Franklin et al., 2000
Amphora sp.	190	Stauber and Adams, unpublished

As algal bioassays are carried out in laboratory conditions, using water with no added organics, such tests may overestimate copper toxicity and do not necessarily provide information on bioavailable copper. However, they can be used as calibration bioassays for assessing the bioavailability of copper in field-collected samples. The toxicity of copper in water samples can be compared with the toxicity in the calibration bioassay to determine whether any processes such as complexation ameliorate the toxicity.

2.4 Deficiencies in Toxicity Tests' Accuracy

2.4.1 Factors affecting the sensitivity of toxicity tests

Increasing knowledge of the biology of aquatic test organisms and the way to measure biological response have improved toxicity tests. Many studies have focused on biological factors affecting sensitivity of laboratory bioassays. The major aspects of toxicity testing using marine algae (test media, test inoculum, pH, salinity, incubation conditions) have been discussed by Walsh (1988) and Hornstrom (1989). Stauber (1995) points out the importance of selection of sensitive and ecologically relevant species. In a recent paper, Stauber and Davies (2000) reviewed all the current use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment.

2.4.2 Adsorptive losses

Adsorptive losses of copper, associated with containers and algal surfaces, are significant in laboratory algal bioassays. Most algal bioassays use borosilicate glass flasks and ecotoxicologists should be aware of the adsorptive losses associated with them (Batley et al., 1999; Stauber and Davies, 2000). Losses of copper above 20 % of the nominal concentrations are frequently recorded after a 72-h bioassay, though losses up to 40% have been reported (Batley et al., 1999). Stauber et al. (2000) showed that mass balances of copper measured at the end of a bioassay in the solution, on the cells, in the cells and on the walls of the bioassay flasks were generally within 10 % of the nominal copper concentration. Approximately 10 % of the nominal concentrations in algal bioassays using *Nitzschia closterium* were adsorbed to the bioassay flasks. Losses to the container surfaces will clearly be greater the larger the ratio of container surface to solution volume in the bioassay (Batley et al., 1999).

Copper losses associated with adsorption on container surfaces may be partly overcome by pre-silanising test flasks or by the use of plastic materials such as polyethylene, polystyrene, and polycarbonate. Presilanizing glass flasks with solutions such as Coatasil (BDH) may reduce copper losses to some extent but they

can still exceed 20% (Stauber and Davies, 2000). Plastic materials, such as polystyrene, polycarbonate or Teflon reduce copper losses. For example, using polycarbonate containers for bacterial bioassays, Davies et al (1998) showed that there was no reduction in dissolved copper concentrations in solutions after 48 h. However, the use of opaque plastic containers may reduce algal growth rates because of reduced light penetration.

2.4.3 Test endpoints and statistical calculations

Algal growth should be exponential over the test period, with no initial lag phase before exponential growth begins. The growth of a control culture can be described by Equation 7 (Nyholm, 1985). Log (cell density) is plotted versus time and the slope of the regression line (the growth rate, μ) at each test concentration is compared to the growth rate in the control. Growth rates, as a percentage of the control growth rate are then plotted versus initial concentration and the resulting sigmoidal plot is used to calculate the IC_{50}.

$$N = N_0 \cdot e^{\mu_m (t-t_0)} \qquad \text{(eq 7)}$$

where

N is the number of cells (cell density) at time t

N_0 is the number of cells at t (t=0)

μ_m is the maximum specific growth rate characteristic of non limited, non toxicant affected growth in the particular test system.

The calculated IC_{50} for any toxicant assumes that the initial concentration and the growth rate (slope, μ) are constant over time. Relationships that describe adsorptive losses of toxicants over the test duration have not been introduced in the calculation of IC_{50}. Yet, because the growth rate depends on toxicant concentration, a significant effect on IC_{50} might be expected.

2.4.4 Remedial actions

In most bioassays, changes in toxicant concentration occur over the test due to adsorption losses to test containers, to the algae themselves, to degradation, to evaporation or pH changes. Although the impact of these copper losses on toxicity test results has not been investigated, few solutions have been introduced to overcome the problem.

A simple way to minimize adsorptive losses and degradation is to reduce the test duration. Standard protocols for algal toxicity tests now recommend short test duration i.e. 48 to 72 hours (Stauber and Davies, 2000).

Static renewal of test solutions had been considered but it is difficult because concentrating the algal cells by centrifugation each day may lead to reduced growth rates and subsequent failure of the test to meet acceptability criteria (Batley, 1999). Flow through systems, in which cell density is controlled, have been designed (Wong et al., 1983). Whereas these systems can find application in research, high cost and practical difficulties have restricted their use for routine toxicity testing.

Copper losses in solution to algal surfaces can also be reduced by the use of lower initial cell densities. Franklin (2001) suggests that standard bioassay using 10^4 to 10^5 algal cells/mL underestimate copper toxicity. For *Selenastrum capricortum*, the author reported a 72h-IC_{50} of 6.2 µg Cu/L at 10^3 cells/mL initial cell density, compared to a 72h-IC_{50} of 17 µg Cu/L at 10^5 cells/mL initial cell density. A significant decrease in the 72h-IC_{50} of copper was observed as well at an initial cell density of 10^4 cells/mL compared to that of 10^3 cells/mL. The cell density dependence of copper toxicity appeared to be related to adsorptive copper losses in testing solutions over the test period. The decrease in copper concentration for the freshwater alga *Chlorella sp.* after 72 h was markedly lower at low initial cell density than at high initial cell densities (Table 5). A greater surface area for binding at high cell density is likely to be responsible for the depletion of copper in solution.

Table 5. Dissolved Cu in solution in bioassays using *Chlorella sp.* after a 72-h exposure to copper.

Initial cell density (cells/mL)	µg/L		
	Added Cu 0 h	Total measured Cu 0 h	Dissolved Cu 72 h
10^3	10	8.9	6.1
10^4	10	8.2	4.7
10^5	10	7.9	2.3

Except for a reduction in test duration, none of the solutions detailed above have been introduced into routine toxicity tests, partly because it is not known whether copper losses alter the overall sensitivity of these tests. To experimentally examine the effect of copper losses on bioassay results, it was first necessary to develop a technique capable of maintaining copper concentrations, and at the same time sustaining algal growth. Alternatively, it may be possible to model the effect that copper losses (from test solutions) have on the bioassay test results, e.g. IC_{50} values.

CHAPTER 3. AIMS AND OBJECTIVES

The accuracy and relevance of environmental studies depends more and more upon the integration of biology, chemistry and modeling. Thus it is important that results of toxicity tests are acceptable from both a biological and chemical perspective, and the theory should be sufficiently sound that modelling can be undertaken. Whereas biological aspects have generally been satisfactorily addressed, this has not always been so with respect to chemistry. Effort has been put into the preparation of metal standards and test solutions to avoid contamination and losses to the container walls. It has been recognised that over a toxicity test period, concentrations of toxicants in test solutions decrease. However, the issue of whether decreasing concentrations affect the sensitivity of laboratory bioassays has not been adequately addressed.

In conducting a laboratory-based toxicity test on a field-collected sample, the assumption is that the toxicant concentration of the collected sample is representative of the toxicant concentration at the collection site. This assumption also includes the idea that natural ecosystems are exposed to constant contaminant concentrations over a certain period of time. These assumptions suggest that in standard laboratory bioassays, in which toxicant depletion is not controlled over the test period, there may be an underestimation of the toxicity of the toxicant in comparison to that occurring at the site of collection. If, on the other hand, the toxicant concentration to which the test organisms are exposed during the initial stage of a test mostly affects the test outcome, then adsorptive losses of toxicants throughout a test would be less likely to influence the final IC_{50} value.

The aim of this study was to investigate the effect that adsorptive losses of toxicants (in this case copper) over a bioassay period (i.e. concentration decreasing during test) have on algal bioassay response and ultimately the test outcome. The specific objectives were:

- To design a bioassay procedure in which the copper concentrations in testing solutions would stay constant over 72 hours.

- To compare the results obtained from a bioassay following this new procedure with the results obtained from a bioassay following the standard procedure.

- To model the effect that decaying toxicant concentrations have on calculated IC_{50} values.

CHAPTER 4. METHODOLOGY

4.1 Chemicals and reagents

Before use, all materials were soaked overnight in 10% (v/v) nitric acid (AnalaR®, 69%, BDH) and rinsed with de-ionized and high purity water (18 MΩ, Milli-Q). Acid used for acidifying samples was nitric acid (Merck, Tracepur®).

Copper spiking solutions were prepared either by dilution of a Spectrosol 1000 mg/L stock standard solution or by dissolution of a copper salt ($CuSO_4 \cdot 5H_2O$) in high purity water and acidification with nitric acid (Suprapur grade, Merck).

Nitrate and phosphate were added to the test solutions as nutrients, required to maintain exponential growth over the toxicity test period. Two nitrate stock solutions of 2.1 g/L and 21 g/Land two phosphate stock solutions of 0.22 g/L and 2.2 g/L were prepared by dissolving sodium nitrate salt and potassium dihydrogen phosphate salt in Milli-Q water.

4.2 Test organisms

4.2.1 Test species

All toxicity tests were performed using the unicellular alga *Phaeodactylum tricornutum*, obtained from the National Research Centre Istituto di Biofisica, Pisa, Italy. *P. tricornutum* was chosen for the study because it has previously been shown to be sensitive to copper (Franklin et al.,2000), easy to count and does not clump or adsorb to the walls of the test containers.

4.2.2 Stock culture maintenance

P. tricornutum was cultured in a half strength f medium (Guillard and Ryther, 1962). Cultures were maintained axenically on a 12:12 h light: dark cycle (Philips TL 40 W fluorescence daylight, 72 µmol photons.m^{-2}.s^{-1}) at 21°C.

4.3 Standard toxicity test procedure

Copper bioassays usually undertaken at the CSIRO Centre for Advanced Analytical Chemistry follow the standard (flask) protocol described in Stauber et al. (1994), as summarized in Figure 2.

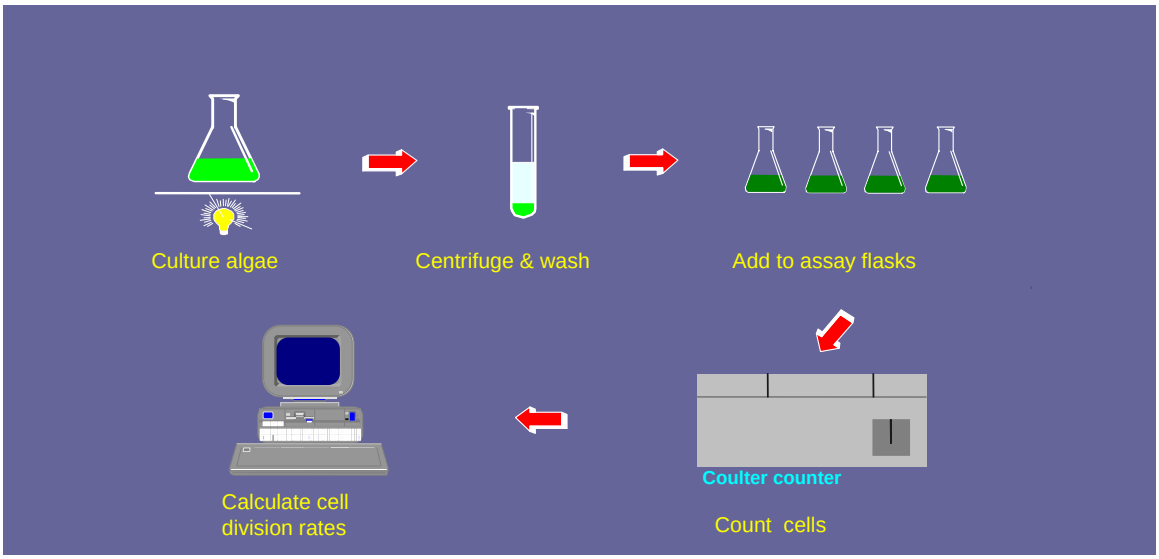

Figure 2. General algal bioassay procedure (Adams, 2000).

4.3.1 Preparation of test inoculum

Exponentially growing cells of *P. tricornutum* from a 4-6 day-old stock culture were used in the bioassays. It is important to use exponentially growing cells to avoid the lag period of growth at the beginning of the test (Stauber et al., 1994). The algal cells were centrifuged in 30 mL glass centrifuge tubes for 7 minutes at 2500 rpm in a

Jouan CR 4.11 centrifuge. The supernatant was decanted and the algal pellet resuspended in about 20 mL of filtered seawater using a vortex mixer. The centrifugation and washing was repeated three times to remove algal culture medium, which may otherwise adsorb or complex metals and reduce the sensitivity of the toxicity test. The final pellet was resuspended in about 15 mL of filtered seawater. Before inoculation, the algal suspension was carefully mixed and homogenized.

4.3.2 Preparation of tests solutions for standard flask bioassays

Controls (no copper) together with five ionic copper concentrations (5-80 µg/L), each in triplicate, were prepared by dispensing 55 mL of filtered seawater into 250 mL glass Erlenmeyer flasks that had been pre-silanized with Coatasil (BDH). Silanisation of the flasks was essential to reduce copper adsorption to the walls of the flasks over the test period. To all flasks, including controls, 0.5 mL of nitrate stock solution and 0.5 mL of phosphate stock solution were added.

Each flask was inoculated with $2\text{-}4\times10^4$ cells.mL^{-1} of a prewashed *P.tricornutum* suspension. The correct inoculum volume was determined in a counting flask, which was then discarded. Flasks were incubated at 21 °C on a 12:12 h light: dark cycle at 140 µmol photons. $m^{-2}.s^{-1}$. At the beginning and end of each experiment, the pH of the test solutions was measured in one of the three replicates, using a Cyberscan 500 pH meter. Subsamples were taken daily from each flask for copper analysis.

4.3.3 Cells counts

Cell density in each flask was determined daily using a Coulter Multisizer II Particle Analyser with 70-µm aperture. Subsamples of cells were taken from each flask, after mixing, homogenized in a tissue grinder to break cell clumps and counted in a plastic counting cup. It was usually necessary to dilute the subsamples taken from the test flasks on day 2 and 3 with seawater prior to counting. Each sample was counted four times and the mean count was then determined. A background count from a test flask containing seawater, nutrients but no algal cells was determined every day. This

coulter blank, arising from small particles other than algal cells in seawater, was subtracted from the mean algal cell count for each flask *(less than* 20%).

4.3.4 Test endpoints and calculations

Growth rate (cell division rate) and cell yield (N, biomass) were calculated to determine how decreasing copper concentrations in test solutions affected these two widely used endpoints.

For growth rate, linear regression analysis was used to fit log cell-density (log N) versus time (t), the regression slope being equivalent to the growth rate per hour (μ). Growth rates per day (doublings per day) were calculated by multiplying μ by 24 x 3.32 (where 3.32 equals ln10/ln2 to convert to cell divisions/day*).* Test results were considered acceptable if the mean growth rate for the controls was in the range 1.5 ± 0.5 doublings per day. The growth rate for each copper concentration was calculated as a percentage of the mean control (no copper) growth rate, from Equation 8.

$$G_c = \mu/\mu_m * 100 \qquad \text{(eq 8)}$$

where

G_c is the percentage of growth of algae in each test-concentration (c) replicate compared with the controls

μ is the growth rate per hour in each test-concentration replicate

μ_m is the mean growth rate for the controls (maximum growth rate)

To calculate cell yield (at time t), the initial cell density was subtracted from the final measured cell density at time t (e.g. 48- or 72-h). Cell yields at each copper concentration were calculated as a percentage of the control from Equation 9.

$$I = \frac{R_c - R}{R_c} \qquad \text{(eq 9)}$$

where

I is the percentage inhibition of algal growth for each test-concentration replicate

R_c is the mean cell yield for the control

R is the cell yield for each test-concentration replicate

The concentration-response curves, in terms of growth rate or cell yield, were then obtained by plotting growth (percentage of control) versus measured initial copper concentration.

4.3.5 Statistical Analysis

Inhibitory concentrations, typically 72h-IC_{50} values and their 95% confidence limits were calculated by linear interpolation using ToxCalc 5.0.23 (Tidepool Software). Measured copper concentrations (at time zero) rather than nominal concentrations were used. Data were tested for normality and homogeneity of variance using the Shapiro Wilk's test. Dunnett's multiple comparison test was then used to determine which concentrations were significantly different to the controls. As Dunnett's test required three replicates, homoscedastic t-test was used when only two replicates were available.

IC values were tested for significant difference using the method described in Sprague and Fogels (1977). Significance levels were tested at p = 0.05 level.

4.4 Tank Toxicity Test Procedure

4.4.1 Principle

To overcome copper losses in bioassay tests over 72-h test duration, a system allowing a continuous replenishment of copper in the testing solutions was designed. Plastic (polystyrene) containers (80 mL) containing the testing solutions (40 mL) were capped with a lid containing a 3-5 µm cellulose nitrate membrane (Millipore or Whatman) of 4.7-cm diameter. These containers were placed in large (39x29x24cm^3) polycarbonate tanks containing an identical concentration of copper (but no cells) to that in the test solutions. The establishment of equilibrium between the testing solutions in the containers and the tank solutions (by diffusion through the membrane) was expected to maintain the copper concentration in the testing solutions

constant over the 72-h test period. Procedures for the preparation of algal test inoculum, cell counts and statistical analysis were the same as that used for the standard (flask) bioassay. The tank bioassay procedure is summarized in Figure 3. The tank bioassays were carried out at the same time as the standard bioassay, matching the algal inoculum and light/temperature conditions as close as possible to the standard conditions.

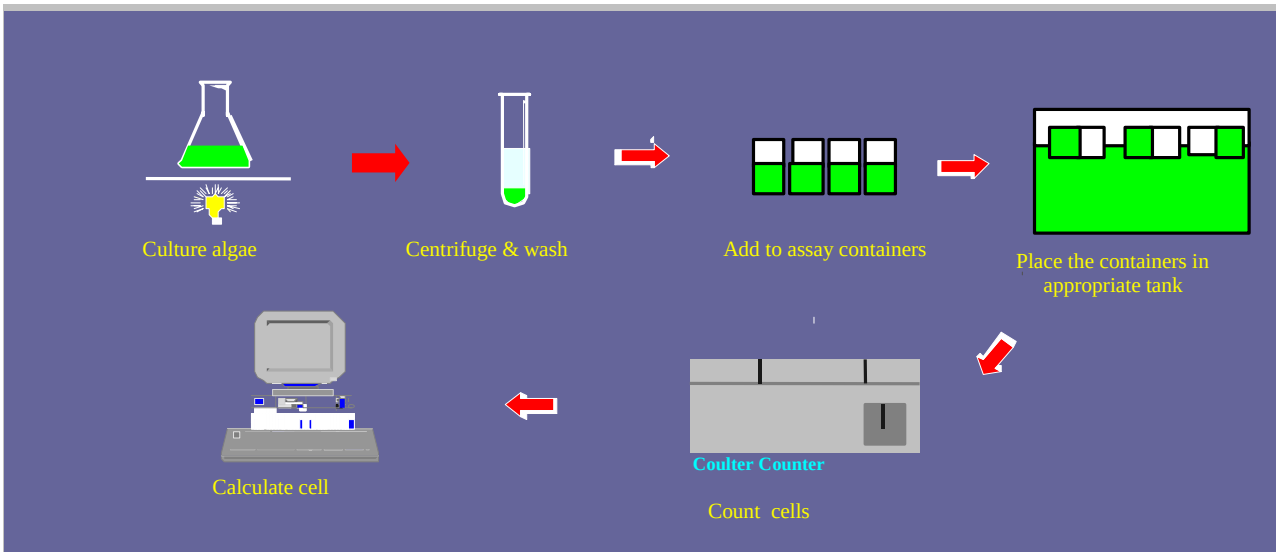

Figure 3. Tank Bioassay Procedure.

4.4.2 Choice of containers and tanks

Polystyrene containers (80 mL) were chosen as test containers. In environmental studies involving copper, plastic materials such as polyethylene, PTFE, polycarbonate, polystyrene and acrylic are better than glass in terms of metal adsorptive losses (Batley and Gardner, 1977). The substitution of plastic in place of glass containers has been demonstrated to reduce copper losses to the container (Davies et al., 1998). The polystyrene containers chosen did not reduce light penetration.

Polycarbonate tanks (39x29x24 cm^3) were used. This material slightly reduced light penetration.

4.4.3 Membranes

Cellulose membrane filters of 47 mm diameter and a pore size of 5 μm (Whatman) or 3 μm (Millipore) were used.

4.4.4 Preparation of tank solutions

To all tanks, including the control, 23 L of clean seawater was added together with 23 mL of 21 g/L $NaNO_3$ solution and 23 mL of 2.2 g/L KH_2PO_4 solution. Copper was added to the respective tanks using a 1000 mg/L standard solution (Spectrosol) to give concentrations ranging from 0 to 50 μg/L.

4.4.5 Preparation of tank bioassay containers and test solutions

Nine to twelve replicate containers were carefully placed in the each of the respective tanks. In preliminary experiments, an equal number of replicates were identically prepared for a bioassay undertaken in normal incubation conditions (i.e. outside the tanks). Replicates (and membranes) used for the tank tests were conditioned by firstly dispensing 41.2 mg (40 mL) of the respective test solutions from the appropriate tank into the polystyrene containers and allowing them to stand overnight with the membrane in the test water. Prior to commencing the tests, containers were capped with the membrane-containing lid.

Each container was inoculated with 2-4x10^4 cells.mL^{-1} of a prewashed *P.tricornutum* suspension. The correct inoculum volume was determined in a counting container, which was then discarded. Containers were incubated in the tanks at 21° C on a 12:12 h light: dark cycle at 124 μmol. $m^{-2}.s^{-1}$.

Two or three replicates from each tank were removed daily for cell counts, subsampled for copper analysis and then discarded. Volumes in the test containers

were measured once at the end of a bioassay and were found to be within 1% of the initial volume. It was thus not necessary to correct the counts for volume changes. At the beginning and end of each experiment, the pH of the test solutions was measured in one of the replicates from each tank, using a Cyberscan 500 pH meter calibrated against standard NBS buffers.

4.5 Total Copper Analysis

4.5.1 Subsampling

Subsamples were taken for total copper analysis at various times throughout the test period, typically 0, 24, 48 and 72 h. Subsamples at day zero were taken after inoculating the algae, unless otherwise indicated. Subsamples were taken using acid-washed 10 mL syringes and filtered into acid-washed polyethylene vials using acid-washed 0.45 μm membrane filters (Minisart®, Sartorius). They were immediately acidified with HNO_3 to 1% (v/v).

4.5.2 Inductively Coupled Plasma Atomic Emission Spectrometry Measurements

Dissolved copper analyses were carried out using a Spectroflame EOP ICP-AES, using the Spectro System Software, Smart Analyser Version 2.10. Lutenium was used to monitor argon flow rates and the wavelength of the copper line was 324.754 nm. Operating conditions are summarised in Table 6.

Table 6. ICP-AES operating conditions

Power settings	1.200 W
Auxiliary gas flow	30 units on instruments flow meter
Cool gas flow	25 units on instruments flow meter
Nebulizer pressure	~ 45 psi
Integration time	5 s
Solution flush time	20 s
Number of integrations taken for each reading	3
Background correction points	SIM 1-900 steps left

The instrument was calibrated using a multi-element standard AES-01 containing the following in 2% nitric acid:

- 0.5 mg/L Ag
- 2 mg/L Al, As,B,Ba,Ca,Cd,Co,Cr,Cu,Fe,Hg,Li,Mg,Mn,Ni,Pb,Se,Sn,Sr,Tl,Ti,V,Zn
- 10 mg/L K,P,SiO_2 (=4.67 mg/L Si).

Seawater standards (0, 5, 10, 15, 20, 30, and 50 µg/L) were prepared in unfiltered seawater from a 5 mg/L standard solution (Spectrosol) and concentrations of copper in samples were calculated using a calibration curve constructed from these standards. In this way the copper concentrations obtained were corrected for signal suppression caused by the high sodium concentrations of the seawater matrix.

4.5.3 Anodic Stripping Voltammetry Measurements

Problems were encountered measuring copper concentrations lower than 10 µg/L by ICP-AES, due to the detection limit of 3 µg/L and related noise. For accurate results, samples with dissolved copper concentrations of 15 µg/L or less were also analyzed by differential pulse ASV. To minimize contamination, all ASV analyses were carried out in a Class-100 clean room.

ASV measurements were performed using a 746 Trace Analyser (Metrohm) attached to a 747 VA stand (Metrohm). Measurements were made in PTFE cells with a three-electrode system including a hanging mercury drop electrode (HMDE), a Ag/AgCl reference system and a Pt auxiliary electrode. Instrumental parameters for the ASV measurements are summarized in Table 7. Prior to use, the PTFE cells were soaked in 10% HNO_3 (v/v) overnight and rinsed with Milli-Q water. Before sample analysis, the electrodes were decontaminated twice by immersing them in 5 % high purity acid (HNO_3) and rinsing twice with Milli-Q water. A blank was then run until a peak area of less than 362 pW was obtained.

Measurements were performed on 9 mL samples. Standard additions of copper to an acidified (1%) seawater sample using a 5 mg Cu/L stock solution, were used to obtain a calibration curve. For quality assurance, duplicate measurements of a quality control

standard (10 μg Cu/L in 0.2% HNO_3) were performed using the instrumental parameters outlined above, except that a deposition time of 300 s was used.

Table 7. ASV operating conditions

Deposition potential	- 600 mV
Sweep rate	3.333 mV/s
Pulse amplitude	50 mV
Pulse period	0.60 s
Voltage step	2 mV
Deposition Time	180 s
Quiescent time	20 s
Stirrer speed	1600 rpm
Purging time	300 s

4.6 Mathematical Model

A mathematical model was developed to calculate the effect that decreasing toxicant concentrations should have on cell growth and cell yield. The key component of the problem associated with decreasing toxicant concentrations over time was the observation that the specific growth rate, μ, itself changes over time through the dependency of this parameter on toxicant concentration. To develop the model, it was assumed that:

a) the decline in concentration of the toxicant over 72 h could be described by (i) an initial drop in concentration ($C_t = C_0 - C_0 \cdot D_1$), (ii) a linear decrease in concentration ($C_t = C_0 - C_0 \cdot D_2 \cdot t/72$), (iii) an exponential decrease in concentration ($C_t = C_0 \cdot e^{(-D3 \cdot t)}$) or an exponential decrease in concentration which slows with time ($C_t = C_0 - C_0 \cdot D_4 \cdot (1-e^{(-t/72)})$), or (iv) any combination of these functions

For these functions:

C_0 is the initial concentration of toxicant (t=0)

C concentration of toxicant at time t

D_1, D_2, D_3, D_4 are constants which were used to alter the extent of the concentration drop or decline (with time).

b) the cell numbers vary exponentially with time following the equation :

$$N_t = N_{t'} \cdot e^{\mu_t (t-t')} \quad \text{(eq.10)}$$

where

N : Number of cells at any given time t

$N_{t'}$: Number of cells at t' (t'<t)

μ_t : growth rate at time t (varying due to changing concentration)

c) the relationship between growth rate μ_t and the toxicant concentration at time t, is described by a sigmoidal function:

$$\mu_t = \beta_0 - [K / (1+\alpha e^{(-\beta 1 Ct)})] \quad \text{(eq.11)}$$

where

β_0 and K were constants chosen (Table 8) so that the growth rate and final biomass calculated for control experiments (zero toxicant concentrations) resembled that of the actual bioassay experiments

β_1 and α were constants chosen so that the effect of toxicant concentration (C_t) resembled that observed for the actual bioassay experiments.

Table 8. Parameters used in calculations.

β_0	K	μ_m	α	β_1
0.07	0.15	0.040	4	0.025

The model could be applied to any toxicants. This study considered copper only. 72h-IC_{50} and 48-h IC_{50} values were calculated with ToxCalc 5.0.23 using the growth rate and cell yield derived from the models.

CHAPTER 5. RESULTS AND DISCUSSION

5.1 Preliminary standard flask bioassay

A preliminary bioassay using the standard protocol was undertaken to estimate copper losses in a 72-h bioassay using *P. tricornutum*. The test system included a control together with 5 copper concentrations (5,10, 20, 40 and 80 µg/L). The mean growth rate in the control was 1.2 doublings/day, meeting the test acceptability criteria. The concentration–response relationship for *P. tricornutum* exposed to Cu for 72 h is shown in Figure 4. The 72 h IC_{50} was 14 µg/L (95 % confidence limits of 12-15 µg/L).

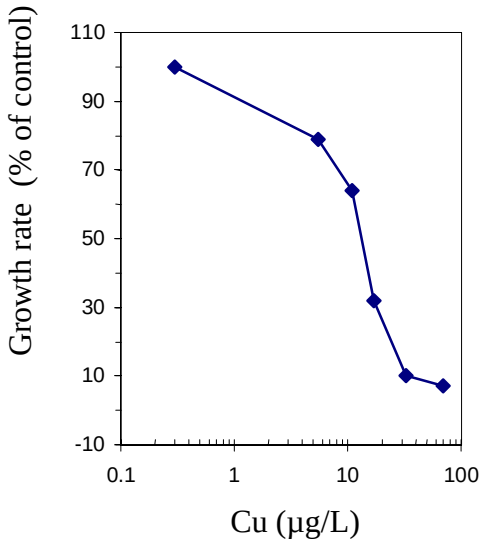

Figure 4. Growth rate inhibition of *Phaeodactylum tricornutum* exposed to copper for 72 h.

Copper concentrations measured in test solutions over the test period are given in Table 9. Copper losses after 72 h were in the range of 24-36 % of the concentration measured at day 0. Most of the decrease occurred within the first 48 h, except at the highest copper concentration.

Table 9. Copper losses in 72-h bioassay using *P. tricornutum*[a].

Initial cell density ($x10^4$cells/L)	Measured Cu µg.L^{-1}			
	0 h	24 h	48 h	72 h
3.7	5	4	4	4
3.7	11	9	7	7
3.2	16	14	11	11
3.7	17	13	12	13
3.7	33	27	25	24
3.2	43	39	36	31

[a] ICP-AES measurements

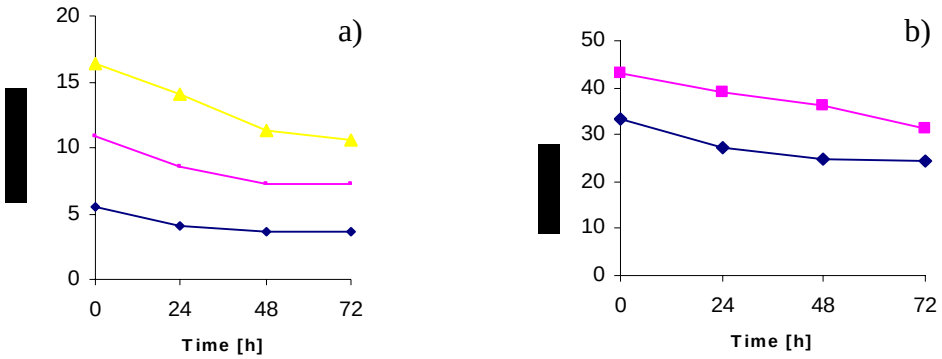

Figure 5. Typical decrease in copper concentration over time. a): Nominal concentrations of 5, 10 and 20 µg/L; b): Nominal concentrations of 40 and 80 µg/L.

5.2 Modelling the Effect of Copper on Algal Growth

Using the mathematical model, algal growth over 72 h was calculated and used to predict the effect of declining copper concentrations on the IC_{50} values. Four different scenarios of concentration decline (over 72 h) are illustrated in Figure 6-9. Figure 6 represents the ideal scenario of a constant copper concentration, whereas the others figures represent (i) a linear decrease of 30 %, (ii) an exponential decrease of about 30% (slowing with time), (iii) an exponential decrease of about 50% (slowing with time) and (iv) an initial drop of 15 % followed by an exponential decrease of 20 % (slowing with time). Plots of (I) cell density (N) versus time (t), (II) the logarithm of cell density ($\log_{10}$ N) versus time (t), and (III) growth rate (% of control) versus copper concentration at time zero are given for each scenario (Figure 6-I,II,III; Figure7,I,II,III; Figure8-I,II,III and Figure9-I,II,III)

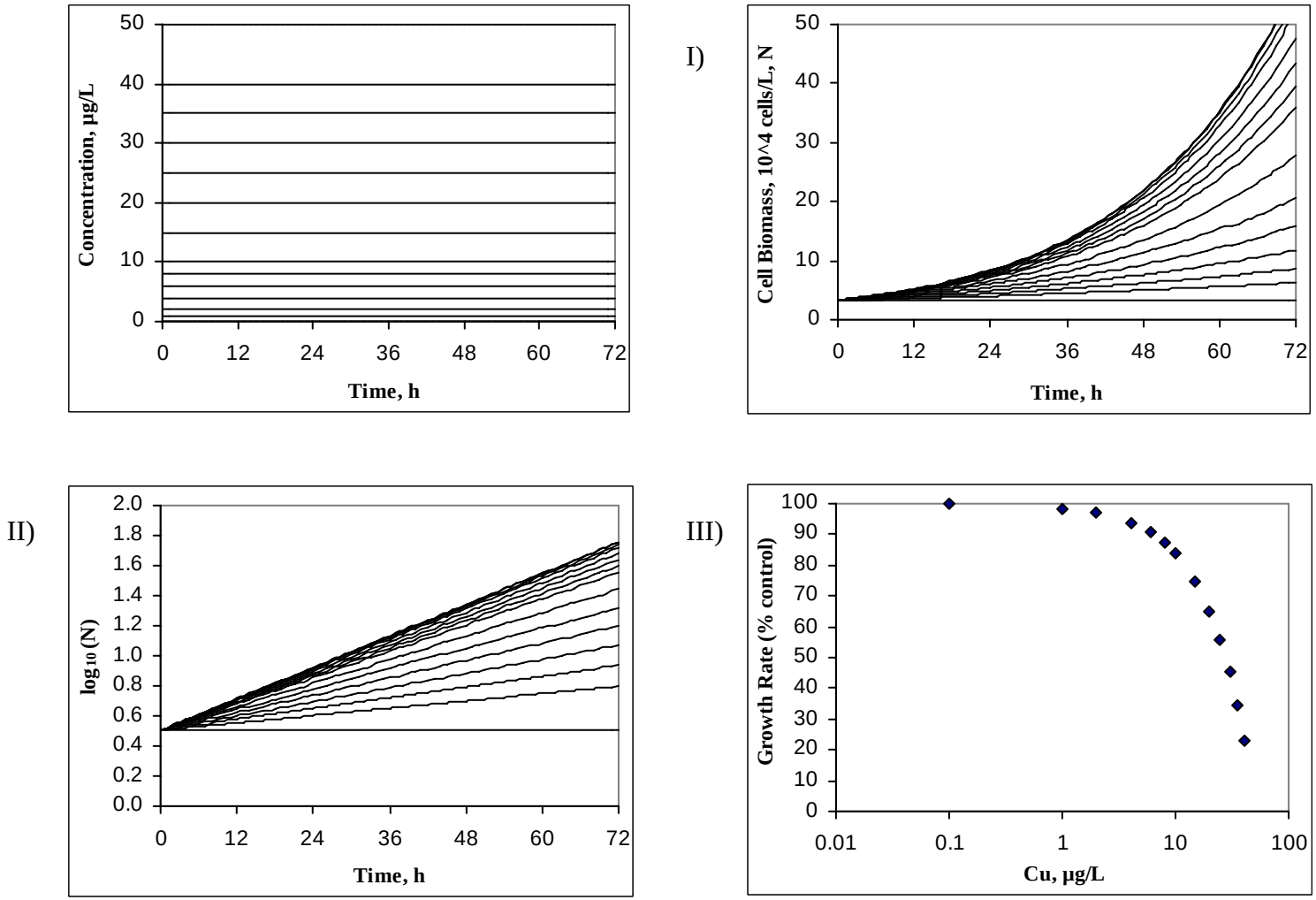

Figure 6. Ideal case of copper concentration constant. I): cell density versus time, II): the logarithm of cell density (log_{10} N) versus time and III): growth rate (% of control) versus copper concentration at time zero.

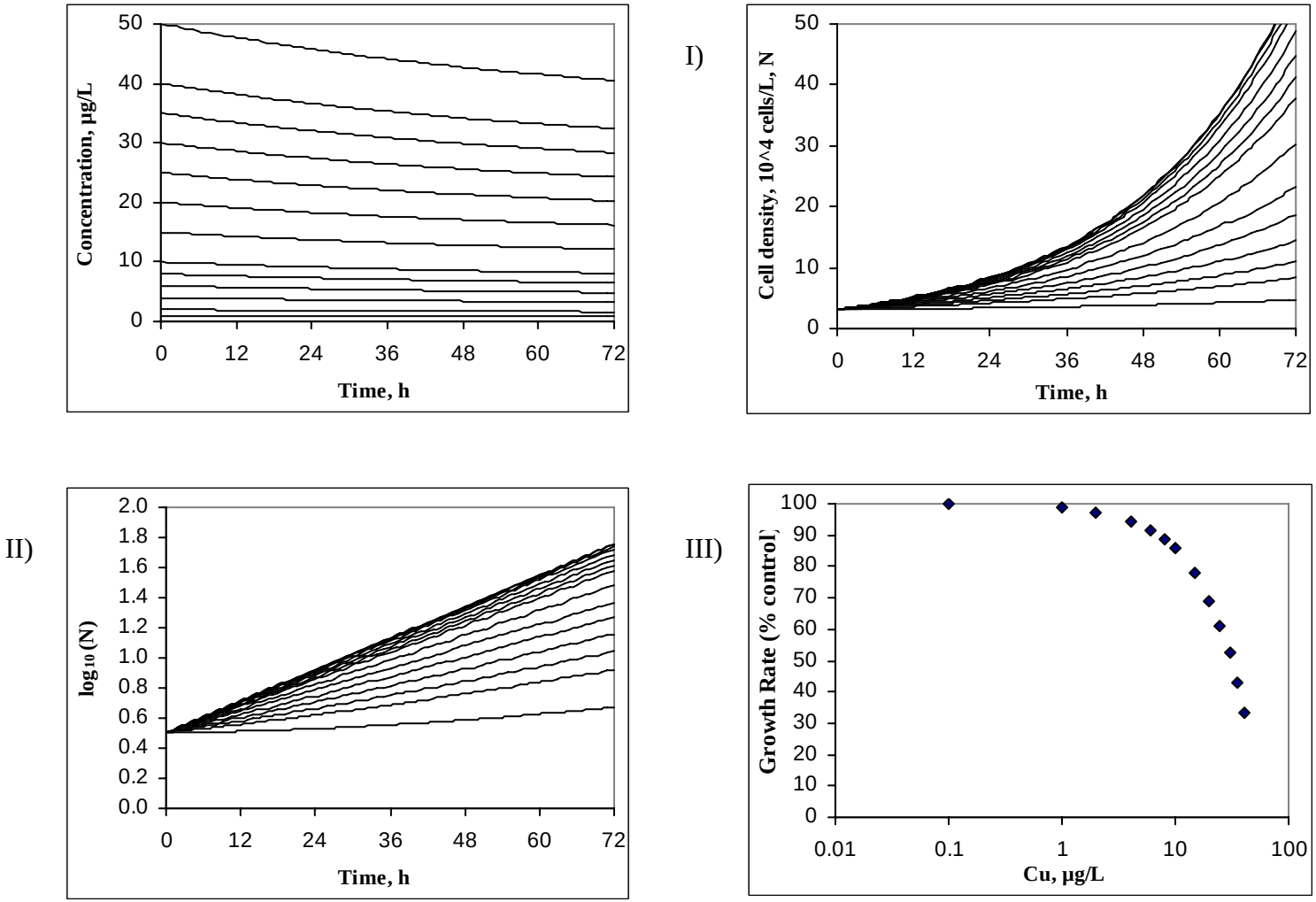

Figure 7. 30% exponential decrease I): cell density versus time, II): the logarithm of cell density (log_{10} N) versus time and III): growth rate (% of control) versus copper concentration at time zero.

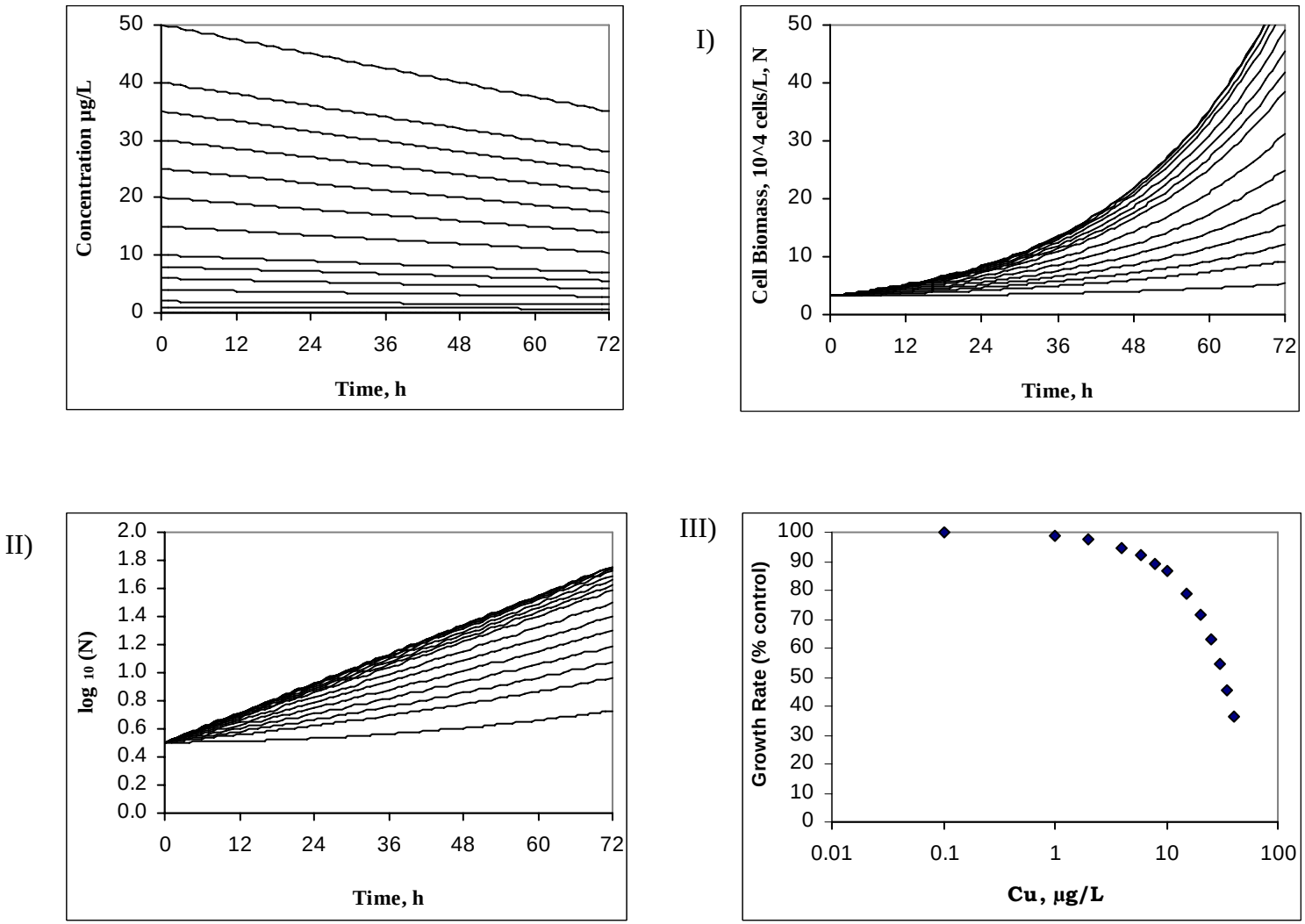

Figure 8. 30% linear decrease I): cell density versus time, II): the logarithm of cell density (log_{10} N) versus time and III): growth rate (% of control) versus copper concentration at time zero.

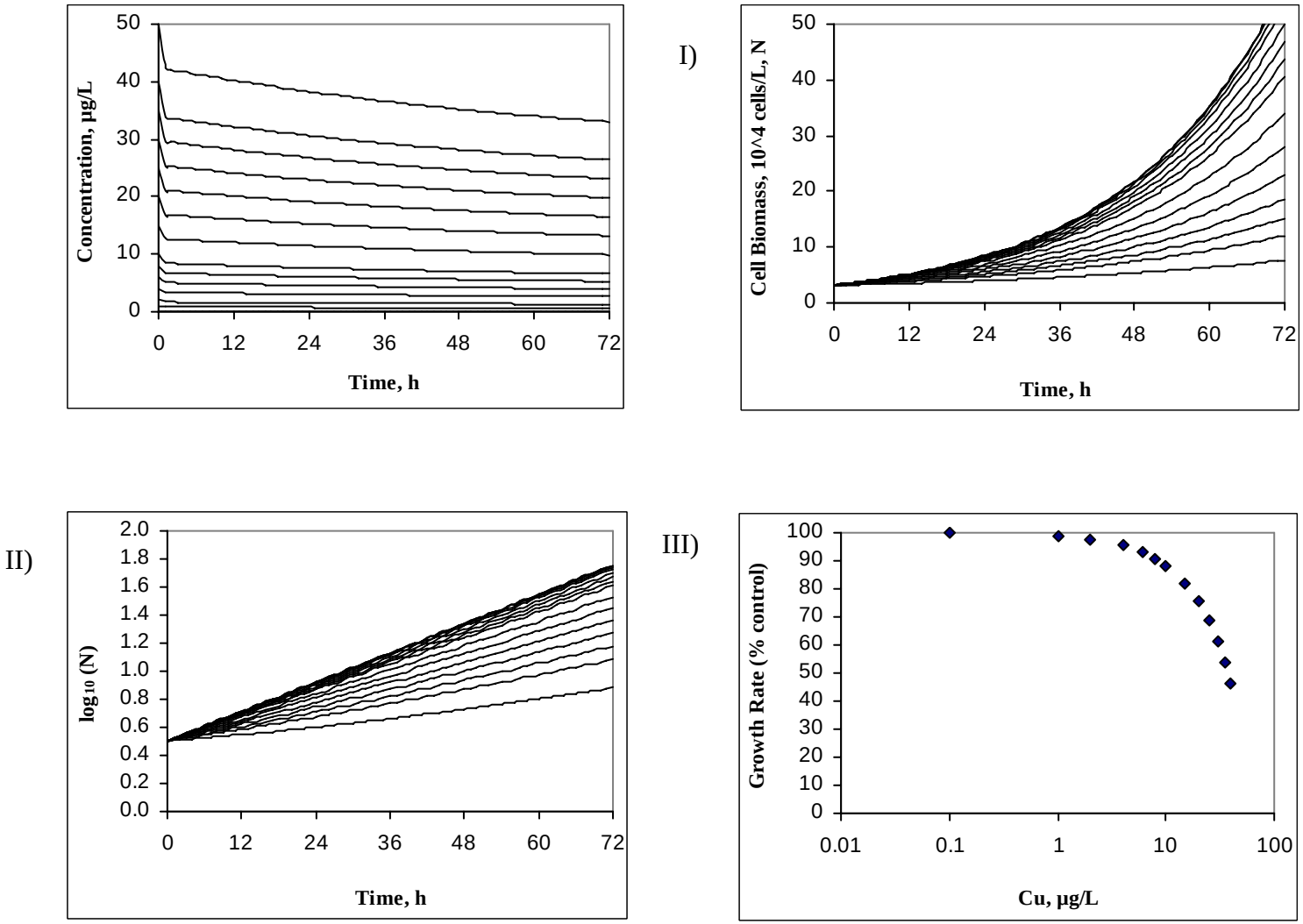

Figure 9. An initial drop of 15 % followed by an exponential decrease of 20 % I): cell density versus time, II): the logarithm of cell density (log_{10} N) versus time and III): growth rate (% of control) versus copper concentration at time zero.

Growth inhibition always increased as the concentration of toxicant increased (Figure 10). With different scenarios of concentration decline, the pattern of growth inhibition changed. Small variations were predicted between growth inhibition at low nominal copper concentrations, with a large difference with increasing copper concentrations. Growth inhibition was greatest when the copper concentration was constant over the 72-h period.

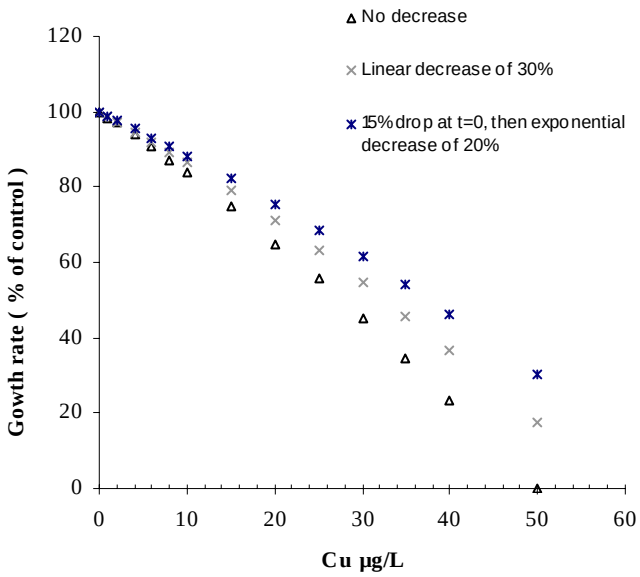

Figure 10. Growth rate inhibition after 72 h exposure for different scenarios.

IC_{50} values (48-h and 72-h), calculated using growth rates and cell yields derived from the model, are shown in Table 10. Copper toxicity after a 72-h exposure decreased with increasing copper losses, as expected. The lowest 72-h $IC_{50,}$ (i.e. greatest toxicity), both for growth rate and cell yield was predicted when the copper concentration was constant over 72 h. The differences between 72-h IC_{50} values were more pronounced for growth rate than for cell yield. For the scenario where the copper decreased exponentially by 30 % over 72 h, the IC_{50} calculated from growth rate increased by 5 µg/L. An exponential decrease in copper concentration of 50 % led to an increase of 11 µg/L. Similar results were obtained for 48-h IC_{50} values. In general, 48-h and 72-h toxicity estimates were very similar. Indeed, for the ideal scenario of no copper decline, the 48-h IC_{50} and 72-h IC_{50} values calculated from growth rate were predicted to be the same.

Table 10. 72 h- IC_{50} calculated from the growth rate obtained by the model, with β_0 = 0.07, K=0.15, α= 4, and β_1=0.025 and μ_c= 1.4 doublings per day.

Type of Concentration Decline	48-h IC_{50} (µg/L)		72-h IC_{50} (µg/L)	
	Growth Rate	Cell Yield	Growth Rate	Cell Yield
None (concentration constant)	28	17	28	14
Linear (30 % over 72 h)	31	19	33	16
Exponential (30% over 72 h)	30	19	31	15
Exponential (50% over 72 h)	32	20	39	19
50% drop at t =0, then exponential (15% over 72 h)	>50	36	43	21
15% drop at t =0, then exponential 20% (over 72 h)	36	22	38	18
15% drop at t =0, then exponential 40% (over 72 h)	42	26	42	20

5.3 Tank Bioassays

5.3.1 Chemistry

To achieve constant copper concentrations in tank bioassays, it was necessary to first test several membrane pore sizes for the interface between the vials and the tank (through which the equilibrium should be maintained). Because algal cells may be as small as 5 µm, there was an upper limit to the membrane sizes that could be tested. Three tank bioassays were undertaken involving a control and a single copper concentration (16 or 11 µg/L) each time. Different membrane pore sizes were tested and for comparison, duplicate experiments, incubated under standard conditions (i.e. not in the tank), were simultaneously run. Mean growth rates of the control were 1.6±0.1 doublings per day, which confirmed that experimental conditions produced acceptable algal growth over 72 h.

Measured copper concentrations in the test solutions over 72 h are summarized in Table 11. On day 0, dissolved copper concentrations were performed before adding

the algal innoculum. Copper concentrations in tank bulk solutions stayed constant over 72 h. At a copper concentration of 16 µg/L, copper concentrations in test solutions after 72 h decreased by 20% compared to the initial measured concentrations. Increasing the pore size did not prevent copper decreases in the test solutions. At a concentration of 11 µg/L, copper decreased in the test solutions by only 6% after 72 h.

Table 11. Dissolved copper measurements over 72 h in the tank bioassays.

Membrane pore size (µm)	Measured Cu (µg/L) [a]			
	0 h	24 h	48 h	72 h
1.5	16±0.3	15	14	13
3	16±0.4	13	13	13
3	11±0.1	11	10	10

[a] ICP measurements

Decreases in copper concentrations in test solutions incubated in plastic containers in the tanks are compared in Table 12 to those in test solutions incubated in standard conditions outside the tanks. Decreases in copper concentrations in the tank test solutions were much less than decreases in the test solutions outside the tank, particularly after 72 h.

Table 12. Comparison of dissolved copper in test solutions between bioassays in and out of tank.

Incubation in tank			Incubation out of tank		
Measured Copper 0 h (µg/L)	Decrease after 48 h	Decrease after 72 h	Measured Copper 0 h (µg/L)	Decrease after 48 h	Decrease after 72 h
16±0.3	13 %	19 %	17±0.6	18 %	41 %
15±0.4	7 %	13 %	16±0.1	33 %	33 %
11±0.1	0 %	9 %	13±2.3	23 %	31 %

5.3.2 Tank Bioassay Versus Standard Bioassay.

A tank bioassay (TB1), using five copper concentrations (0, 5, 10, 15, 20 and 40 µg/L) and three pseudoreplicates in each tank was undertaken together with a bioassay following the flask standard protocol (SB1). A tank bioassay (TB2), using

four copper concentrations (0, 5, 10, 20 and 40 µg/L) and two replicates was previously carried out together with a bioassay following the standard protocol. Because growth rate in this standard bioassay did not match the required criteria (>1 doublings per day), the results of both tank bioassays were compared to the acceptable standard flask bioassay (SB1) only.

5.3.2.1 Chemistry

Measured copper concentrations in test solutions SB1 and TB1 are shown in Table 13. It was not possible to completely prevent copper losses in the tank bioassays. Decreases in copper concentrations occurred both in SB1 and in TB1 and after 48 h were of similar magnitude, although considering each nominal concentration, the decrease was lower in TB1 than in SB1. After 72 h, copper losses were in the range of 34-85% for SB1 and 14-66% for TB1 (Table 14). At each initial copper concentration, copper losses were lower for the tank than for the standard bioassay. Similarly, copper losses for TB2 were 0-33%, based on ICP-AES measurements only (Table 13).

Table 13. Measured copper in testing solutions for TB2 by ICP-AES.

Time		**Measured Cu (µg/L)**				
Nominal [Cu]		0	5	10	20	40
0 h		2	6 ± 0.0	10 ± 0.4	17 ± 0.0	36 ± 0.3
24 h		1	5 ± 0.6	10 ± 0.2	17 ± 1.0	31 ± 0.6
48 h		2	5 ± 0.3	9 ± 0.3	18 ± 0.5	33 ± 0.7
72 h		1	4 ± 0.2	7 ± 1.0	17 ± 0.5	33 ± 0.9
Decrease %	**48 h**	**-**	**17**	**10**	**0**	**8**
	72 h		**33**	**30**	**0**	**8**

Table 14. Measured copper for SB1 and TB1 by ASV, unless specified.

Time	Measured copper µg/L											
	Standard Flask Bioassay 1						Tank Bioassay 1					
Nominal concentraion	0	5	10	15	20	40	0	5	10	15	20	40
0 h	0 ±0.0	6 ±0.1	10 ±0.1	16 ±0.2	17 ±0.8*	35 ±0.7*	1 ±0.5	6 ±0.1	9 ±0.3	13 ±1	19 ±0.1[a]	35 ±0.5[a]
24 h	0 ±0.0	4 ±0.1	10 ±0.4	15 ±0.2	13 ±0.4*	29 ±0.9*	1 ±0.5	2 ±0.1	7 ±0.3	9 ±0.7	17 ±0.3[a]	33 ±0.4[a]
48 h	0 ±0.0	3 ±0.1	9 ±0.3	9 ±0.5	11 ±0.3*	25 ±0.5*	1 ±0.5	2 ±0.1	7 ±0.4	9 ±0.5	16 ±0.3[a]	32 ±0.6[a]
72 h	0 ±0.0	1 ±0.2	5 ±0.2	6 ±0.3	10 ±0.3*	23 ±0.9*	0 ±1	2 ±0.2	8 ±0.5	7 ±0.1	14 ±0.5[a]	30 ±0.2[a]
Decrease % 48h	**-**	**50**	**10**	**44**	**31**	**29**	**-**	**66**	**22**	**31**	**16**	**8**
72h		**85**	**50**	**62**	**37**	**34**		**66**	**11**	**46**	**26**	**14**

[a] ICP-AES measurements

5.3.2.2 Effect of copper on Phaeodactylum tricornutum growth rate

Algal growth rates were higher under standard bioassay conditions than under tank bioassay conditions, probably due to different light and temperature conditions. The mean growth rate of the controls after 72 h were 1.61 ± 0.03 doublings per day in SB1, 1.45 ± 0.04 doublings per day in TB1 and 1.37 ± 0.09 doublings per day in TB2. The mean growth rate of the controls after 48 h, were 1.52 ± 0.01 doublings per day in SB1, 1.28 ± 0.02 doublings per day in TB1 and 1.05 ± 0.05 doublings per day in TB2. In TB2 the growth rate after 48 h was at the lower limit of the acceptable growth rate, which indicated a lag phase.

The concentration-response relationships for *P.tricornutum* exposed to Cu for 48 h and 72 h are shown in Figure 11. In each case, growth rate decreased as the concentration of copper increased. LOEC and IC_{50} values calculated from growth rates are summarized in Table 15. The standard bioassay gave a 48-h IC_{50} of 12 µg/L, which was significantly lower than the 72-h IC_{50} of 15 µg. L^{-1}. The LOEC was 6 µg. L^{-1} for both 48-h and 72-h exposures. For the tank bioassay 1, the 48-h IC_{50} for cell yield (9 µg/L) was not significantly different from the 72-h IC_{50} (10 µg/L). The LOEC (5.6 µg/L) was similar to the standard bioassay. Because only two replicates were used in TB2, the normality of the data could not be confirmed. The 48-h IC_{50} of 12 µg/L was not statistically different from the 72-h IC_{50} of 7 µg/L, largely due to the wide 95 % confidence limits.

Based on 48-h IC_{50} values, there was no change in toxicity between the standard bioassay and the tank bioassays. This was to be expected, as copper losses after 48 h were in similar range.

After 72-h exposure, there was also no statistically difference between SB1 and TB2. However for TB1, there was a significant increase in toxicity, i.e. a lower IC_{50} (10 µg/L) than for SB1 (15 µg/L). This is in agreement with the copper losses over 72 h, which were much larger for SB1.

Table 15. Growth rate endpoint for *Phaeodactylum tricornutum* exposed to copper for 48 h and 72 h.

Test Endpoints	Standard bioassay 1 (SB1)	Tank Bioassay 1 (TB1)	Tank Bioassay 2 (TB2)
48h			
Mean growth rate (Doublings/ day)	1.52 ± 0.01	1.28 ± 0.02	1.05 ± 0.05
IC_{50} (95% CI)	11.8 (11.0-12.6)	9.1 (8.5-12.1)	11.6 (10.3-13.0)
LOEC	6.4	5.6	-[a]
72 h			
Meangrowthrate(Doublings/day)	1.61 ± 0.03	1.45 ± 0.04	1.37 ± 0.09
IC_{50} (95% Confidence limit)	14.9(13.6-16.2)	10.1 (7.5-13.4)	5.6 (4.4-26.0)
LOEC	6.4	5.6	-[a]

[a] not calculated: too few replicates

5.3.2.3 Effect of copper on Phaeodactylum tricornutum cell yield

Algal cell yield followed the same trend as growth rate, being larger under standard bioassay conditions. The mean control cell yield after 48 h was 22 ± 0 in SB1, 15 ± 1 in TB1 and 9 ±1 in TB2. The mean control cell yield after 72 h was 77 ± 5 in SB1, 56 ± 4 in TB1 and 46 ± 11 in TB2.

The concentration-cell yield curves are shown in Figure 12. LOEC and IC_{50} values calculated from cell yield are given in Table 16. In the standard bioassay, both the 48-h IC_{50} and 72-h IC_{50} values were 7.8 µg/L. TB1 gave a 48-h IC_{50} of 6.4 µg/L and a 72-h IC_{50} of 4.5 µg/L, which were significantly different despite overlapping confidence limits. TB2 gave a 48-h IC_{50} of 5 µg/L and a 72-h IC_{50} of 3.5 µg/L, and these values were not significantly different.

Based on both 72-h IC_{50} and 48-h IC_{50} values, the toxicity of copper to P.*tricornutum* was significantly greater in TB1 and TB2 than in SB1. Decreases in the IC_{50} values were more pronounced after a 72-h than after a 48-h exposure to copper.

Table 16. Cell yield endpoint for *Phaeodactylum tricornutum* exposed to copper for 48 h and 72h.

Test Endpoints	Standard bioassay1 (SB1)	Tank Bioassay 1 (TB1)	Tank Bioassay 2 (TB2)
48h			
Mean control yield(x10^4 cells/L)	22±0.4	15±0.7	9±0.8
IC_{50} (95% CI)	7.8 (6.9-8.4)	6.4 (5.3-7.3)	4.7 (3.8-5.8)
LOEC	6.4	5.6	- [a]
72 h			
Mean control yield (x10^4 cells/L)	77±5	156±4	46±11
IC_{50} (95% CI)	7.8 (7.1-8.5)	4.5 (3.8-5.4)	3.5 (2.7-4.7)
LOEC	6.4	5.6	- [a]

[a] not calculated: too few replicates

5.3.2.4 Alternative statistical endpoints.

From Figure 11, differences in growth rate inhibition between the standard bioassay and the tank bioassays were more evident at low copper concentrations and after a 72-h exposure. Additional inhibitory concentrations, IC_{25} (IC to give 25 % effect) and IC_{10} (IC to give 10 % effect) were calculated to see if these differences could be quantified (Table 17 and Table 18). Calculated values from the tank bioassays were tested for significant difference with corresponding values in the standard bioassay, and the results are summarized in Table 19.

After a 48-h exposure, IC_{25} values based on growth rate were significantly lower for the tank bioassays than the standard bioassay, whereas IC_{25} values, based on cell yield, were not. No general pattern was found for 48-h IC_{10}. After a 72-h exposure, IC_{25} and IC_{10} values based on growth rate and cell yield were all significantly lower in the tank bioassays than in the standard bioassays.

Table 17. Endpoints calculated from growth rate.

µg Cu/L				
Endpoint	Time	Standard bioassay	Tank Bioassay 1	Tank Bioassay 2
IC_{50}	48	12	9	12
IC_{25}	48	7	6	3
IC_{10}	48	3	2	1
IC_{50}	72	15	10	6
IC_{25}	72	8	5	3
IC_{10}	72	4	2	1

Table 18. Endpoints calculated from cell yield.

µg Cu/L				
Endpoint	Time	Standard bioassay 1	Tank Bioassay 1	Tank Bioassay 2
IC_{50}	48	8	6	4
IC_{25}	48	4	3	2
IC_{10}	48	2	1	1
IC_{50}	72	8	5	5
IC_{25}	72	4	2	2
IC_{10}	72	2	1	1

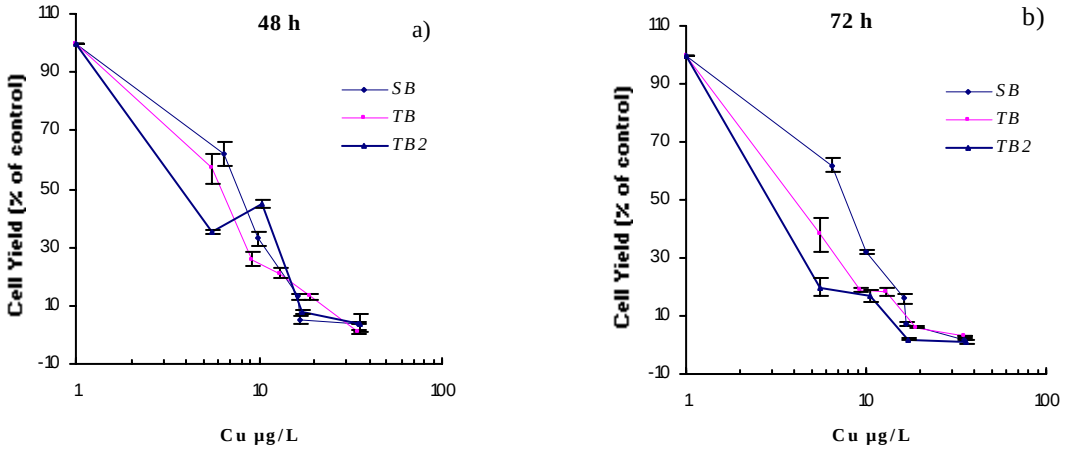

Figure 11. Growth rate inhibition on *Phaeodactylum tricornutum* exposed to copper for a) 48 h and b) 72 h. Data points represent the mean value ± standard deviation, from at least two replicates.

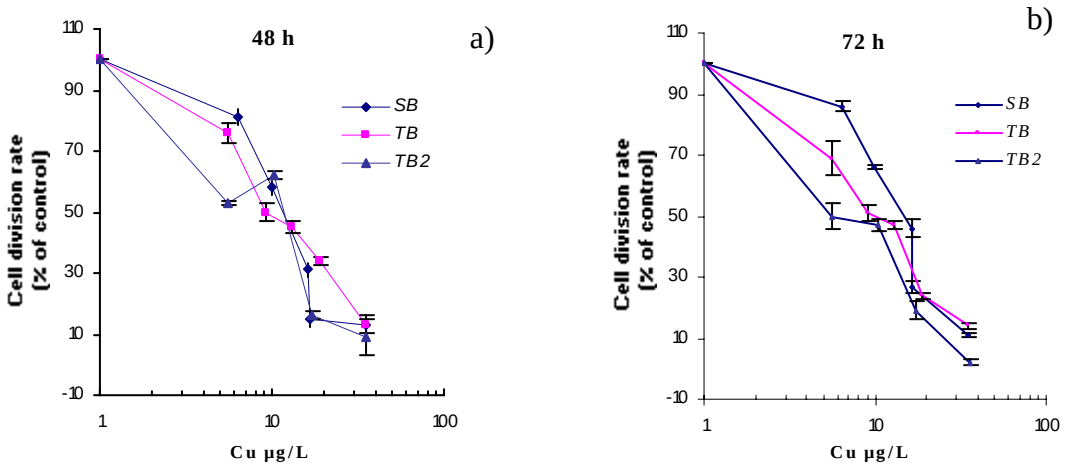

Figure 12. Cell yield inhibition on *Phaeodactylum tricornutum* exposed to copper for a) 48 h and b) 72 h. Data points represent the mean value ± standard deviation, from at least two replicates.

Table 19. Significant difference tests for alternative endpoints (SIG= Significant difference NOT SIG = No significant difference).

		GROWTH RATE Standard Bioassay 1					
		48-h IC_{50}	72-h IC_{50}	48-h IC_{25}	72-h IC_{25}	48-h IC_{10}	72-h IC_{10}
GROWTH RATE Tank Bioassay 1	48-h IC_{50}	**NOT SIG**	.	.	.	.	.
	72-h IC_{50}	.	**SIG**	.	.	.	.
	48-h IC_{25}	.	.	**SIG**	.	.	.
	72-h IC_{25}	.	.	.	**SIG**	.	.
	48-h IC_{10}	.	.	.	.	**NOT SIG**	.
	72-h IC_{10}	.	.	.	.	.	**SIG**

		GROWTH RATE Standard Bioassay 2					
		48-h IC_{50}	72-h IC_{50}	48-h IC_{25}	72-h IC_{25}	48-h IC_{10}	72-h IC_{10}
GROWTH RATE Tank Bioassay 2	48-h IC_{50}	**NOT SIG**	.	.	.	.	.
	72-h IC_{50}	.	**NOT SIG**	.	.	.	.
	48-h IC_{25}	.	.	**SIG**	.	.	.
	72-h IC_{25}	.	.	.	**SIG**	.	.
	48-h IC_{10}	.	.	.	.	**SIG**	.
	72-h IC_{10}	.	.	.	.	.	**SIG**

		Cell YIELD Standard Bioassay 1					
		48-h IC_{50}	72-h IC_{50}	48-h IC_{25}	72-h IC_{25}	48-h IC_{10}	72-h IC_{10}
CELL YIELD Tank Bioassay 1	48-h IC_{50}	**SIG**	.	.	.	.	.
	72-h IC_{50}	.	**SIG**	.	.	.	.
	48-h IC_{25}	.	.	**NOT SIG**	.	.	.
	72-h IC_{25}	.	.	.	**SIG**	.	.
	48-h IC_{10}	.	.	.	.	**NOT SIG**	.
	72-h IC_{10}	.	.	.	.	.	**SIG**

		Cell YIELD Standard Bioassay 2					
		48-h IC_{50}	72-h IC_{50}	48-h IC_{25}	72-h IC_{25}	48-h IC_{10}	72-h IC_{10}
CELL YIELD Tank Bioassay 2	48-h IC_{50}	**SIG**	.	.	.	.	.
	72-h IC_{50}	.	**SIG**	.	.	.	.
	48-h IC_{25}	.	.	**NOT SIG**	.	.	.
	72-h IC_{25}	.	.	.	**SIG**	.	.
	48-h IC_{10}	.	.	.	.	**SIG**	.
	72-h IC_{10}	.	.	.	.	.	**SIG**

5.4 Do toxicant losses in test solutions affect the sensitivity of bioassays?

5.4.1 Predictions from modeling

Modeling the effect of copper on algal growth correctly predicted that growth inhibition in a test system would increase with increasing copper concentration. Growth rates were predicted based on copper losses in test solutions over 72 h described either by a mathematical function or by measured data. In both cases, calculated IC_{50} values for growth rate were higher than those calculated from the experimental bioassays. The modeling was based on an ideal sigmoidal concentration-response curve, whereas the experimentally derived concentration-response relationships were more variable in slope. This suggested that the functional relationship (Equation 11) between μ and the toxicant concentration, rather than the equation describing copper losses,was inacurate.

Although the modelled toxicity data gave higher IC_{50} values, the model was useful to illustrate the effect different rates of contaminant losses may have on toxicity. If copper was constant over 72 h, a lower IC_{50} was predicted than if copper decreased over time.

5.4.2 Confirmation by Experimental Work.

Tank bioassays, in which copper in the test solutions would be in equilibrium with copper in the external solution, via a 3μm membrane, were developed in a attempt to keep copper concentrations constant over 72 h. Unfortunately it was not possible to keep the copper concentrations constant. Decreases up to 46% could still be recorded in the bioassays using the tank procedures. In addition, from one experiment to another, wide variations in copper decrease were obtained for the same nominal concentration. These variations may have resulted from variations in temperature and light conditions. Indeed, cell density in test solutions affects the extent of copper losses over the test period. Cell density is in turn affected by growth rate, which is determined by incubation conditions, i.e. light and temperature. Copper losses in tank bioassays were yet lower compared to the standard flask bioassays, particularly over 72 h.

By minimising copper losses in the tank bioassays, it was found that copper was more toxic to *P.tricornutum* (i.e. lower IC_{50}) after 72 h. However, after a 48-h exposure, toxicity of copper was not significantly different.

Bioassays based on growth rate or cell yield inhibition of *P. tricornutum* exposed to copper, in which concentrations drop over the test period, will underestimate copper toxicity over 72 h. The difference between the standard bioassay and the tank bioassays would be even more pronounced if it had been possible to completely prevent copper concentrations decreasing with time. Differences in toxicity were even more obvious at low copper concentrations and particularly after 72 h, where 10-25 % inhibition of growth rate and cell yield was calculated (i.e. IC_{25} and IC_{10}). Based on 72-h IC_{10} and 72-h IC_{25}, toxicity was in some cases increased by a factor of two when the bioassay was undertaken using the tank protocol. Risk assessments, which often use IC_{25} or IC_{10} values as estimates of threshold effects concentrations, are likely to underestimate the potential effect of copper.

5.4.3 72-h versus 48-h exposure.

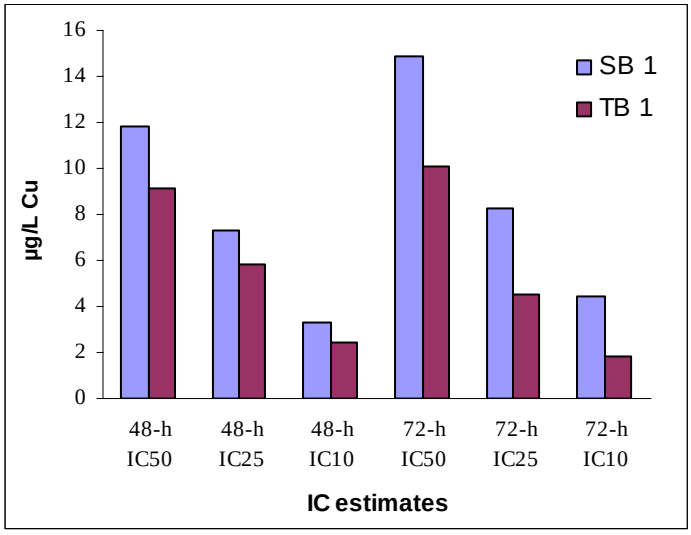

Figure 13. Comparison between 48-h and 72-h IC values calculated from growth rate for standard bioassay and tank bioassay.

Figure 13 illustrates the increased toxicity of copper to *P. tricornutum* in tank bioassays (TB1) compared to standard bioassays for 72-h versus 48-h. The increase in toxicity is significantly greater after a 72-h exposure than after a 48-h exposure. It is surprising that copper losses affect the 72-h IC values for growth rate more than 48-h IC_{50} values, considering that most of the copper losses generally occur within the first 48 hours (Table 13 and Table 14).

5.5 Conclusion

Following the tank bioassay procedure, although copper losses still occurred, losses were lower than in the standard bioassay and a significant difference in toxicity was shown based on a 72-h exposure. It thus confirmed the hypothesis that decreasing copper concentrations over the test period affect the estimation of toxicity. This has important implications for toxicity testing with other compounds (e.g. some organic compounds) that may be lost from solution more quickly.

This study indicates that the error in the estimated toxicity will depend upon the exposure duration. Whereas 72-h copper toxicity tests clearly underestimate copper toxicity, 48-h toxicity test results may not be affected by copper losses over the test period. It thus reinforced the importance of test duration as a factor influencing the accuracy of toxicity test results.

The results also indicate that mathematical correction of results from toxicity tests with decaying toxicant concentrations may allow prediction of expected results in the absence of decay.

CHAPTER 6. RECOMMENDATION FOR FUTURE BIOASSAYS

Standard laboratory bioassays that ignore metal losses in test solutions potentially underestimate metal toxicity. For toxicity data to be accepatable. toxicant losses over the period of a bioassay must be minimized. One recommendation is that toxicant losses can be minimized by lowering the initial cell density. To avoid depletion of dissolved copper in solution, initial cell densities should not exceed 10^3 cells/ mL. The exposure time is also critical. This study clearly suggests that toxicity tests should be undertaken for a 48-h exposure period. Using 48-h exposure, rather than 72-h, only slightly decreased copper losses but this was sufficient to remove the effect of copper losses on toxicity estimates.

None of the above recommendationscompletely eliminates toxicant losses. Although losses will be reduced, they will still potentially affect the quality of toxicity test data. This study also highlight the need of better models which incorporate time-varying concentration of metals into calculations of toxicity (IC) values.

In this ways, laboratory toxicity tests should more closely estimate metal bioavailability and toxicity in natural waters.

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