



## ABSTRACT

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Heavy metals are ubiquitous contaminants of the marine environment and can accumulate and persist in sediments. The toxicity of metal contaminants in sediments to organisms is dependent on the bioavailability of the metals in both the water and sediment phases and the sensitivity of the organism to the metal exposure. This study investigated the effects of two metal contaminants of concern ( $\text{CdCl}_2$  and  $\text{CuCl}_2$ ) on a battery of marine bioassays employed for sediment assessment. Cadmium, a known carcinogen and widespread marine pollutant, was found to be the least toxic of the two assayed metals in all *in vivo* tests. However  $\text{CdCl}_2$  was found to be more toxic to the fish cell lines PLHC-1 and RTG-2 than  $\text{CuCl}_2$ . *Tisbe battagliai* was the most sensitive species to both metals and the Microtox<sup>®</sup> and cell lines were the least sensitive (cadmium was found to be three orders of magnitude less toxic to *Vibrio fischeri* than to *T. battagliai*). The sensitivity of *Tetraselmis suecica* to the two metals varied greatly. Marine microalgae are among the organisms that can tolerate higher levels of cadmium. This hypothesis is demonstrated in this study where it was not possible to derive an  $\text{EC}_{50}$  value for  $\text{CdCl}_2$  and the marine prasinophyte, *T. suecica*. Conversely,  $\text{CuCl}_2$  was observed to be highly toxic to the marine alga,  $\text{EC}_{50}$  of  $1.19 \text{ mg l}^{-1}$ . The genotoxic effect of Cu on the marine phytoplankton was evaluated using the Comet assay. Copper concentrations ranging from  $0.25$  to  $2.50 \text{ mg l}^{-1}$  were used to evaluate the effects. DNA damage was measured as percent number of comets and normal cells. There was no significant DNA damage observed at any concentration of  $\text{CuCl}_2$  tested and no correlation with growth inhibition and genetic damage was found.

**Keywords:** heavy metal toxicity; battery of bioassays; fish cell lines; Comet assay.

1

## 2 **1. Introduction**

3 Metals are among the most intensely studied contaminants in estuarine and marine  
4 environments. Heavy metals are elements with atomic weights ranging from 63.456 to  
5 200.590 and are characterised by having similar electronic distribution in their external  
6 shell (e.g. copper, cadmium, zinc) (Viarengo, 1989). Several heavy metals are essential  
7 to life at very low concentrations, but at higher doses most of them are toxic (Warnau et  
8 al., 1995).

9 Although heavy metals exist in dissolved, colloidal and particulate phases in  
10 seawater, the concentration of dissolved forms in aquatic systems is low. As they are  
11 particle reactive they readily sorb onto suspended particulate matter (SPM) as they enter  
12 riverine, coastal or estuarine waters. Ultimately heavy metals are removed to bottom  
13 sediments in estuarine systems which serve as a repository for these elements  
14 (Niencheski et al., 1994).

15 Metal toxicity in seawater is affected by many factors of which the  
16 physiochemical state of the metal is one of the most important. Adsorption to particles  
17 or complexation with dissolved organics will reduce the toxicity. Unfortunately the  
18 form in which a metal exists is hard to characterise and most toxicity studies measure  
19 the total concentration of a particular metal, which in practice does not correlate well  
20 with the observed toxicity (Florence et al., 1984). Other important parameters when  
21 dealing with metals are pH and redox potential. At a low pH, metals generally exist as  
22 free cations but at an alkaline pH, as in seawater, they tend to precipitate out as  
23 insoluble hydroxides, carbonates, oxides or phosphates (reduced toxicity). The  
24 bioavailability of metals may also be affected by the presence of natural organic matter  
25 e.g. humic acids. Humic acids are produced by the degradation of organic matter by

1 microorganisms and are able to bind a variety of metals at their carboxylic groups,  
2 altering the bioavailability and consequently affecting the toxicity (Tsiridis et al., 2006).

3 Copper is present in oceanic waters at concentrations of about  $0.1 \mu\text{g l}^{-1}$ .  
4 However, higher levels  $2.0$  to  $> 100 \mu\text{g l}^{-1}$  are found in estuaries and a large fraction of  
5 this may sorb to particulates and concentrate in the bottom sediment [ranging from  $10$   
6  $\mu\text{g g}^{-1}$  dry weight in pristine areas to  $2000 \mu\text{g g}^{-1}$  dry weight at impacted sites] (Bryan  
7 and Langston, 1992; Kennish, 1997). Estimates of total anthropogenic discharge of  
8 copper to surface waters range from  $35 \times 10^3$  to  $90 \times 10^3$  metric tons per year worldwide  
9 (Nriagu and Pacyna, 1988). Levels of cadmium are much lower in open ocean waters  
10 ( $0.2 - 60 \text{ ng l}^{-1}$ ) and coastal waters ( $1 - 100 \text{ ng l}^{-1}$ ). While, cadmium concentrations in  
11 estuarine sediments typically range from  $0.2$  to  $10 \mu\text{g g}^{-1}$  dry weight (Kennish, 1997).

12 Above a threshold bioavailability all trace metals are potentially toxic (Rainbow,  
13 1993). According to Abel (1989) an approximate order of decreasing toxicity of  
14 common heavy metals in aquatic organisms is as follows: mercury, cadmium, copper,  
15 zinc, nickel, lead, chromium, aluminium, and cobalt. However, the toxicity of a given  
16 metal can vary greatly from one species to another. Some estuarine and marine  
17 organisms have the capacity to store, remove or detoxify metal contaminants using  
18 proteins (e.g. metallothioneins, phytochelatins) or cellular structures (e.g. lysosomes).

19 A battery of marine bioassays has been developed and optimised within the  
20 Radiation and Environmental Science Centre (RESC) for use in the routine monitoring  
21 of Irish marine sediments. It is important to evaluate the sensitivities of the battery  
22 species to a wide variety of contaminants. Therefore during the development process  
23 the sensitivity of the battery to a variety of organic contaminants was evaluated  
24 (Macken et al., 2007). Similarly this paper investigates the effects of two inorganic  
25 contaminants on the battery of species. The test battery consists of species

1 representative of several trophic levels: *Vibrio fischeri* (decomposer), *Tetraselmis*  
2 *suecica* (primary producer), *Tisbe battagliai* (primary consumer) and the fish cell lines  
3 PLHC-1 (*Poeciliopsis lucida* hepatoma cell line) and RTG-2 (*Oncorhynchus mykiss*  
4 gonad cells) representing the secondary consumer trophic level. Endpoints employed  
5 include light inhibition (*V. fischeri*), growth inhibition (*T. suecica*), mortality (*T.*  
6 *battagliai*), cell viability as assessed by the alamar blue and neutral red assays (fish  
7 cells) and the investigation of sublethal DNA damage with the Comet assay (*T.*  
8 *suecica*). Table 1 presents the summary toxicity data from the literature for the species  
9 and chemicals employed in this study.

10 Alkaline single-cell gel electrophoresis, also known as the Comet assay,  
11 developed by Singh et al. (1988), allows for the investigation of damage to genomic  
12 DNA caused by genotoxic agents. The Comet assay is a valuable method that has  
13 grown in popularity in recent years. Although mammalian cells are commonly  
14 employed in the Comet assay, the test can be applied to many other cell types (Tice et  
15 al., 2000). The assay has been successfully adapted for many aquatic species such as  
16 clams (Hartl et al., 2004), mussels (Nigro et al., 2006), dog-whelks (Hagger et al., 2006)  
17 among others. Erbes et al. (1997) detected DNA damage in the green algae  
18 *Chlamydomonas reinhardtii* exposed to 4-nitroquinoline-1-oxide, *N-*  
19 *nitorodimethylamine* and H<sub>2</sub>O<sub>2</sub>. As phytoplankton are the primary producers in marine  
20 ecosystems, sub-lethal effects on these organisms are of great importance. Cadmium is  
21 a known carcinogen and its toxic mechanisms have been well documented. The  
22 genotoxic effects of Cd on phytoplankton has been investigated (Desai et al., 2006),  
23 however, there is little known about the genotoxic effects of Cu especially in  
24 phytoplankton. Therefore the genotoxic effects of Cu to *T. suecica* were investigated in  
25 this study.

1           The main aim of this study was to investigate the sensitivity of the battery to two  
2 recognised metal pollutants of the marine environment and rank species sensitivity  
3 accordingly.

## 1 **2. Materials and Methods**

### 2 *2.1 Test Substances*

3 Copper chloride (CAS Registry No. 7447-39-4) and cadmium chloride (CAS  
4 Registry No. 10108-64-2) were obtained from Sigma Aldrich (UK). Analytical grade  
5 potassium dichromate (CAS Registry No 778-50-9) and phenol (CAS Registry No. 108-  
6 95-2) were obtained from Sigma Aldrich (UK) and BDH (UK) respectively and were  
7 employed as reference toxicants to validate the test procedures. All test compounds  
8 chosen are known/detectable pollutants of the estuarine/marine environment in Ireland  
9 (Kilemade et al., 2004; Davoren et al., 2005; Cronin et al., 2006).

10

### 11 *2.2 Ecotoxicity tests*

12 For each chemical stock solutions of 1000 mg l<sup>-1</sup> were prepared using deionised  
13 water and suitable dilution series were prepared employing appropriate test media. For  
14 all tests, and for each chemical, testing was performed in two stages. A preliminary  
15 range finding test was conducted to determine the range of concentrations to be tested in  
16 the definitive test. All definitive testing was conducted in at least triplicate on three  
17 independent occasions. All tests, blanks and positive controls (reference chemicals)  
18 with the exception of the Microtox<sup>®</sup> test were conducted with natural seawater collected  
19 from the Bull Lagoon, Co. Dublin, Ireland (53°22'N 006°08'W) with a salinity range of  
20 29 – 32 ‰. *T. suecica* and *T. battagliai* were maintained in the laboratory in  
21 accordance with standard methods (BS EN ISO 10253, 1998; ISO/DIS 14669, 1997).  
22 Maintenance temperatures for both species were 20 °C ± 2 °C. Fish cell lines were also  
23 maintained and cultured in the RESC according to Ní Shúilleabháin et al. (2004). The  
24 selection of test species was based on their standardisation and frequent employment in

1 toxicity testing, reported sensitivity to a wide range of pollutants and their relevance to  
2 an Irish environment (algae and invertebrate).

3

#### 4 2.2.1 *Microtox*<sup>®</sup> assay

5 Lyophilised *V. fischeri* bacteria (NRRL B-11177) and all *Microtox*<sup>®</sup> reagents  
6 were obtained from SDI Europe, Hampshire, UK. The *Microtox*<sup>®</sup> assay was performed  
7 in accordance with operational procedures from Azur Environmental Ltd. (Azur  
8 Environmental Ltd, 1998). Five, fifteen and thirty-minute EC<sub>50</sub> tests were performed  
9 using the 90 % basic test for aqueous extracts (nine concentrations). Bioluminescent  
10 responses were measured using a *Microtox*<sup>®</sup> Model 500 analyser and acute toxicity data  
11 were obtained and analyzed using the *MicrotoxOmni*<sup>®</sup> software (SDI Europe,  
12 Hampshire, UK). A basic test was conducted with the reference standard phenol for  
13 each fresh vial of bacteria opened to ensure validity of test method.

14

#### 15 2.2.2 *Microalgal toxicity test*

16 *T. suecica* (Kylin) Butcher (CCAP66/4) was obtained from the Culture  
17 Collection of Algae and Protozoa (CCAP) (Argyll, Scotland). Toxicity tests were  
18 conducted according to the International Organization for Standardization (ISO)  
19 Guideline 10253 (BS EN ISO 10253, 1998). All microalgal growth inhibition tests  
20 were conducted at 20 ± 1 °C with continuous shaking at 100 rpm, illumination of 10,000  
21 lux and a continuous photoperiod. The initial algal density of all flasks was 1 x 10<sup>4</sup> cell  
22 ml<sup>-1</sup> in a final volume of 20 ml. Negative controls were incorporated for each test  
23 containing only algal growth media and algal inoculum. The cell density of each  
24 replicate was measured after 72 h using a Neubauer Improved (Bright-Line) chamber



1 (Brand, Germany). Average specific growth rate and percentage inhibition of average  
2 specific growth rate relative to controls were calculated for each concentration. The  
3 reference chemical potassium dichromate was employed as a positive control to ensure  
4 validity of test method.

5

### 6 2.2.3 *Copepod toxicity test*

7 A starting culture of *T. battagliai* was kindly supplied by Shannon Acute  
8 Toxicity Laboratory (SATL), Ireland. *T. battagliai* toxicity tests were conducted with  
9 slight modifications according to the ISO method (ISO/DIS 14669, 1997). Toxicity  
10 tests with *T. battagliai* were conducted with copepodids  $6 \pm 2$  days old. During testing  
11 copepodids were incubated in a temperature controlled room at  $20 \pm 2$  °C and under a  
12 16:8 h light:dark photoperiod. A positive control using potassium dichromate was run  
13 alongside tests in order to verify the sensitivity of the copepods. Lethality for each  
14 chemical at each concentration was recorded and the percentage mortality ( $LC_{50}$ )  
15 compared to the controls was determined after 24 h and 48 h.

16

### 17 2.2.4 *Cell culture*

18 RTG-2 cells (Catalogue number 90102529) derived from rainbow trout gonads,  
19 were obtained from the European Collection of Cell Cultures (Salisbury, UK). The  
20 PLHC-1 cell line (CRL-2406) derived from a hepatocellular carcinoma in the  
21 topminnow were from the American Type Culture Collection and purchased from  
22 Promochem (UK). Both cell types were maintained in Dulbecco's Modified Medium  
23 Nutrient Mixture/ F-12 Ham (DMEM) supplemented with either 10 % (RTG-2) or 5 %  
24 (PLHC-1) foetal calf serum (FCS) and 45 IU ml penicillin, 45 µg ml streptomycin.

1 Cultures were maintained in a refrigerated incubator (Leec, Nottingham, U.K.) at either  
2 20 °C (RTG-2) or 30 °C (PLHC-1) under a normoxic atmosphere.

3

#### 4 2.2.4.1 Cytotoxicity testing: Metal exposure

5 Individual wells of a 96-well microplate (Nunc, Denmark) were seeded with 100  
6 µl of cell suspension at a seeding density of  $2 \times 10^5$  cells per ml for RTG-2 cells and  $8 \times$   
7  $10^5$  cells per ml for PLHC-1 cells for 24 h exposure periods. For 96 h exposure periods  
8 cells were seeded at  $1.6 \times 10^5$  cells per ml for RTG-2 cells and  $2 \times 10^5$  cells per ml for  
9 PLHC-1 cells. Test chemicals were prepared in a reduced serum medium (5 % FCS).  
10 Range finding tests were first conducted with the metal compounds to select the  
11 concentrations for definitive testing. Six replicate wells were used for each control and  
12 test concentration per microplate. Following exposure of the cells, the test medium was  
13 removed; cell monolayers washed with phosphate buffered saline (PBS) and  
14 cytotoxicity assessed using the Alamar Blue (AB) and Neutral Red (NR) assays  
15 conducted subsequently on the same set of plates as previously described (Davoren and  
16 Fogarty, 2006).

17

#### 18 2.2.5 Comet Assay

19 Three test concentrations of copper were selected based on the previous toxicity  
20 tests with *T. suecica* (section 2.2.2) and  $\text{CuCl}_2$ . These concentrations (0.25, 0.75 and  
21  $2.5 \text{ mg l}^{-1}$ ) along with a control were set up in duplicate as per the ISO standard method  
22 (BS EN ISO 10253, 1998). The initial algal density of all flasks was  $1 \times 10^4 \text{ cell ml}^{-1}$  in  
23 a final volume of 20 ml. Cell density of each flask was measured after 72 h to ensure  
24 similar growth to initial toxicity tests.

1           The Comet assay was performed according to modified procedures based on  
2 Singh et al. (1988) and Hagger et al. (2006). Five millilitres of culture was harvested  
3 from each replicate after 72. All sub-samples were centrifuged at 2000 rpm for 5 min at  
4 4 °C. After centrifugation supernatant was discarded and cell pellets were re-suspended  
5 in 180 µl of low melting point agarose (LMP) and aspirated. Immediately 80 µl of cell  
6 suspension/LMP agarose were dropped onto frosted microscopic slides which were  
7 previously coated with 1 % normal melting point agarose (NMP) dissolved in PBS.  
8 Coverslips were gently placed over the cell suspension and slides transferred to an  
9 icepack to allow the agarose to set. When the gels had set the slides were placed into  
10 freshly prepared lysing solution at 4 °C for 1 h (2.5 M NaCl, 100 mM Na<sub>2</sub>-EDTA, 10  
11 mM Tris [pH 10.0]). One percent Triton X-100 and 10 % DMSO were added to the  
12 lysis solution after the pH was adjusted and immediately prior to adding the slides. The  
13 lysis and all following steps were conducted in the dark to prevent any further damage  
14 to cell DNA.

15           Slides were removed from the lysis buffer and placed in a horizontal  
16 electrophoresis tank (Compact L/XL, Biometra, Germany). The tank was filled with  
17 electrophoresis buffer ( 300 mM NaOH, 1 mM Na<sub>2</sub>-EDTA [pH 13]) until all slides were  
18 covered, and the DNA was allowed to unwind for 40 min before electrophoresis.  
19 Electrophoresis was performed at 25 V, 300 amps for 30 min. After electrophoresis,  
20 slides were removed from the tank and rinsed three times (for 5 min each time) in  
21 neutralising buffer (0.4 M Tris, pH 7.5). Finally, slides were stained with 20 µl of 5 µg  
22 ml<sup>-1</sup> ethidium bromide solution and viewed under ultraviolet fluorescence light (400x).  
23 Slides were scored using the Comet software (version 5.0; Kinetic imaging Ltd, Wirral,  
24 UK). Twenty five cells were scored per slide and 2 slides per treatment were scored (50

1 cells per treatment in total). Finally, cell viability was tested by means of the trypan-  
2 blue exclusion method (Absolom, 1986).

3

#### 4 2.2.6 *Statistical analysis*

5 The EC<sub>50</sub> (concentration that elicits an estimated 50 % toxic effect e.g. growth  
6 inhibition, mortality) values for all chemicals were calculated using REGTOX-EV6.xls  
7 (Èric Vindimian <http://eric.vindimian.9online.fr/>), a curve fitting macro for  
8 Microsoft® Excel. For each definitive test, each concentration was tested in triplicate  
9 (microtox, microalgae, fish cells) or quadruplicate (copepod tests) and three  
10 independent experiments were performed. The acute toxicity data for the Microtox®  
11 assays was analysed using the MicrotoxOmni® software (SDI Europe, Hampshire, UK).  
12 Toxicity data for the algal and copepod tests were fitted to a sigmoidal curve and the  
13 Weibull (algal assays) and Hill (copepods and bacterial assays) models were used to  
14 calculate Effective Concentration (EC) and Lethal Concentration (LC) values  
15 respectively.

16 For all cell assays fluorescence as fluorescent units (AB and NR assays) was  
17 measured using a microplate reader (TECAN GENios, Grödig, Austria). Cytotoxicity  
18 was expressed as mean percentage inhibition relative to the unexposed control ±  
19 standard error of the mean (SEM), which was calculated using the formula [100-((Mean  
20 Experimental data/Mean Control data) x 100)]. Control values were set at 0 %  
21 cytotoxicity. Cytotoxicity data (where appropriate) was fitted and the Hill model  
22 (REGTOX-EV6.xls) used to calculate the 50 % Effective Concentration (EC<sub>50</sub>), which  
23 was the concentration of test compound which caused a 50% inhibition in comparison  
24 to untreated controls. The EC<sub>50</sub> values are reported ± 95 % Confidence Intervals (±  
25 95% CI).

1           Statistical analyses were carried out using a one-way analyses of variance  
2 (ANOVA) followed by Dunnett's multiple comparison test. These data analyses were  
3 performed using MINITAB<sup>®</sup> release 14 (MINITAB Inc. PA, USA). Statistical  
4 significance was accepted at  $p \leq 0.05$ . Percentage inhibition data generated by the  
5 MicrotoxOmni<sup>®</sup> software were Arcsin transformed prior to statistical analysis to  
6 improve normality and homogeneity of variances and reduce the influence of outliers.  
7 To confirm the precision of tests, the coefficient of variation (CV) was calculated for all  
8 controls.  
9

### 1 **3. Results**

2           After initial range finding tests final concentration ranges of 0.065 – 16.670 mg  
3  $\text{l}^{-1}$  and 1.758 – 450  $\text{mg l}^{-1}$  for  $\text{CuCl}_2$  and  $\text{CdCl}_2$  respectively were employed in the  
4 definitive testing with the Microtox<sup>®</sup> system. Bioluminescence of *V. fischeri*  
5 (Microtox<sup>®</sup>) decreased after exposure to both  $\text{CdCl}_2$  and  $\text{CuCl}_2$  indicating that both  
6 chemicals are toxic to the marine bacterium. Figure 1 shows the experimental values of  
7 the relative light intensity (normalized with respect to the initial light intensity) at  
8 various concentrations of (a)  $\text{CuCl}_2$  and (b)  $\text{CdCl}_2$  (only five concentrations graphed for  
9 clarity purposes). Toxicity of both metals to *V. fischeri* was observed to increase with  
10 time and copper was an order of magnitude more toxic than cadmium for all time  
11 intervals (Table 2).

12           Initial range finding tests with  $\text{CdCl}_2$  and *T. suecica* (0.001 – 100  $\text{mg l}^{-1}$ ) failed  
13 to identify a suitable concentration range for definitive testing. Significant toxicity was  
14 observed at 100  $\text{mg l}^{-1}$  but the level of growth inhibition was well below 50 %. Further  
15 testing with  $\text{CdCl}_2$  and *T. suecica* were therefore not performed as higher concentrations  
16 were not deemed relevant to known environmental levels (Kilemade et al., 2004;  
17 Davoren et al., 2005; Cronin et al., 2006). Copper chloride was considerably more toxic  
18 to the marine prasinophyte and showed significant inhibition of growth at  
19 concentrations as low as 0.25  $\text{mg l}^{-1}$  (Figure 2) and yielded an  $\text{EC}_{50}$  of 1.19  $\text{mg l}^{-1}$  (Table  
20 2).

21           Results of toxicity testing with *T. battagliai* and the two metals are shown in  
22 Table 2. Copper chloride was more toxic than  $\text{CdCl}_2$  with 24 and 48 h  $\text{LC}_{50}$  values of  
23 0.19  $\text{mg l}^{-1}$  and 0.08  $\text{mg l}^{-1}$  respectively. However, *T. battagliai* was more sensitive to  
24 both metals than either the bacterial or algal tests. There was significant toxicity at all

1 concentrations of CuCl<sub>2</sub> after 48 h exposure (0.05 – 1.00 mg l<sup>-1</sup>). Significant effects  
2 were only seen with CdCl<sub>2</sub> at 0.2 – 1.0 mg l<sup>-1</sup> after 48 h exposure (Figure 3).

3 The degree of DNA structural integrity was evaluated in *T. suecica* cells by  
4 single cell gel electrophoresis (Comet assay) after 72 hours of exposure to CuCl<sub>2</sub>. Fifty  
5 comets per concentration were measured, 25 per replicate. Results showed that there  
6 was no significant DNA damage at the concentrations tested (Figure 4) compared to the  
7 control. In the control percentage tail DNA in the algal cells was 9.93 ± 1.59 % (SEM)  
8 and at the top concentration (2.5 mg l<sup>-1</sup>) percentage tail DNA was 8.45 ± 1.89 % (SEM).  
9 These concentrations were selected based on acute toxicity results so that a comparison  
10 could be made between growth inhibition and genotoxicity. The cell viability for the  
11 Comet assay was recorded for each concentration employed (Control, 0.25 mg l<sup>-1</sup>, 0.75  
12 mg l<sup>-1</sup>, 2.5 mg l<sup>-1</sup>) resulting in cell viabilities of over 90 % at all concentrations apart  
13 from 2.5 mg l<sup>-1</sup> (> 80 % viability).

14 In the cell assays a toxic effect was observed with both CdCl<sub>2</sub> and CuCl<sub>2</sub> on both  
15 cell lines tested with both the NR and AB assays. All calculated cytotoxicity values are  
16 presented in Table 3. Cadmium was observed to be the most toxic compound to both  
17 cell lines. There was significant toxicity ( $p \leq 0.05$ ) with cadmium at all concentrations  
18 with the PLHC-1 cell line (24 and 96 h) for both the NR and AB assays. For the RTG-2  
19 cells there was significant toxicity at all concentrations after 96 h with cadmium as  
20 determined by NR and AB. The 24 h EC<sub>50</sub> with AB and the PLHC-1 cell line and  
21 CdCl<sub>2</sub> was 11.31 (11.04 – 11.48) mg l<sup>-1</sup>, while for the RTG-2 cell line it was 20.90  
22 (18.33 – 23.14) therefore indicating that the RTG-2 cells were less sensitive than the  
23 PLHC-1.

24 For copper significant toxicity was observed ( $p \leq 0.05$ ) at between 40 – 100 mg  
25 l<sup>-1</sup> depending on the assay and duration of exposure for PLHC-1 cells. RTG-2 cells

1 were only significantly affected by copper at the top two concentrations with both  
2 assays. The same difference in sensitivity between cell lines was observed for copper as  
3 were for cadmium. The 96 h EC<sub>50</sub> with PLHC-1 cells and CuCl<sub>2</sub> as determined by AB  
4 was 56.28 (51.82 – 62.38) mg l<sup>-1</sup> while for RTG-2 cells it was 92.04 (86.98 – 100.47)  
5 mg l<sup>-1</sup>. In the case of copper the AB assay was more sensitive for both cell types after  
6 96 h e.g. 96 h NR EC<sub>50</sub> was 65.43 (62.30 – 68.24) mg l<sup>-1</sup> and the 96 h AB EC<sub>50</sub> was  
7 56.28 (51.82 – 62.38) mg l<sup>-1</sup> for PLHC-1 cells.

8           With the exception of the EC values for CuCl<sub>2</sub> and the PLHC-1 cell line as  
9 determined by NR, toxicity of both metals was observed to increase with lengthened  
10 exposure time for all endpoints. Based on these values the toxicity ranking was  
11 identical for both NR and AB (EC<sub>10</sub> and EC<sub>50</sub>) in the order CdCl<sub>2</sub> > CuCl<sub>2</sub> for PLHC-1  
12 and RTG-2 cells. However, there was a significant difference in the sensitivities of the  
13 two cell lines employed. In general the PLHC-1 cells were more sensitive than the  
14 RTG-2 cells and AB was the most sensitive end point employed.

15           Tables 2 and 3 summarise the ecotoxicity and cytotoxicity data respectively for  
16 all species and both metals. Copper chloride was the most toxic of the two to *V.*  
17 *fischeri*, *T. suecica* and *T. battalgiai*. In contrast to this CdCl<sub>2</sub> was the most toxic  
18 chemical to the fish cell lines. Of all the assays employed the acute lethality test with *T.*  
19 *battalgiai* was the most sensitive.



1

## 2 **4. Discussion**

3       The investigation into the toxicity of cadmium and copper to a battery of  
4 bioassays showed varying toxicity between test species. In the Microtox<sup>®</sup> assay the  
5 light production in *V. fischeri* is directly proportional to the metabolic activity of the  
6 bacterial population and inhibition of enzymatic activity correspondingly decreases  
7 bioluminescence. The use of this assay provides a measure of sub-lethal response. In  
8 this study both metals elicited a toxic effect in the Microtox<sup>®</sup> assay, however, the assay  
9 was one of the least sensitive of the battery tests employed. Copper chloride was an  
10 order of magnitude more toxic than CdCl<sub>2</sub> to the bacteria and the toxicity of both metals  
11 was observed to increase with lengthened exposure time (Table 2). This increase in  
12 toxicity with time is indicative of metal contamination and is a well documented effect  
13 in *V. fischeri* (Azur Environment Ltd., 1998). Codina et al. (2000) obtained EC<sub>50</sub> values  
14 of 150 μm l<sup>-1</sup> (34.25 mg l<sup>-1</sup>) and 7.2 μmol l<sup>-1</sup> (1.23 mg l<sup>-1</sup>) for CdCl<sub>2</sub>·2.5H<sub>2</sub>O and  
15 CuCl<sub>2</sub>·2H<sub>2</sub>O respectively. These results concur with the values obtained in this study  
16 employing the anhydrous metal salts of cadmium and copper. Newman and McCloskey  
17 (1996) investigated total EC<sub>50</sub> values for nine metals (added as chloride salts) and the  
18 values for both agreed with our study and other studies within the literature. Their EC<sub>50</sub>  
19 value for CuCl<sub>2</sub> was 2.78 μmol l<sup>-1</sup> (4.8 mg l<sup>-1</sup>) and their value for CdCl<sub>2</sub> was 195 μmol l<sup>-1</sup>  
20 (35.75 mg l<sup>-1</sup>). In all studies cadmium toxicity to *V. fischeri* was found to be low  
21 compared to copper. A lack of sensitivity of Gram negative bacteria towards cadmium  
22 has previously been reported (Bitton and Freihoffer, 1978; Morozzi et al., 1986; Bauda  
23 and Block, 1990). These authors attributed the low toxicity of cadmium to the presence  
24 of exopolysaccharides on the outer layer of the bacterial membrane, which have been

1 found to adsorb and trap cadmium. Fulladosa et al. (2005) also found low toxicity of  
2 Cd (II) to *V. fischeri*.

3 The order of metal toxicity to algae varies with species and experimental  
4 conditions, but generally the order can be considered to be  $Hg > Cu > Cd > Ag > Pb >$   
5  $Zn$  (Rice et al. 1973; and Rai et al. 1981). In this study  $CuCl_2$  was far more toxic than  
6  $CdCl_2$  to the prasinophyte *T. suecica*. Ismail et al. (2002) reported  $IC_{50}$  values for Cd(II)  
7 ( $0.05 - 7.5 \text{ mg l}^{-1}$ ) and Cu(II) ( $0.03 - 0.41 \text{ mg l}^{-1}$ ) for the marine microalgal species *T.*  
8 *tetrahele* and *Tetraselmis* sp. after 96 h based on Optical Density (OD) measurements  
9 and cell counting. Satoh et al. (2005) reported similar  $IC_{50}$  values for both Cu and Cd of  
10  $7.4 \text{ mg l}^{-1}$  and  $9.8 \text{ mg l}^{-1}$ , respectively. This is in stark contrast to our study where  
11 cadmium was only observed to have a significant effect at  $100 \text{ mg l}^{-1}$  and no  $EC_{50}$  value  
12 was derived (as testing at higher concentrations was deemed to be unrealistic to  
13 environmentally relevant levels). The  $EC_{50}$  value for  $CuCl_2$  was determined at  $1.19 \text{ mg}$   
14  $l^{-1}$  which was similar to the values generated by other authors (Ismail et al., 2002; Satoh  
15 et al., 2005), however, de Kuhn et al. (2006) found  $Cu^{2+}$  ions to be an order of  
16 magnitude less toxic than was observed in the present study ( $EC_{50}$  value of  $40 \text{ mg l}^{-1}$ ).  
17 Differing physio-chemical parameters (e.g. pH) during experimental procedures may  
18 explain some of the differences observed between studies employing the same algae and  
19 metal.

20 The mechanism of metal toxicity (specifically copper) in microalgae has been  
21 described by Stauber and Florence (1987). They stated that the initial form of Cu that  
22 binds to the cell may bind to the carboxylic and amino residues in the membrane  
23 protein, rather than the thiol group. At the cell membrane Cu may interfere with cell  
24 permeability or the binding of essential metals (in low concentrations). Copper is then  
25 transported into the cytosol and may react with  $-SH$  enzyme groups and free thiols,

1 disrupting enzyme active sites and cell division. Copper may also exert toxicity on  
2 subcellular organelles interfering with mitochondrial electron transport, respiration,  
3 ATP production and photosynthesis. Nassiri et al., (1996) noted the presence of copper  
4 in the walls of a multilayered cell in *T. suecica* suggesting that these structures  
5 constitute an adsorbing area for this element. They also reported drastic damaging  
6 effects of copper contamination such as the depletion of starch which would perturb  
7 photosynthesis and the loss of flagella leading to a lack of motility in *T. suecica* after  
8 ultrastructure examination. Conversely there was no ultrastructural damage evident in  
9 the algae exposed to cadmium. In this study surviving *T. suecica* exposed to higher  
10 levels of  $\text{CuCl}_2$  ( $< 1.0 \text{ mg l}^{-1}$ ) were observed to have reduced motility compared to the  
11 controls but there was no obvious reduction in motility in the higher concentrations of  
12  $\text{CdCl}_2$ . Copper reduces growth as well as photosynthetic and respiratory activities  
13 (Nalewajko and Olaveson, 1995). The photosynthetic machinery is particularly  
14 susceptible to copper and may result in a decrease in the activity of photosystem II and  
15 electron transfer rates within the algae (Fernades and Henriques, 1991; Mallick and  
16 Mohn, 2003). Cid et al. (1995) also observed copper to interfere with algal  
17 photosynthesis and ATP production, with  $0.5 \text{ mg l}^{-1}$  causing a 50 % decrease in  
18 photosynthesis.

19 Nassiri et al (1996) also found that the toxic effects of copper to *T. suecica* were  
20 more pronounced than those of cadmium. They only observed toxicity with cadmium  
21 in the latency phase of growth, which suggests an adaptation phenomenon of *T. suecica*  
22 to this metal. However, the sensitivity of *T. suecica* to Cd in our study was far less ( $<$   
23  $40 \%$  effect at  $100 \text{ mg l}^{-1}$ ) than that of Nassiri et al. (1996) who observed an  $\text{IC}_{50}$  value  
24 of  $9.38 \text{ mg l}^{-1}$ . Marine microalgae are among the organisms that can tolerate higher  
25 levels of cadmium and *T. suecica* is a good example (Pérez-Rama et al., 2006). The

1 biosynthesis of phytochelatins (small, thiol containing peptides) seems to be one of the  
2 main tolerance mechanisms to metals, with cadmium being one of the main inductors  
3 (Scarano and Morelli, 2002; Hu et al., 2001). Cadmium although considered to be  
4 highly toxic to algae was found to be several orders of magnitude less toxic to *T.*  
5 *suecica* than copper. As mentioned copper has the ability to free thiols in the cystol of  
6 the cell. Pérez-Rama et al. (2001) showed that approximately 87 % of bioaccumulated  
7 cadmium in *T. suecica* was bound by phytochelatins after exposure to 6 mg l<sup>-1</sup> for 8  
8 days. Phytochelatins and cysteine in *T. suecica* are important cellular components  
9 involved in mechanisms of tolerance to cadmium, with the intracellular level of these  
10 molecules being regulated by the concentration of this metal in its medium (Pérez-Rama  
11 et al., 2006). The ability of an organism to synthesise phytochelatins with a greater  
12 number of subunits allows it to tolerate a higher level of cadmium and therefore reduces  
13 the toxicity of the cadmium to the organism. In their study Pérez-Rama et al. (2006)  
14 detected phytochelatins with up to seven subunits in *T. suecica*.

15         The toxicities of CdCl<sub>2</sub> and CuCl<sub>2</sub> to *T. battagliai* were very similar (Table 2).  
16 Very little data exist in the literature for *T. battagliai* and the heavy metals Cd and Cu.  
17 However, comparable 96 h EC<sub>50</sub> values were generated by Hutchinson et al. (1994)  
18 when assaying CdCl<sub>2</sub> and Cu[NO<sub>3</sub>]<sub>2</sub> with *T. battagliai* (Tables 1 and 2). Bechmann  
19 (1999) observed that the sensitivity to copper varies between copepod species. LC<sub>50</sub>  
20 values for copepods (including eight different species, differing life stages and different  
21 test conditions) ranged from 19 to 762 µg Cu l<sup>-1</sup> (O'Brian et al., 1988).

22         This study employed two cell lines and two endpoints as an additional  
23 test system to evaluate their ability to assess potential cytotoxicity of the two metal  
24 contaminants. Segner and Braunbeck (1998) advocated the use of *in vitro* cell culture  
25 techniques for the ecotoxicological assessment of the early and sensitive detection of

1 chemical exposure. Although they were not as sensitive as other assays employed (*T.*  
2 *suecica* and *T. battagliai*) they are still valuable tools for the screening of environmental  
3 samples (Ní Shúilleabháin et al., 2004). In this study the sensitivity of the cell lines  
4 differed and the PLHC-1 cells were observed to be the most sensitive to the two metals  
5 assayed.

6 It is well established that *in vitro* studies on fish cell lines are less sensitive than  
7 *in vivo* fish cell studies (Babich et al., 1986; Babich et al., 1990; Babich and  
8 Borenfreund, 1987; Saito et al., 1991; Fent and Hunn, 1996). Castaño et al. (1996)  
9 found that the RTG-2 cell line was between 20 to 200 times less sensitive than *in vivo*  
10 trout bioassays. In this study corresponding *in vivo* toxicity assays with fish were not  
11 conducted to validate the toxicity of these chemicals however comparative values exist  
12 within the literature. Besser et al. (2007) reported 96 h LC<sub>50</sub> values of 5.2 (4.7 – 5.9)  
13 and 42 (39 – 46) µg l<sup>-1</sup> for Cd and Cu respectively. In this study 96 h EC<sub>50</sub> values for  
14 RTG-2 cells with Cd and Cu were three orders of magnitude less sensitive than the  
15 reported *in vivo* results with a 96 h EC<sub>50</sub> for CdCl<sub>2</sub> of 7.12 (4.96 – 9.47) mg l<sup>-1</sup> (7120  
16 [4960 – 9470] µg l<sup>-1</sup>) and 96 h EC<sub>50</sub> for CuCl<sub>2</sub> of 46.74 (38.58 – 54.83) mg l<sup>-1</sup> (46740  
17 [38580 - 54830] µg l<sup>-1</sup> with the AB assay. However, there is increasing pressure to  
18 reduce the numbers of fish employed in regulatory testing. Therefore there is a need to  
19 find alternative ways in which to accurately assess the potential hazard of a chemical or  
20 environmental sample (e.g. relocation of dredged sediment). The reduced sensitivity of  
21 *in vitro* cell line methods would make it unfeasible to employ these assays in a  
22 regulatory capacity to assess the pollution status of environmental samples. However  
23 there is a possibility of employing *in vitro* cells lines as screening tools for the ranking  
24 of environmental samples. Although *in vitro* assays do not reflect the true *in vivo*  
25 situation and absolute toxicities have been observed to differ, good correlation in terms

1 of the ranking order of chemicals has been observed (Castaño et al., 1996; Ní  
2 Shúilleabháin. et al., 2004).

3         The genotoxic effect of copper on *T. suecica* was investigated using single cell  
4 gel electrophoresis (or Comet assay). Although cadmium has been observed to cause  
5 DNA damage in phytoplankton (Desai et al., 2006) it was not assayed with *T. suecica* as  
6 it was not possible in this study to obtain significant effects on the growth of the alga at  
7 the concentrations assayed (i.e. environmentally relevant concentrations). Unlike  
8 cadmium, the possible genotoxic effects of copper have not been fully investigated.  
9 From this study it is apparent that CdCl<sub>2</sub> has no significant genotoxic effect on *T.*  
10 *suecica* at the concentrations tested. The concentrations tested (0.25, 0.75 and 2.5 mg l<sup>-1</sup>  
11 <sup>1</sup>) were selected because at concentrations greater than 2.5 mg l<sup>-1</sup> inhibition of growth  
12 was too great to guarantee sufficient cell survival and viability. Although there was no  
13 genotoxic effect observed in this study, the genotoxic potential of copper has been  
14 observed in the literature. Guecheva et al. (2001) observed significant DNA damage  
15 post exposure to copper in planarians (24 h or 7 d). In this study the algae were only  
16 exposed for 72 h therefore it may be that a longer period of incubation may be required  
17 before an effect is observed. Other studies have exposed the algae for longer durations.  
18 Desai et al. (2006) exposed *Chaetoceros tenuissimus* to CdCl<sub>2</sub>.H<sub>2</sub>O for 20 days in total.

19         Recent studies have shown that metals, including iron, copper, chromium, and  
20 vanadium undergo redox cycling resulting in the production of reactive oxygen species  
21 (ROS) (Stohs and Bagchi, 1995). Over the last decade, evidence is emerging for  
22 copper-induced mutagenesis via ROS production (Reid et al., 1994; Anderson et al.,  
23 1994). However, little is known about the genotoxic effects of copper on marine  
24 organisms and there are no data in the literature about the effects of copper in the Comet  
25 assay. Therefore it is recommended that the method described in this chapter can be

1 employed in future studies with single compounds and environmental samples (e.g.  
2 porewaters, effluents) to assess the genotoxic potential of these compounds/mixtures on  
3 phytoplankton, one of the most vital components of aquatic food webs.

4 Toxicity tests used for water quality criteria only examine exposure to metals via  
5 the dissolved phase, express results relative to ambient concentrations only, and do not  
6 consider the mechanism of toxicity. These tests may not mimic environmental  
7 conditions closely enough to accurately predict the impact of anthropogenic  
8 contamination. Therefore it is suggested that further studies comparing the response of  
9 copepods to sublethal levels of metals accumulated from food and from water be  
10 assessed through radiotracer and feeding (on metal exposed algae) experiments and  
11 toxic impact and metal body burdens be compared. This may allow for the  
12 characterisation of the mechanisms by which any toxic effects occur.

13 The results of this study contribute to the understanding of the problems  
14 associated with assessing metal contamination and the associated complexities involved  
15 in metal toxicity. It is obvious from this study that all species do not react in a similar  
16 manner to potentially hazardous pollutants such as the heavy metals. Therefore no  
17 single screening tool is sufficient to safely monitor the environmental effects of heavy  
18 metal pollution. Instead several biological assays incorporating multiple endpoints  
19 should be used in tandem in order to get a more complete picture of possible  
20 environmental or health effects.

21

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17 **Figure 4** DNA integrity, evaluated as electrophoretic DNA migration (% Tail DNA), in  
18 *Tetrasmis suecica* exposed to concentrations of CdCl<sub>2</sub>.

**Table 1**

Trophic level	Test Species	Exposure Time	End point	Chemical	Toxicity value	Reference
Bacteria	<i>Vibrio fischeri</i>	15 min	EC <sub>50</sub>	Cu (II)	0.5 ± 0.1 mg l <sup>-1</sup>	Utgikar et al., 2004
		15 min	EC <sub>50</sub>	Cd <sup>2+</sup>	27 ± 5 µmol l <sup>-1</sup>	Newman, 1995
		15 min	EC <sub>50</sub>	Cu <sup>2+</sup>	1.62 ± 0.13 µmol l <sup>-1</sup>	Newman, 1995
		15 min	EC <sub>50</sub>	CdCl <sub>2</sub>	195 ± 18.8 µmol l <sup>-1</sup>	Newman & McCloskey, 1996
		15 min	EC <sub>50</sub>	CuCl <sub>2</sub>	2.78 ± 0.52 µmol l <sup>-1</sup>	Newman & McCloskey, 1996
			EC <sub>50</sub>	CdCl <sub>2</sub>	9.4 mg l <sup>-1</sup>	Peinado et al., 2002
			IC <sub>50</sub>	Cd in HNO <sub>3</sub>	50.4 ± 7.61 µg l <sup>-1</sup>	Hsieh et al., 2004
			IC <sub>50</sub>	Cu[NO <sub>3</sub> ] <sub>2</sub>	7.08 ± 0.352 µg l <sup>-1</sup>	Hsieh et al., 2004
		5 min	EC <sub>50</sub>	Cu	1.3 mg l <sup>-1</sup>	Toussaint et al., 1995
		15 min	EC <sub>50</sub>	CdCl <sub>2</sub> ·2.5H <sub>2</sub> O	150 µmol l <sup>-1</sup>	Codina et al. 2000
		15 min	EC <sub>50</sub>	CuCl <sub>2</sub> ·2H <sub>2</sub> O	7.2 µmol l <sup>-1</sup>	Codina et al. 2000
Algae	<i>Tetraselmis suecica</i>	96 h	EC <sub>50</sub>	CdCl <sub>2</sub>	5.8 mg l <sup>-1</sup>	Pérez-Rama et al., 2001
		6 d	EC <sub>50</sub>	CdCl <sub>2</sub>	7.9 ± 1 mg l <sup>-1</sup>	Pérez-Rama et al., 2002
			IC <sub>50</sub>	CdCl <sub>2</sub>	9.38 mg l <sup>-1</sup>	Nassiri et al., 1996
			IC <sub>50</sub>	CuCl <sub>2</sub>	0.172 mg l <sup>-1</sup>	Nassiri et al., 1996
			EC <sub>50</sub>	CuSO <sub>4</sub> ·5H <sub>2</sub> O	40 mg l <sup>-1*</sup>	De Kuhn et al., 2006*
Copepods	<i>Tisbe battagliai</i>	96 h	LC <sub>50</sub>	CdCl <sub>2</sub>	0.34 mg l <sup>-1</sup>	Hutchinson et al., 1994
		96 h	LC <sub>50</sub>	Cu[NO <sub>3</sub> ] <sub>2</sub>	0.088 mg l <sup>-1</sup>	Hutchinson et al., 1994
Fish Cells	RTG-2	48 h	NR <sub>50</sub>	Cd in HNO <sub>3</sub>	0.055 mmol l <sup>-1</sup>	Castaño et al. 1996
		48 h	NR <sub>50</sub>	Cu in HNO <sub>3</sub>	0.150 mmol l <sup>-1</sup>	Castaño et al. 1996
	PLHC-1	24 h	NR <sub>50</sub>	CuSO <sub>4</sub> ·5H <sub>2</sub> O	32.4 µg ml <sup>-1</sup>	Ryan & Hightower, 1994
		24 h	NR <sub>50</sub>	CdCl <sub>2</sub> ·2.5H <sub>2</sub> O	µg ml <sup>-1</sup>	Ryan & Hightower, 1994

EC<sub>50</sub> = Effective Concentration of 50 % of sample population, LC<sub>50</sub> = Lethal concentration of 50 % of sample population, NOEC = No Observed Effects Concentration, NR<sub>50</sub> = NR50 endpoint is the concentration of test agent that reduces Neutral Red uptake by 50 %. It is equivalent to 50 % viability. \* Inhibition of the motility of *Tetraselmis suecica*

**Table 2**

Test chemical	Test Species/ cell line	Exposure time	Endpoint	Concentration range (mg l <sup>-1</sup> )	EC <sub>10</sub> <sup>a</sup> (mg l <sup>-1</sup> )	EC <sub>50</sub> <sup>a</sup> (mg l <sup>-1</sup> )	NOEC <sup>b</sup> (mg l <sup>-1</sup> )	LOEC <sup>c</sup> (mg l <sup>-1</sup> )
CdCl <sub>2</sub>	<i>Vibrio fischeri</i>	5 min	Reduction in bioluminescence	1.758 – 450.0	25.97 (17.07 – 48.65)	105.82 (95.01 – 148.56)	56.250	112.500
	<i>Vibrio fischeri</i>	15 min	Reduction in bioluminescence	1.758 – 450.0	8.25 (6.13 – 13.35)	47.28 (43.14 – 61.81)	14.063	28.125
	<i>Vibrio fischeri</i>	30 min	Reduction in bioluminescence	1.758 – 450.0	4.56 (3.47 – 7.90)	23.22 (22.61 – 33.54)	7.031	14.063
CuCl <sub>2</sub>	<i>Vibrio fischeri</i>	5 min	Reduction in bioluminescence	0.065 – 16.67	1.87 (1.86 – 4.85)	10.65 (10.43 – 17.47)	2.084	4.168
	<i>Vibrio fischeri</i>	15 min	Reduction in bioluminescence	0.065 – 16.67	0.61 (0.49 – 1.28)	3.12 (3.03 – 5.05)	0.521	1.042
	<i>Vibrio fischeri</i>	30 min	Reduction in bioluminescence	0.065 – 16.67	0.25 (0.15 – 0.47)	1.32 (1.10 – 1.88)	0.521	1.042
CuCl <sub>2</sub>	<i>Tetraselmis suecica</i>	72 h	Growth inhibition	0.100 – 5.0	0.26 (0.20 – 0.30)	1.19 (1.04 – 1.26)	0.100	0.250
CdCl <sub>2</sub>	<i>Tisbe battagliai</i>	24 h	lethality	0.050 – 1.0	0.13 (0.06 – 0.21)	0.84 (0.66 – 1.07)	0.400	0.800
	<i>Tisbe battagliai</i>	48 h	lethality	0.050 – 1.0	0.02 (0.01 – 0.06)	0.19 (0.14 – 0.24)	0.100	0.200
CuCl <sub>2</sub>	<i>Tisbe battagliai</i>	24 h	lethality	0.050 – 1.0	0.07 (0.05 – 0.09)	0.19 (0.16 – 0.21)	0.100	0.200
	<i>Tisbe battagliai</i>	48 h	lethality	0.050 – 1.0	0.02 (0.01 – 0.04)	0.08 (0.07 – 0.11)	< 0.050	0.050

<sup>a</sup>EC<sub>50</sub> values and corresponding 95 % confidence intervals in parentheses

<sup>b</sup>NOEC, no observed effect concentration, the highest observed concentration at which no significant effect ( $p \leq 0.05$ ) was detected

<sup>c</sup>LOEC, lowest observed effect concentration, the lowest concentration of the tested concentration at which a significant ( $p \leq 0.05$ ) effect was detected.

**Table 3**

Test chemical	Test Species/ cell line	Exposure time	Endpoint	Concentration range (mg l <sup>-1</sup> )	EC <sub>10</sub> <sup>a</sup> (mg l <sup>-1</sup> )	EC <sub>50</sub> <sup>a</sup> (mg l <sup>-1</sup> )	NOEC <sup>b</sup> (mg l <sup>-1</sup> )	LOEC <sup>c</sup> (mg l <sup>-1</sup> )
CdCl <sub>2</sub>	PLHC - 1	24 h	NR	10 – 40	10.29 (9.78 – 10.98)	14.09 (14.12 – 14.73)	< 10	10
	PLHC - 1	96 h	NR	10 – 40	5.15 (0.46 – 7.08)	6.67 (1.56 – 8.13)	< 10	10
	PLHC – 1	24 h	AB	10 – 40	7.86 (7.50 – 8.18)	11.31 (11.04 – 11.48)	< 10	10
	PLHC – 1	96 h	AB	10 – 40	< 10	< 10	< 10	10
	RTG – 2	24 h	NR	10 – 40	14.40 (10.98 – 17.55)	25.88 (23.96 – 28.39)	20	25
	RTG – 2	96 h	NR	10 – 40	4.61 (2.31 – 6.54)	11.47 (9.32 – 13.46)	< 10	10
	RTG – 2	24 h	AB	10 – 40	12.47 (8.86 – 16.03)	20.90 (18.33 – 23.14)	15	20
	RTG – 2	96 h	AB	10 – 40	7.12 (4.93 – 9.47)	10.26 (9.10 – 11.70)	< 10	10
CuCl <sub>2</sub>	PLHC - 1	24 h	NR	10 – 100	41.82 (33.54 – 57.36)	56.28 (51.97 – 62.23)	20	40
	PLHC - 1	96 h	NR	10 – 100	50.25 (44.04 – 54.48)	65.43 (62.30 – 68.24)	40	60
	PLHC – 1	24 h	AB	10 – 100	69.10 (63.70 – 78.86)	---	60	80
	PLHC – 1	96 h	AB	10 – 100	41.82 (34.59 – 57.19)	56.28 (51.82 – 62.38)	40	60
	RTG – 2	24 h	NR	10 – 100	98.90 (93.50 – 130.78)	---	> 100	> 100
	RTG – 2	96 h	NR	10 – 100	67.17 (56.98 – 88.93)	---	80	100
	RTG – 2	24 h	AB	10 – 100	74.85 (69.57 – 86.58)	---	80	100
	RTG – 2	96 h	AB	10 – 100	46.74 (38.58 – 54.83)	92.04 (86.98 – 100.47)	60	80

<sup>a</sup>EC<sub>50</sub> values and corresponding 95 % confidence intervals in parentheses

<sup>b</sup>NOEC, no observed effect concentration, the highest observed concentration at which no significant effect ( $p \leq 0.05$ ) was detected

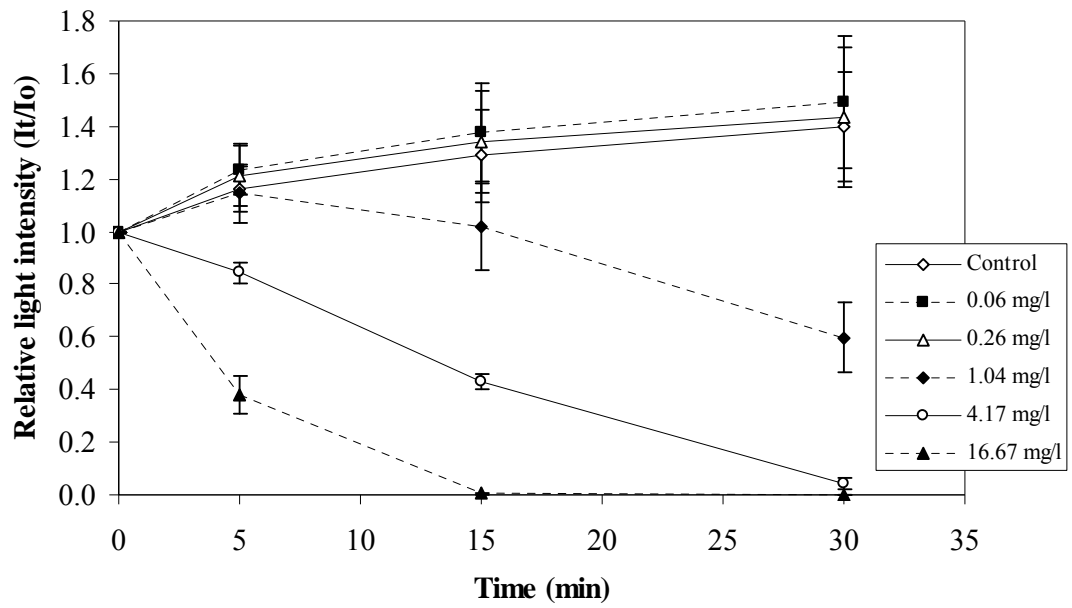
<sup>c</sup>LOEC, lowest observed effect concentration, the lowest concentration of the tested concentration at which a significant ( $p \leq 0.05$ ) effect was detected.

NR = Neutral Red,

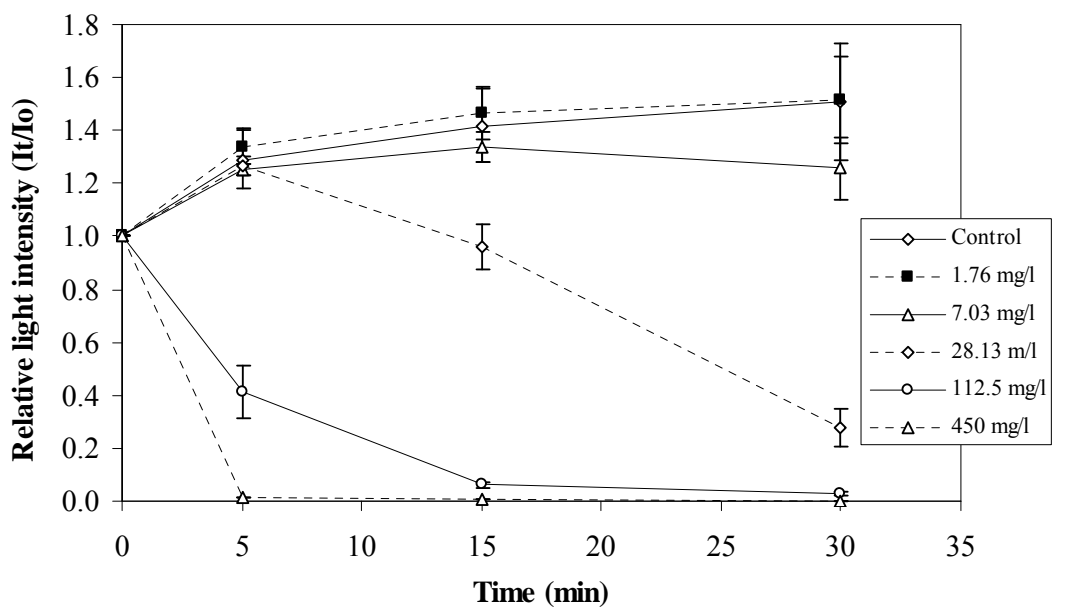
AB = Alamar Blue



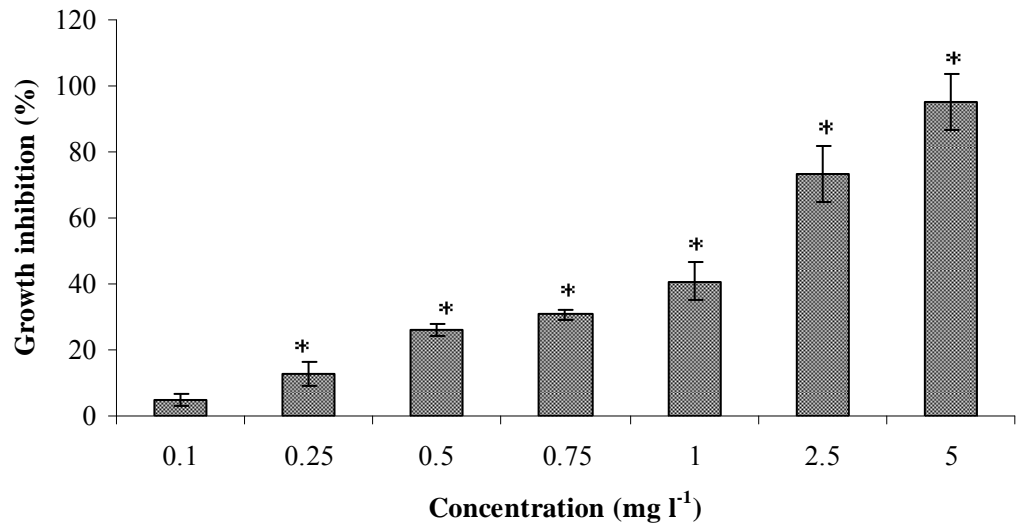
(a)



(b)

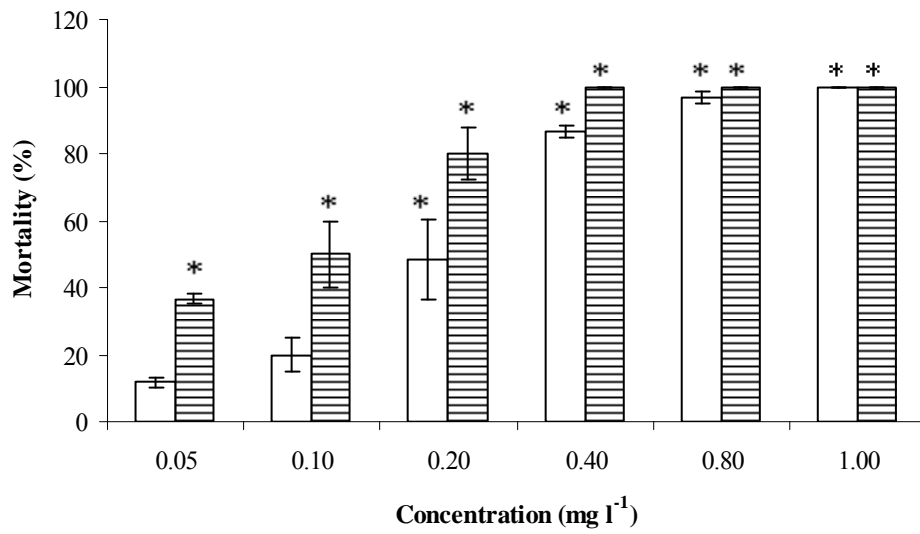


**Figure 1** Light attenuation of *Vibrio fischeri* following exposure to (a) various copper chloride concentrations, and (b) various cadmium chloride concentrations.

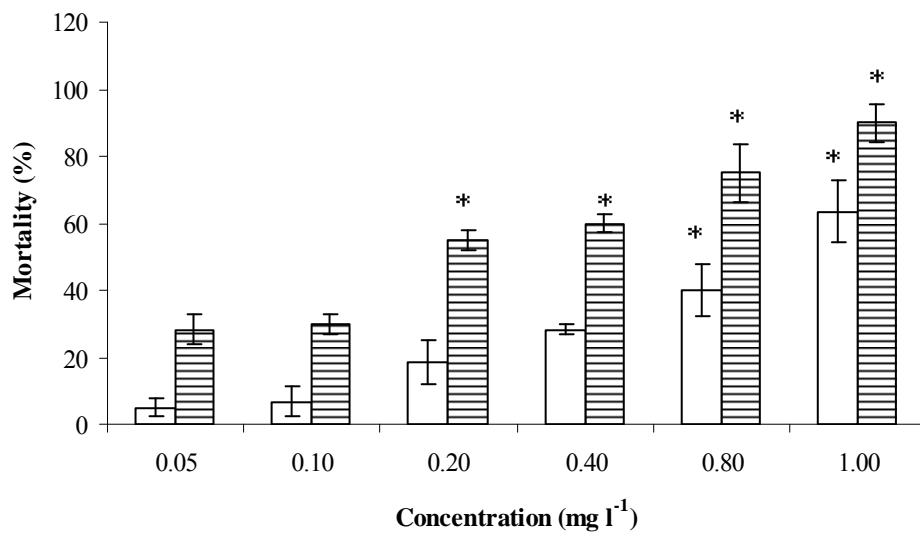


**Figure 2** Percentage growth inhibition of *T. suecica* after 72 h exposure to CuCl<sub>2</sub>. Data are expressed as a percentage of unexposed controls  $\pm$  SEM of three independent experiments. \* denotes significance from the control ( $p \leq 0.05$ ). C.V. for the controls ranged from 1.18 – 8.89 %.

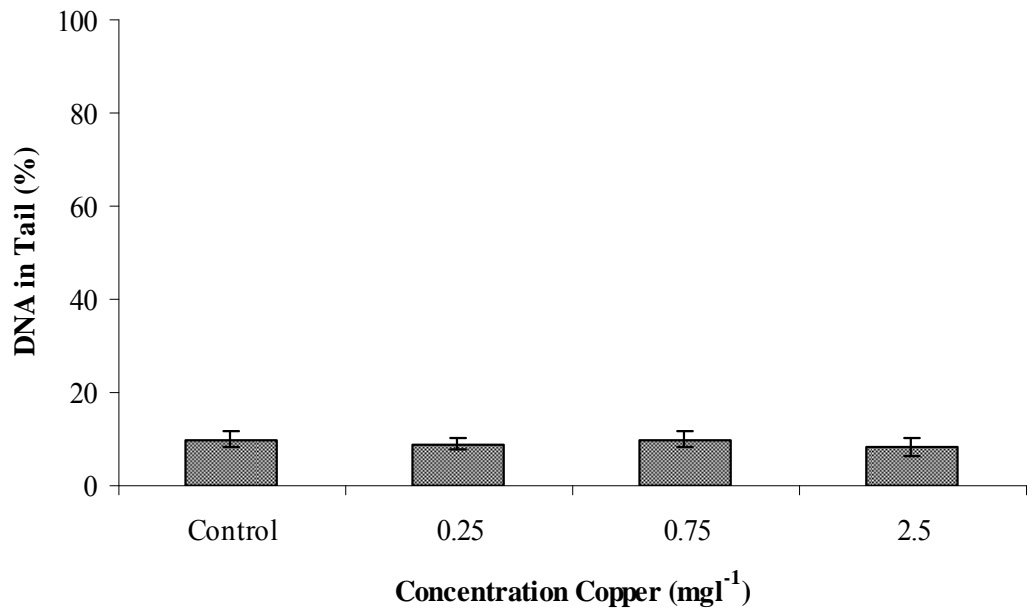
(a)



(b)



**Figure 3.** Effects of CuCl<sub>2</sub> (a), and CdCl<sub>2</sub> (b) on *T. battagliai* after 24 h (□) and 48 h (▨) exposure. Data is expressed as a percentage of unexposed controls ± SEM of three replicates for each exposure concentration. \* denoted significant difference from the control ( $p \leq 0.05$ ).



**Figure 4** DNA integrity, evaluated as electrophoretic DNA migration (% Tail DNA), in *Tetraselmis suecica* exposed to concentrations of CdCl<sub>2</sub>.