A test battery approach to the ecotoxicological evaluation of cadmium and copper employing a battery of marine bioassays

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ABSTRACT

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 ($CdCl_2$ and $CuCl_2$) 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 $CdCl_2$ was found to be more toxic to the fish cell lines PLHC-1 and RTG-2 than $CuCl_2$. *Tisbe battagliai* was the most sensitive species to both metals and the Microtox® 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 EC_{50} value for $CdCl_2$ and the marine prasinophyte, *T. suecica.* Conversely, $CuCl_2$ was observed to be highly toxic to the marine alga, EC_{50} of 1.19 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 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 $CuCl_2$ 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. Introduction

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

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

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

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

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

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

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

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

Alkaline single-cell gel electrophoresis, also known as the Comet assay, developed by Singh et al. (1988), allows for the investigation of damage to genomic DNA caused by genotoxic agents. The Comet assay is a valuable method that has grown in popularity in recent years. Although mammalian cells are commonly employed in the Comet assay, the test can be applied to many other cell types (Tice et al., 2000). The assay has been successfully adapted for many aquatic species such as clams (Hartl et al., 2004), mussels (Nigro et al., 2006), dog-whelks (Hagger et al., 2006) among others. Erbes et al. (1997) detected DNA damage in the green algae *Chlamydomonas reinhardtii* exposed to 4-nitroquinoline-1-oxide, *N-nitorodiumethylamine* and H_2O_2. As phytoplankton are the primary producers in marine ecosystems, sub-lethal effects on these organisms are of great importance. Cadmium is a known carcinogen and its toxic mechanisms have been well documented. The genotoxic effects of Cd on phytoplankton has been investigated (Desai et al., 2006), however, there is little known about the genotoxic effects of Cu especially in phytoplankton. Therefore the genotoxic effects of Cu to *T. suecica* were investigated in this study.

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

2. Materials and Methods

2.1 Test Substances

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

2.2 Ecotoxicity tests

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

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

2.2.1 Microtox® assay

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

2.2.2 Microalgal toxicity test

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

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

2.2.3 Copepod toxicity test

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

2.2.4 Cell culture

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

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

2.2.4.1 Cytotoxicity testing: Metal exposure

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

2.2.5 Comet Assay

Three test concentrations of copper were selected based on the previous toxicity tests with *T. suecica* (section 2.2.2) and $CuCl_2$. These concentrations (0.25, 0.75 and 2.5 mg l^{-1}) along with a control were set up in duplicate as per the ISO standard method (BS EN ISO 10253, 1998). The initial algal density of all flasks was 1 x 10^4 cell ml^{-1} in a final volume of 20 ml. Cell density of each flask was measured after 72 h to ensure similar growth to initial toxicity tests.

The Comet assay was performed according to modified procedures based on Singh et al. (1988) and Hagger et al. (2006). Five millilitres of culture was harvested from each replicate after 72. All sub-samples were centrifuged at 2000 rpm for 5 min at 4 °C. After centrifugation supernatant was discarded and cell pellets were re-suspended in 180 µl of low melting point agarose (LMP) and aspirated. Immediately 80 µl of cell suspension/LMP agarose were dropped onto frosted microscopic slides which were previously coated with 1 % normal melting point agarose (NMP) dissolved in PBS. Coverslips were gently placed over the cell suspension and slides transferred to an icepack to allow the agarose to set. When the gels had set the slides were placed into freshly prepared lysing solution at 4 °C for 1 h (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris [pH 10.0]). One percent Triton X-100 and 10 % DMSO were added to the lysis solution after the pH was adjusted and immediately prior to adding the slides. The lysis and all following steps were conducted in the dark to prevent any further damage to cell DNA.

Slides were removed from the lysis buffer and placed in a horizontal electrophoresis tank (Compact L/XL, Biometra, Germany). The tank was filled with electrophoresis buffer (300 mM NaOH, 1 mM Na_2-EDTA [pH 13]) until all slides were covered, and the DNA was allowed to unwind for 40 min before electrophoresis. Electrophoresis was performed at 25 V, 300 amps for 30 min. After electrophoresis, slides were removed from the tank and rinsed three times (for 5 min each time) in neutralising buffer (0.4 M Tris, pH 7.5). Finally, slides were stained with 20 µl of 5 µg ml^{-1} ethidium bromide solution and viewed under ultraviolet fluorescence light (400x). Slides were scored using the Komet software (version 5.0; Kinetic imaging Ltd, Wirral, UK). Twenty five cells were scored per slide and 2 slides per treatment were scored (50

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

2.2.6 Statistical analysis

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

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

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

3. Results

After initial range finding tests final concentration ranges of 0.065 – 16.670 mg l^{-1} and 1.758 – 450 mg l^{-1} for $CuCl_2$ and $CdCl_2$ respectively were employed in the definitive testing with the Microtox® system. Bioluminescence of *V. fischeri* (Microtox®) decreased after exposure to both $CdCl_2$ and $CuCl_2$ indicating that both chemicals are toxic to the marine bacterium. Figure 1 shows the experimental values of the relative light intensity (normalized with respect to the initial light intensity) at various concentrations of (a) $CuCl_2$ and (b) $CdCl_2$ (only five concentrations graphed for clarity purposes). Toxicity of both metals to *V. fischeri* was observed to increase with time and copper was an order of magnitude more toxic than cadmium for all time intervals (Table 2).

Initial range finding tests with $CdCl_2$ and *T. suecica* (0.001 – 100 mg l^{-1}) failed to identify a suitable concentration range for definitive testing. Significant toxicity was observed at 100 mg l^{-1} but the level of growth inhibition was well below 50 %. Further testing with $CdCl_2$ and *T. suecica* were therefore not performed as higher concentrations were not deemed relevant to known environmental levels (Kilemade et al., 2004; Davoren et al., 2005; Cronin et al., 2006). Copper chloride was considerably more toxic to the marine prasinophyte and showed significant inhibition of growth at concentrations as low as 0.25 mg l^{-1} (Figure 2) and yielded an EC_{50} of 1.19 mg l^{-1} (Table 2).

Results of toxicity testing with *T. battagliai* and the two metals are shown in Table 2. Copper chloride was more toxic than $CdCl_2$ with 24 and 48 h LC_{50} values of 0.19 mg l^{-1} and 0.08 mg l^{-1} respectively. However, *T. battalgiai* was more sensitive to both metals than either the bacterial or algal tests. There was significant toxicity at all

concentrations of $CuCl_2$ after 48 h exposure (0.05 – 1.00 mg l^{-1}). Significant effects were only seen with $CdCl_2$ at 0.2 – 1.0 mg l^{-1} after 48 h exposure (Figure 3).

The degree of DNA structural integrity was evaluated in *T. suecica* cells by single cell gel electrophoresis (Comet assay) after 72 hours of exposure to $CuCl_2$. Fifty comets per concentration were measured, 25 per replicate. Results showed that there was no significant DNA damage at the concentrations tested (Figure 4) compared to the control. In the control percentage tail DNA in the algal cells was 9.93 ± 1.59 % (SEM) and at the top concentration (2.5 mg l^{-1}) percentage tail DNA was 8.45 ± 1.89 % (SEM). These concentrations were selected based on acute toxicity results so that a comparison could be made between growth inhibition and genotoxicity. The cell viability for the Comet assay was recorded for each concentration employed (Control, 0.25 mg l^{-1}, 0.75 mg l^{-1}, 2.5 mg l^{-1}) resulting in cell viabilities of over 90 % at all concentrations apart from 2.5 mg l^{-1} (> 80 % viability).

In the cell assays a toxic effect was observed with both $CdCl_2$ and $CuCl_2$ on both cell lines tested with both the NR and AB assays. All calculated cytotoxicity values are presented in Table 3. Cadmium was observed to be the most toxic compound to both cell lines. There was significant toxicity ($p \leq 0.05$) with cadmium at all concentrations with the PLHC-1 cell line (24 and 96 h) for both the NR and AB assays. For the RTG-2 cells there was significant toxicity at all concentrations after 96 h with cadmium as determined by NR and AB. The 24 h EC_{50} with AB and the PLHC-1 cell line and $CdCl_2$ was 11.31 (11.04 – 11.48) mg l^{-1}, while for the RTG-2 cell line it was 20.90 (18.33 – 23.14) therefore indicating that the RTG-2 cells were less sensitive than the PLHC-1.

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

were only significantly affected by copper at the top two concentrations with both assays. The same difference in sensitivity between cell lines was observed for copper as were for cadmium. The 96 h EC_{50} with PLHC-1 cells and $CuCl_2$ as determined by AB was 56.28 (51.82 – 62.38) mg l^{-1} while for RTG-2 cells it was 92.04 (86.98 – 100.47) mg l^{-1}. In the case of copper the AB assay was more sensitive for both cell types after 96 h e.g. 96 h NR EC_{50} was 65.43 (62.30 – 68.24) mg l^{-1} and the 96 h AB EC_{50} was 56.28 (51.82 – 62.38) mg l^{-1} for PLHC-1 cells.

With the exception of the EC values for $CuCl_2$ and the PLHC-1 cell line as determined by NR, toxicity of both metals was observed to increase with lengthened exposure time for all endpoints. Based on these values the toxicity ranking was identical for both NR and AB (EC_{10} and EC_{50}) in the order $CdCl_2$ > $CuCl_2$ for PLHC-1 and RTG-2 cells. However, there was a significant difference in the sensitivities of the two cell lines employed. In general the PLHC-1 cells were more sensitive than the RTG-2 cells and AB was the most sensitive end point employed.

Tables 2 and 3 summarise the ecotoxicity and cytotoxicity data respectively for all species and both metals. Copper chloride was the most toxic of the two to *V. fischeri, T. suecica* and *T. battagliai.* In contrast to this $CdCl_2$ was the most toxic chemical to the fish cell lines. Of all the assays employed the acute lethality test with *T. battalgiai* was the most sensitive.

4. Discussion

The investigation into the toxicity of cadmium and copper to a battery of bioassays showed varying toxicity between test species. In the Microtox® assay the light production in *V. fischeri* is directly proportional to the metabolic activity of the bacterial population and inhibition of enzymatic activity correspondingly decreases bioluminescence. The use of this assay provides a measure of sub-lethal response. In this study both metals elicited a toxic effect in the Microtox® assay, however, the assay was one of the least sensitive of the battery tests employed. Copper chloride was an order of magnitude more toxic than $CdCl_2$ to the bacteria and the toxicity of both metals was observed to increase with lengthened exposure time (Table 2). This increase in toxicity with time is indicative of metal contamination and is a well documented effect in *V. fischeri* (Azur Environment Ltd., 1998). Codina et al. (2000) obtained EC_{50} values of 150 μm l^{-1} (34.25 mg l^{-1}) and 7.2 μmol l^{-1} (1.23 mg l^{-1}) for $CdCl_2 \cdot 2.5H_2O$ and $CuCl_2 \cdot 2H_2O$ respectively. These results concur with the values obtained in this study employing the anhydrous metal salts of cadmium and copper. Newman and McCloskey (1996) investigated total EC_{50} values for nine metals (added as chloride salts) and the values for both agreed with our study and other studies within the literature. Their EC_{50} value for $CuCl_2$ was 2.78 μmol l^{-1} (4.8 mg l^{-1}) and their value for $CdCl_2$ was 195 μmol l^{-1} (35.75 mg l^{-1}). In all studies cadmium toxicity to *V. fischeri* was found to be low compared to copper. A lack of sensitivity of Gram negative bacteria towards cadmium has previously been reported (Bitton and Freihoffer, 1978; Morozzi et al., 1986; Bauda and Block, 1990). These authors attributed the low toxicity of cadmium to the presence of exopolysaccharides on the outer layer of the bacterial membrane, which have been

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

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

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

disrupting enzyme active sites and cell division. Copper may also exert toxicity on subcellular organelles interfering with mitochondrial electron transport, respiration, ATP production and photosynthesis. Nassiri et al., (1996) noted the presence of copper in the walls of a multilayered cell in *T. suecica* suggesting that these structures constitute an adsorbing area for this element. They also reported drastic damaging effects of copper contamination such as the depletion of starch which would perturb photosynthesis and the loss of flagella leading to a lack of motility in *T. suecica* after ultrastructure examination. Conversely there was no ultrastructural damage evident in the algae exposed to cadmium. In this study surviving *T. suecica* exposed to higher levels of $CuCl_2$ (< 1.0 mg l^{-1}) were observed to have reduced motility compared to the controls but there was no obvious reduction in motility in the higher concentrations of $CdCl_2$. Copper reduces growth as well as photosynthetic and respiratory activities (Nalewajko and Olaveson, 1995). The photosynthetic machinery is particularly susceptible to copper and may result in a decrease in the activity of photosystem II and electron transfer rates within the algae (Fernades and Henriques, 1991; Mallick and Mohn, 2003). Cid et al. (1995) also observed copper to interfere with algal photosynthesis and ATP production, with 0.5 mg l^{-1} causing a 50 % decrease in photosynthesis.

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

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

The toxicities of $CdCl_2$ and $CuCl_2$ to *T. battagliai* were very similar (Table 2). Very little data exist in the literature for *T. battalgiai* and the heavy metals Cd and Cu. However, comparable 96 h EC_{50} values were generated by Hutchinson et al. (1994) when assaying $CdCl_2$ and $Cu[NO_3]_2$ with *T. battagliai* (Tables 1 and 2). Bechmann (1999) observed that the sensitivity to copper varies between copepod species. LC_{50} values for copepods (including eight different species, differing life stages and different test conditions) ranged from 19 to 762 μg Cu l^{-1} (O'Brian et al., 1988).

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

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

It is well established that *in vitro* studies on fish cell lines are less sensitive than *in vivo* fish cell studies (Babich et al., 1986; Babich et al., 1990; Babich and Borenfreund, 1987; Saito et al., 1991; Fent and Hunn, 1996). Castaño et al. (1996) found that the RTG-2 cell line was between 20 to 200 times less sensitive than *in vivo* trout bioassays. In this study corresponding *in vivo* toxicity assays with fish were not conducted to validate the toxicity of these chemicals however comparative values exist within the literature. Besser et al. (2007) reported 96 h LC_{50} values of 5.2 (4.7 – 5.9) and 42 (39 – 46) μg l^{-1} for Cd and Cu respectively. In this study 96 h EC_{50} values for RTG-2 cells with Cd and Cu were three orders of magnitude less sensitive than the reported *in vivo* results with a 96 h EC_{50} for $CdCl_2$ of 7.12 (4.96 – 9.47) mg l^{-1} (7120 [4960 – 9470] μg l^{-1}) and 96 h EC_{50} for $CuCl_2$ of 46.74 (38.58 – 54.83) mg l^{-1} (46740 [38580 - 54830] μg l^{-1} with the AB assay. However, there is increasing pressure to reduce the numbers of fish employed in regulatory testing. Therefore there is a need to find alternative ways in which to accurately assess the potential hazard of a chemical or environmental sample (e.g. relocation of dredged sediment). The reduced sensitivity of *in vitro* cell line methods would make it unfeasible to employ these assays in a regulatory capacity to assess the pollution status of environmental samples. However there is a possibility of employing *in vitro* cells lines as screening tools for the ranking of environmental samples. Although *in vitro* assays do not reflect the true *in vivo* situation and absolute toxicities have been observed to differ, good correlation in terms

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

The genotoxic effect of copper on *T. suecica* was investigated using single cell gel electrophoresis (or Comet assay). Although cadmium has been observed to cause DNA damage in phytoplankton (Desai et al., 2006) it was not assayed with *T. suecica* as it was not possible in this study to obtain significant effects on the growth of the alga at the concentrations assayed (i.e. environmentally relevant concentrations). Unlike cadmium, the possible genotoxic effects of copper have not been fully investigated. From this study it is apparent that $CdCl_2$ has no significant genotoxic effect on *T. suecica* at the concentrations tested. The concentrations tested (0.25, 0.75 and 2.5 mg l^{-1}) were selected because at concentrations greater than 2.5 mg l^{-1} inhibition of growth was too great to guarantee sufficient cell survival and viability. Although there was no genotoxic effect observed in this study, the genotoxic potential of copper has been observed in the literature. Guecheva et al. (2001) observed significant DNA damage post exposure to copper in planarians (24 h or 7 d). In this study the algae were only exposed for 72 h therefore it may be that a longer period of incubation may be required before an effect is observed. Other studies have exposed the algae for longer durations. Desai et al. (2006) exposed *Chaetoceros tenuissimus* to $CdCl_2.H_2O$ for 20 days in total.

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

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

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

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

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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 $CuCl_2$. Data are expressed as a percentage of unexposed controls ± 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 %.

Figure 3. Effects of $CuCl_2$ (a), and $CdCl_2$ (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 $CdCl_2$.

Table 1

Trophic level	Test Species	Exposure Time	End point	Chemical	Toxicity value	Reference
Bacteria	*Vibrio fischeri*	15 min	EC_{50}	Cu (II)	0.5 ± 0.1 mg l^{-1}	Utgikar et al.,2004
		15 min	EC_{50}	Cd^{2+}	27 ± 5 µmol l^{-1}	Newman, 1995
		15 min	EC_{50}	Cu^{2+}	1.62 ± 0.13 µmol l^{-1}	Newman, 1995
		15 min	EC_{50}	$CdCl_2$	195 ± 18.8 µmol l^{-1}	Newman & McCloskey, 1996
		15 min	EC_{50}	$CuCl_2$	2.78 ± 0.52 µmol l^{-1}	Newman & McCloskey, 1996
			EC_{50}	$CdCl_2$	9.4 mg l^{-1}	Peinado et al., 2002
			IC_{50}	Cd in HNO_3	50.4 ± 7.61 µg l^{-1}	Hsieh et al., 2004
			IC_{50}	$Cu[NO_3]_2$	7.08 ± 0.352 µ g l^{-1}	Hsieh et al., 2004
		5 min	EC_{50}	Cu	1.3 mg l^{-1}	Toussaint et al., 1995
		15 min	EC_{50}	$CdCl_2 \cdot 2.5H_2O$	150 µmol l^{-1}	Codina et al. 2000
		15 min	EC_{50}	$CuCl_2 \cdot 2H_2O$	7.2 µmol l^{-1}	Codina et al. 2000
Algae	*Tetraselmis suecica*	96 h	EC_{50}	$CdCl_2$	5.8 mg l^{-1}	Pérez-Rama et al., 2001
	Tetraselmis suecica	6 d	EC_{50}	$CdCl_2$	7.9 ± 1 mg l^{-1}	Pérez-Rama et al., 2002
	Tetraselmis suecica		IC_{50}	$CdCl_2$	9.38 mg l^{-1}	Nassiri et al., 1996
	Tetraselmis suecica		IC_{50}	$CuCl_2$	0.172 mg l^{-1}	Nassiri et al., 1996
	Tetraselmis suecica		EC_{50}	$CuSO_4.5H_2O$	40 mg l^{-1}*	De Kuhn et al., 2006*
Copepods	*Tisbe battagliai*	96 h	LC_{50}	$CdCl_2$	0.34 mg l^{-1}	Hutchinson et al., 1994
	Tisbe battagliai	96 h	LC_{50}	$Cu[NO_3]_2$	0.088 mg l^{-1}	Hutchinson et al., 1994
Fish Cells	RTG-2	48 h	NR_{50}	Cd in HNO_3	0.055 mmol l^{-1}	Castaño et al. 1996
		48 h	NR_{50}	Cu in HNO_3	0.150 mmol l^{-1}	Castaño et al. 1996
	PLHC-1	24 h	NR_{50}	$CuSO_4.5H_2O$	32.4 µg ml^{-1}	Ryan & Hightower, 1994
		24 h	NR_{50}	$CdCl_2.2.5H_2O$	µg ml^{-1}	Ryan & Hightower, 1994

EC_{50} = Effective Concentration of 50 % of sample population, LC_{50} = Lethal concentration of 50 % of sample population, NOEC = No Observed Effects Concentration, NR_{50} = 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^{-1})	EC_{10}[a] (mg l^{-1})	EC_{50}[a] (mg l^{-1})	NOEC[b] (mg l^{-1})	LOEC[c] (mg l^{-1})
$CdCl_2$	*Vibrio fischeri*	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
	Vibrio fischeri	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
	Vibrio fischeri	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
$CuCl_2$	*Vibrio fischeri*	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
	Vibrio fischeri	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
	Vibrio fischeri	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
$CuCl_2$	*Tetraselmis suecica*	72 h	Growth inhibition	0.100 – 5.0	0.26 (0.20 – 0.30)	1.19 (1.04 – 1.26)	0.100	0.250
$CdCl_2$	*Tisbe battagliai*	24 h	lethality	0.050 – 1.0	0.13 (0.06 – 0.21)	0.84 (0.66 – 1.07)	0.400	0.800
	Tisbe battagliai	48 h	lethality	0.050 – 1.0	0.02 (0.01 – 0.06)	0.19 (0.14 – 0.24)	0.100	0.200
$CuCl_2$	*Tisbe battagliai*	24 h	lethality	0.050 – 1.0	0.07 (0.05 – 0.09)	0.19 (0.16 – 0.21)	0.100	0.200
	Tisbe battagliai	48 h	lethality	0.050 – 1.0	0.02 (0.01 – 0.04)	0.08 (0.07 – 0.11)	< 0.050	0.050

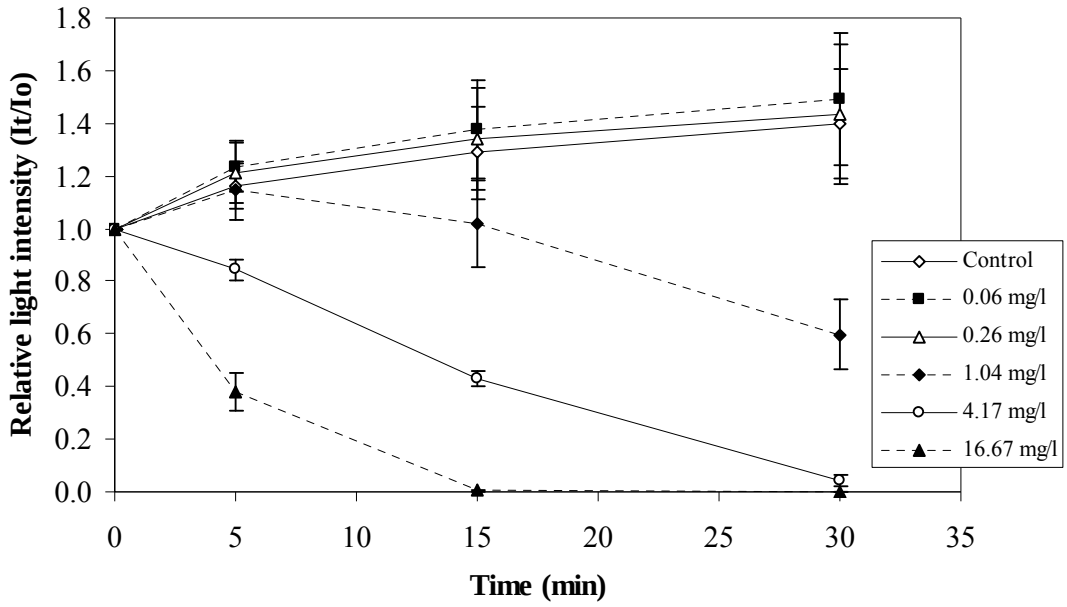
[a]EC_{50} values and corresponding 95 % confidence intervals in parentheses
[b]NOEC, no observed effect concentration, the highest observed concentration at which no significant effect ($p \leq 0.05$) was detected
[c]LOEC, lowest observed effect concentration, the lowest concentration of the tested 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^{-1})	EC_{10}[a] (mg l^{-1})	EC_{50}[a] (mg l^{-1})	NOEC[b] (mg l^{-1})	LOEC[c] (mg l^{-1})
$CdCl_2$	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
$CuCl_2$	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

[a]EC_{50} values and corresponding 95 % confidence intervals in parentheses
[b]NOEC, no observed effect concentration, the highest observed concentration at which no significant effect ($p \leq 0.05$) was detected
[c]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)

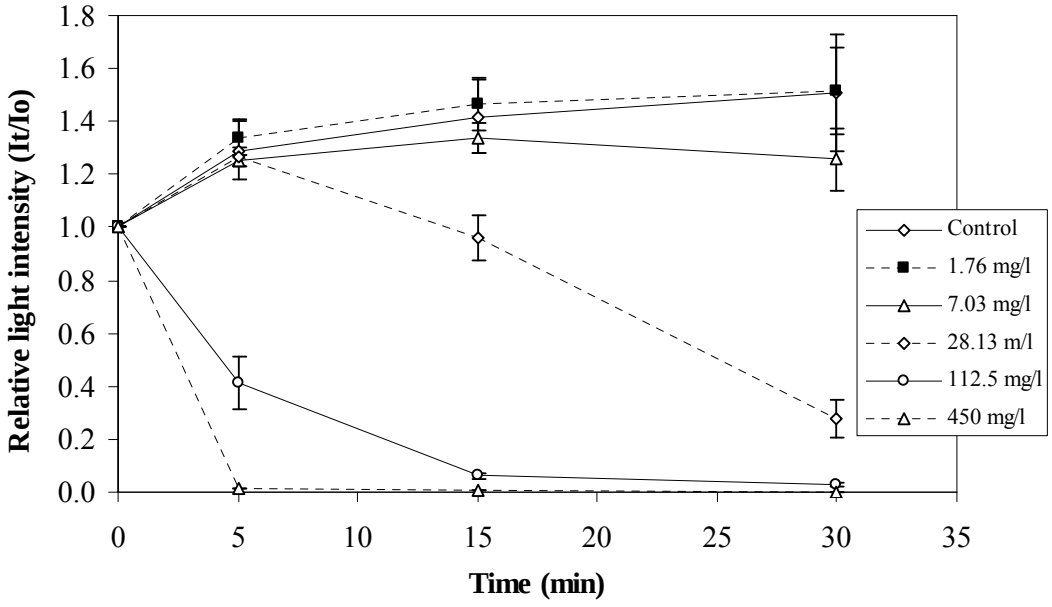


Figure 1 Light attenuation of *Vibrio fischeri* following exposure to (a) various copper chloride concentrations, and (b) various cadmium chloride concentrations.

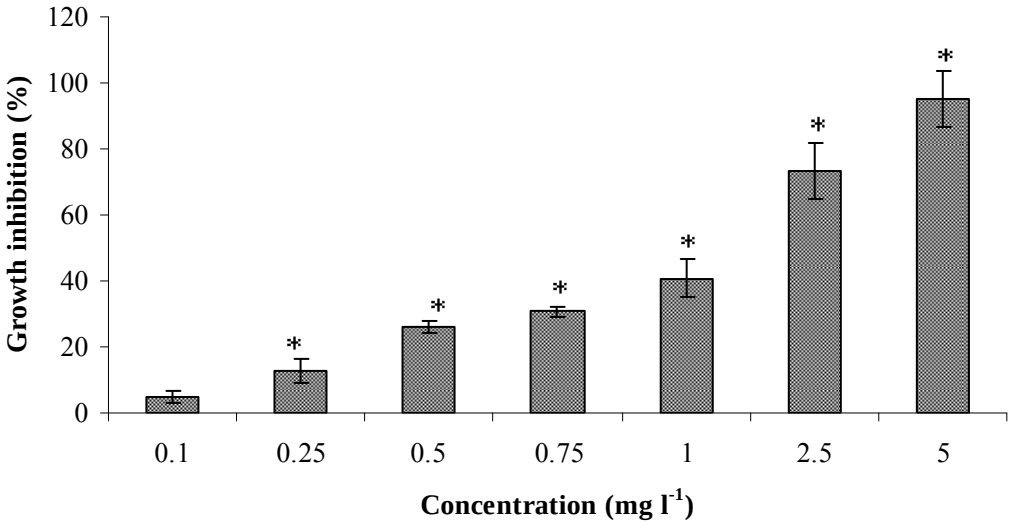
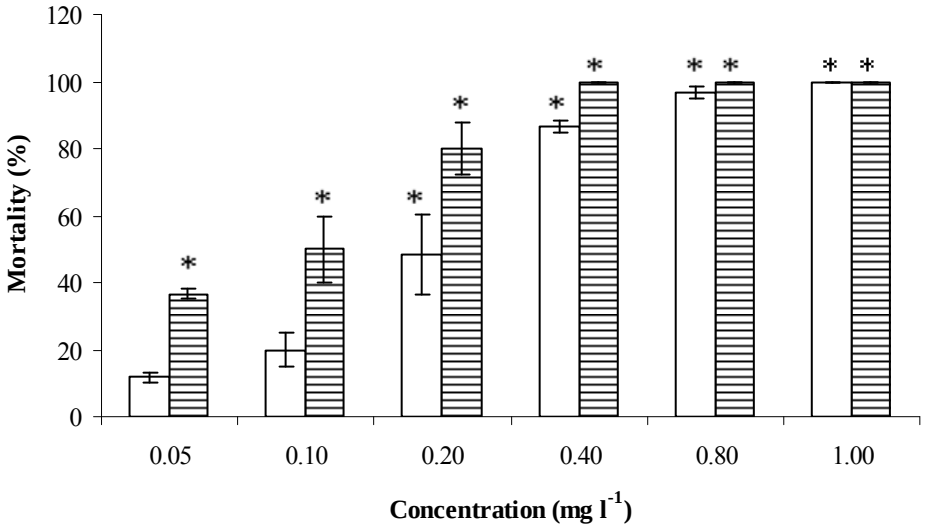


Figure 2 Percentage growth inhibition of *T. suecica* after 72 h exposure to $CuCl_2$. Data are expressed as a percentage of unexposed controls ± SEM of three independent experiments. * denotes significance from the control ($p \leq 0.05$). C.V. for the controls ranged from 1.18 – 8.89 %.

(a)



(b)

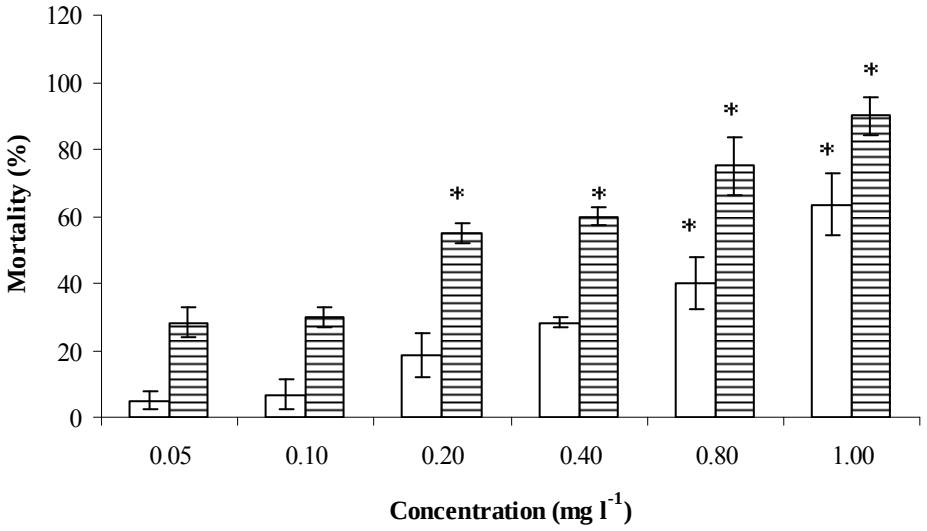


Figure 3. Effects of $CuCl_2$ (a), and $CdCl_2$ (b) on *T. battagliai* after 24 h (□) and 48 h (⊟) exposure. Data is expressed as a percentage of unexposed controls ± SEM of three replicates for each exposure concentration. * denoted significant difference from the control ($p \leq 0.05$).

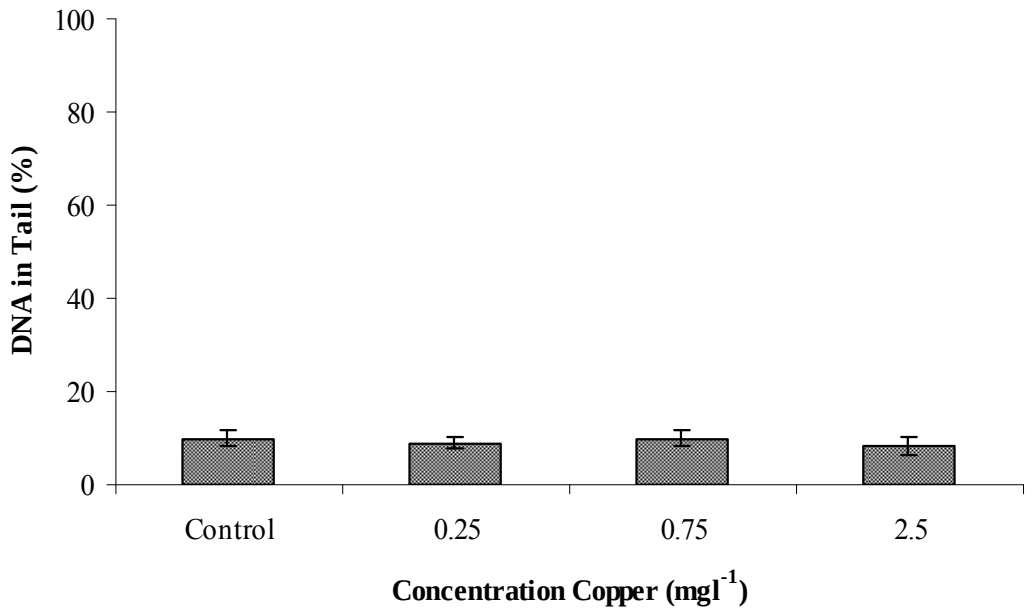


Figure 4 DNA integrity, evaluated as electrophoretic DNA migration (% Tail DNA), in *Tetraselmis suecica* exposed to concentrations of $CdCl_2$.