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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

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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 (Rainbow 1993; Warnau et al. 1995). 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.

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). 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 2,000 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 35×10^3 to 90×10^3 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).

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. The bioavailability of metals may also be affected by the presence of natural organic matter e.g. humic acids. Humic acids are able to bind a variety of metals at their carboxylic groups, altering the bioavailability and consequently affecting the toxicity (Tsiridis et al. 2006).

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. 2008). 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: Proteobacteria; Gammaproteobacteria), *Tetraselmis suecica* (primary producer: Chlorophyta; Prasinophyceae), *Tisbe battagliai* (primary consumer: Arthropoda; Maxillopoda) and the fish cell lines PLHC-1 (*Poeciliopsis lucida* hepatoma cell line: Chordata; Actinopterygii) and RTG-2 (*Oncorhynchus mykiss* gonad cells: Chordata; Actinopterygii) 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. 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.

Materials and methods

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).

Ecotoxicity tests

For each chemical stock solutions of 1,000 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 ± 2°C. 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).

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 (1989). About 5, 15 and 30 min EC_{50} tests were performed using the 90% basic test for aqueous

Table 1 Ecotoxicity data from the literature for the species and chemicals employed in this study

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 and McCloskey (1996)
		15 min	EC_{50}	$CuCl_2$	2.78 ± 0.52 µmol l^{-1}	Newman and 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}	$CuSO_4 \cdot 5H_2O$	40 mg l^{-1}[a]	De Kuhn et al. (2006)[a]
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}	$CuSO_4 \cdot 5H_2O$	32.4 µg ml^{-1}	Ryan and Hightower (1994)
		24 h	NR_{50}	$CdCl_2 \cdot 2.5H_2O$	µg ml^{-1}	Ryan and 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

[a] Inhibition of the motility of *Tetraselmis suecica*

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.

Microalgal toxicity test

Tetraselmis 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°C with continuous shaking at 100 rpm, illumination of 10,000 lux and a continuous photoperiod. The initial algal density of all flasks was 1×10^4 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.

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°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 and 48 h.

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, UK) at either 20°C (RTG-2) or 30°C (PLHC-1) under a normoxic atmosphere.

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 2×10^5 cells per ml for RTG-2 cells and 8×10^5 cells per ml for PLHC-1 cells for 24 h exposure periods. For 96 h exposure periods cells were seeded at 1.6×10^5 cells per ml for RTG-2 cells and 2×10^5 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).

Comet assay

Three test concentrations of copper were selected based on the previous toxicity tests with *T. suecica* (see section "Microalgal toxicity test") 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 1×10^4 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). Comet slides were scored using the Komet software (version 5.0; Kinetic imaging Ltd, Wirral, UK). Twenty five cells were scored per slide and two slides per treatment were scored (50 cells per treatment in total). DNA damage was reported as percentage tail DNA for the algal cells. Finally, cell viability was tested by means of the trypan-blue exclusion method (Absolom 1986).

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) × 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% 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.

Results

After initial range finding tests final concentration ranges of 0.065–16.670 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 (1) $CuCl_2$ and (2) $CdCl_2$ (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} (Fig. 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 and Fig. 3. Copper chloride was more toxic than $CdCl_2$ with 24 and 48 h LC_{50} values

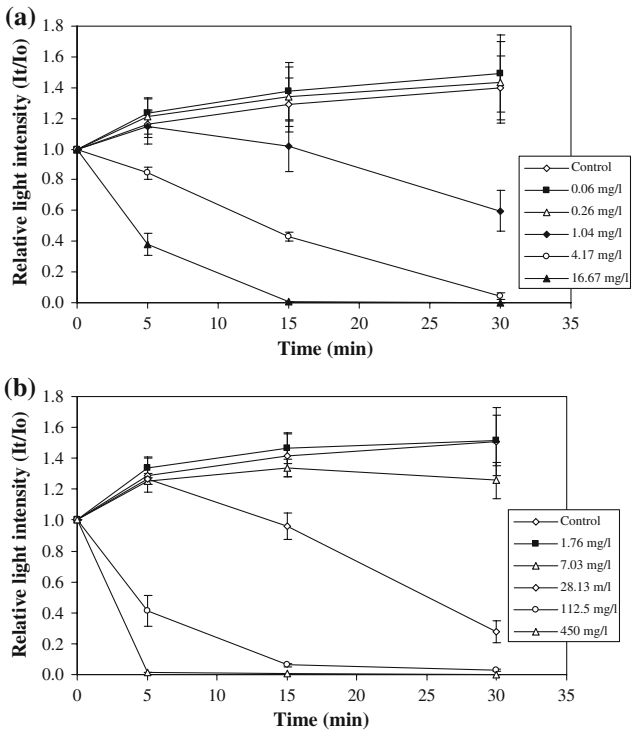

Fig. 1 Light attenuation of *Vibrio fischeri* following exposure to **a** various copper chloride concentrations, and **b** various cadmium chloride concentrations

of 0.19 mg and 0.08 mg l^{-1}, respectively. However, *T. battalgiai* was more sensitive to both metals than either the bacterial or algal tests.

The degree of DNA structural integrity was evaluated in *T. suecica* cells by single cell gel electrophoresis (Comet assay) after 72 h of exposure to $CuCl_2$. Results showed that there was no significant DNA damage at the concentrations tested (Fig. 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). The cell viability for the Comet assay was recorded for each concentration employed (Control, 0.25, 0.75, 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 mg l^{-1}, while for the RTG-2 cell line it was 20.90 therefore indicating that the RTG-2 cells were less sensitive than the PLHC-1.

The same difference in sensitivity between cell lines was observed for both metals. The 96 h EC_{50} with PLHC-1 cells and $CuCl_2$ as determined by AB was 56.28 mg l^{-1} while for RTG-2 cells it was 92.04 mg l^{-1}. In the case of copper the AB assay was more sensitive for both cell types after 96 h. Based on EC_{50} values the toxicity ranking for the fish cell lines were 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.

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

Table 2 Results of ecotoxicty testing with all species for $CdCl_2$ and $CuCl_2$

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

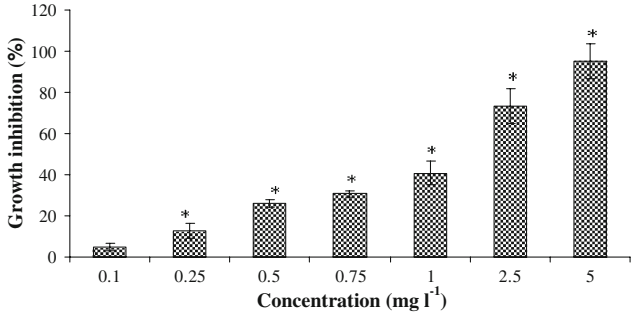

Fig. 2 Percentage growth inhibition of *T. suecica* after 72 h exposure to $CuCl_2$. Data are expressed as a percentage of unexposed controls ± SEM of three independent experiments. *Significance from the control ($P \leq 0.05$). CV for the controls ranged from 1.18 to 8.89%

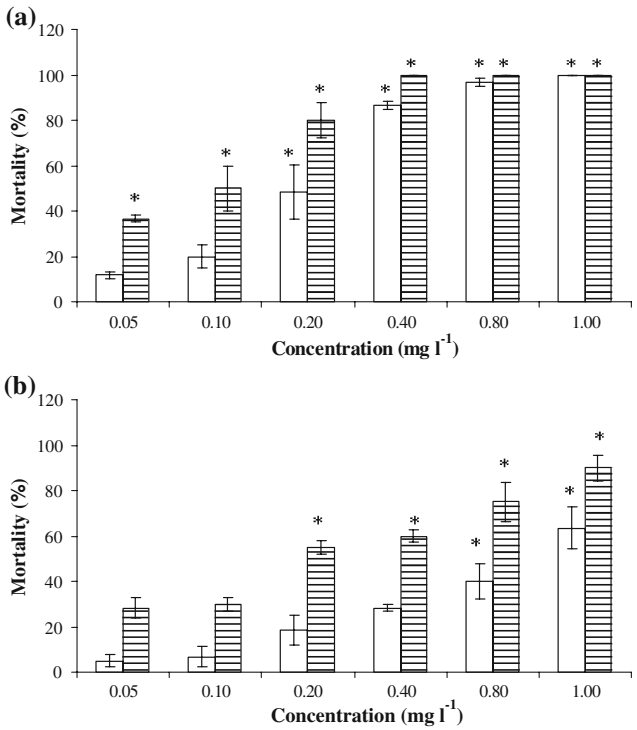

Fig. 3 Effects of $CuCl_2$ (**a**), and $CdCl_2$ (**b**) on *T. battagliai* after 24 h (□) and 48 h ⊟ exposure. Data is expressed as a percentage of unexposed controls ± SEM of three replicates for each exposure concentration. *Significant difference from the control ($P \leq 0.05$)

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*

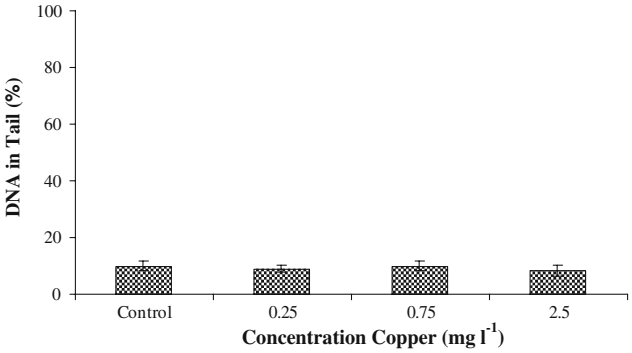

Fig. 4 DNA integrity, evaluated as electrophoretic DNA migration (% Tail DNA), in *Tetraselmis suecica* exposed to concentrations of $CdCl_2$

(Azur Environmental Ltd 1989). 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; Rai et al. 1981). In this study $CuCl_2$ was 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 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.

Table 3 Results of cytotoxicty testing with PLHC-1 and RTG-2 for $CdCl_2$ and $CuCl_2$

Test chemical	Test Species/ cell line	Exposure time (h)	Endpoint	Concentration range (mg l^{-1})	EC^a_{10} (mg l^{-1})	EC^a_{50} (mg l^{-1})	$NOEC^b$ (mg l^{-1})	$LOEC^c$ (mg l^{-1})
$CdCl_2$	PLHC-1	24	NR	10–40	10.29 (9.78–10.98)	14.09 (14.12–14.73)	<10	10
	PLHC-1	96	NR	10–40	5.15 (0.46–7.08)	6.67 (1.56–8.13)	<10	10
	PLHC-1	24	AB	10–40	7.86 (7.50–8.18)	11.31 (11.04–11.48)	<10	10
	PLHC-1	96	AB	10–40	<10	<10	<10	10
	RTG-2	24	NR	10–40	14.40 (10.98–17.55)	25.88 (23.96–28.39)	20	25
	RTG-2	96	NR	10–40	4.61 (2.31–6.54)	11.47 (9.32–13.46)	<10	10
	RTG-2	24	AB	10–40	12.47 (8.86–16.03)	20.90 (18.33–23.14)	15	20
	RTG-2	96	AB	10–40	7.12 (4.93–9.47)	10.26 (9.10–11.70)	<10	10
$CuCl_2$	PLHC-1	24	NR	10–100	41.82 (33.54–57.36)	56.28 (51.97–62.23)	20	40
	PLHC-1	96	NR	10–100	50.25 (44.04–54.48)	65.43 (62.30–68.24)	40	60
	PLHC-1	24	AB	10–100	69.10 (63.70–78.86)	–	60	80
	PLHC-1	96	AB	10–100	41.82 (34.59–57.19)	56.28 (51.82–62.38)	40	60
	RTG-2	24	NR	10–100	98.90 (93.50–130.78)	–	>100	>100
	RTG-2	96	NR	10–100	67.17 (56.98–88.93)	–	80	100
	RTG-2	24	AB	10–100	74.85 (69.57–86.58)	–	80	100
	RTG-2	96	AB	10–100	46.74 (38.58–54.83)	92.04 (86.98–100.47)	60	80

NR neutral red, *AB* alamar blue

[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

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.

Nassiri et al (1996) 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. 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 presence of these phytochelatins, along with the potential effects of differing physio-chemical parameters, may explain the low toxicity observed in this study.

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, 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 and 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} [7,120 (4,960–9,470) μg l^{-1}] and 96 h EC_{50} for $CuCl_2$ of 46.74 (38.58–54.83) mg l^{-1} [46,740 (38,580–54,830) 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 days). 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 $CdCl_2 \cdot H_2O$ 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.

The results of this study contribute to the understanding of the problems associated with assessing metal contamination and highlight some of 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. The findings of this study highlight the importance of employing a battery of species in the ecotoxicological assessment of single compounds as well as complex environmental samples. In this study the acute lethality test with *T. battagliai* was found to be the most sensitive of the test species. Copper was found to be the most toxic of the two metals to the bacteria, alga and copepod. In contrast cadmium was the most toxic in the in vitro fish cell line assays. Therefore, it is recommended that employing these tests in tandem is the most appropriate strategy for future testing of environmental samples, as their differing sensitivities, trophic status, mode of living (e.g. benthic copepod, pelagic microalgae) and potential effects [e.g. genotoxic (algal Comet assay), acute toxicity (copepod lethality), sublethal (algal growth inhibition)] will aid in the full interpretation of the effect of an "unknown" environmental mixture.

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References

Abel PD (1989) Water pollution biology. Ellis Horwood, Chichester

Absolom DR (1986) Basic methods for the study of phagocytosis. Methods Enzymol 132:95–182. doi:10.1016/S0076-6879(86)32005-6

Anderson LM, Kasprzak KS, Rice JM (1994) Preconception exposure of males and neoplasia in their progeny: effects of metals and consideration of mechanisms. In: Olshan AF, Mattison DR (eds) Male-mediated development toxicity. Plenum Press, NY, pp 129–140

Azur Environmental Ltd (1989) Microtox acute toxicity basic test procedures. Azur Environmental, Carlsbad

Babich H, Borenfreund E (1987) In vitro cytotoxicity of organic pollutants to bluegill sunfish (BF-2) cells. Environ Res 42:229–237. doi:10.1016/S0013-9351(87)80024-5

Babich H, Puerner JA, Borenfreund E (1986) In vitro cytoxicity of metals to bluegill (BF-2) cells. Arch Environ Contam Toxicol 15:31–37. doi:10.1007/BF01055246

Babich H, Goldstein SH, Borenfreund E (1990) In vitro cyto- and genotoxicity of organomercurials to cells in culture. Toxicol Lett 50:143–149. doi:10.1016/0378-4274(90)90004-6

Bauda P, Block JC (1990) Role of envelops of Gram negative bacteria in cadmium binding and toxicity. Toxicol Assess 5:47–60. doi:10.1002/tox.2540050105

Bechmann RK (1999) Effects of the endocrine disrupter nonylphenol on the marine copepod *Tisbe battagliai*. Sci Total Environ 233:33–46. doi:10.1016/S0048-9697(99)00177-1

Besser JM, Mebane CA, Mount DR, Ivey CD, Kunz JL, Greer IE, May TW, Ingersoll CG (2007) Sensitivity of mottled sculpins (*Cottus bairdi*) and rainbow trout (*Onchorhynchus mykiss*) to acute and chronic toxicity of cadmium, copper, and zinc. Environ Toxicol Chem 26:1657–1665. doi:10.1897/06-571R.1

Bitton G, Freihoffer V (1978) Influence of extracellular polysaccharides on the toxicity of copper and cadmium towards *Klebsiella aerogenes*. Microb Ecol 4:199–225. doi:10.1111/j.1574-6968.1978.tb02864.x

Bryan GW, Langston WJ (1992) Bioavailability, accumulation, and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. Environ Pollut 76:89–131. doi:10.1016/0269-7491(92)90099-V

BS EN ISO 10253 (1998) water quality-marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*. The British Standard Institute, London

Castaño A, Cantarino MJ, Castillo P, Tarazona JV (1996) Correlations between the RTG-2 cytotoxicity tests EC_{50} and in vivo LC_{50} rainbow trout bioassay. Chemosphere 32:2141–2157. doi:10.1016/0045-6535(96)00126-9

Codina JC, Cazorla FM, Pérez-García A, De Vicente A (2000) Heavy metal toxicity and genotoxicity in water and sewage determined by microbiological methods. Environ Toxicol Chem 19:1552–1558. doi:10.1897/1551-5028(2000)019<1552:HMTAGI>2.3.CO;2

Cronin M, McGovern E, McMahon T, Boelens R (2006) Guidelines for the assessment of dredge material for disposal in Irish waters. Marine Environmental and Health Series, No. 24. Marine Institute, Galway

Davoren M, Fogarty AM (2006) In vitro cytotoxicity assessment of the biocidal agents sodium *o*-phenylphenol, sodium *o*-benzyl-*p*-chlorophenol, and sodium *p*-tertiary amylphenol using established fish cell lines. Toxicol In Vitro 20:1190–1201. doi:10.1016/j.tiv.2006.03.005

Davoren M, Ní Shúilleabháin S, Hartl MGJ, Sheehan D, O'Brien NM, O'Halloran J, Van Pelt FNAM, Mothersill C (2005) Assessing the potential of fish cell lines as tools for the cytotoxicity of estuarine sediment aqueous elutriates. Toxicol In Vitro 19:421–431. doi:10.1016/j.tiv.2004.12.002

De Kuhn RM, Streb C, Breiter R, Richter P, Neeße T, Häder DP (2006) Screening for unicellular algae as possible bioassay organisms for monitoring marine water samples. Water Res 40:2695–2703. doi:10.1016/j.watres.2006.04.045

Desai SR, Verlecar XN, Nagarajappa Goswami U (2006) Genotoxicity of cadmium in marine diatom *Chaetoceros tenussimus* using the alkaline Comet assay. Ecotoxicology 15:359–363. doi:10.1007/s10646-006-0076-2

Fent K, Hunn J (1996) Cytotoxicity of organic environmental chemicals to fish liver cells (PLHC-1). Mar Environ Res 42:377–382. doi:10.1016/0141-1136(95)00043-7

Fulladosa E, Murat JC, Martínez M, Villaescusa I (2005) Patterns in metals and arsenic poisoning in *Vibrio fischeri* bacteria. Chemosphere 60:43–48. doi:10.1016/j.chemosphere.2004.12.026

Guecheva T, Henriques JAP, Erdtmann B (2001) Genotoxic effects of copper sulphate in freshwater planarian in vivo, studied with the single-cell gel test (comet assay). Mutat Res 497:19–27

Hagger JA, Depledge MH, Oehlmann J, Jobling S, Galloway TS (2006) Is there a causal association between genotoxicity and the imposex effect? Environ Health Perspect 114:20–26

Hsieh CY, Tsai MH, Ryan DK, Pancorbo OC (2004) Toxicity of the 13 priority pollutant metals to *Vibrio fischeri* in the Microtox® chronic toxicity test. Sci Total Environ 320:37–50. doi:10.1016/S0048-9697(03)00451-0

Hu SX, Lau KWK, Wu M (2001) Cadmium sequestration in *Chlamydomonas reinhardtii*. Plant Sci Limerick 161:987–996. doi:10.1016/S0168-9452(01)00501-5

Hutchinson TH, Williams TD, Eales GJ (1994) Toxicity of cadmium, hexavalent chromium and copper to marine fish larvae (*Cyprinodon variegates*) and copepods (*Tisbe battagliai*). Mar Environ Res 38:275–290. doi:10.1016/0141-1136(94)90028-0

Ismail M, Phang S, Tong S, Brown MT (2002) A modified toxicity testing method using tropical marine microalgae. Environ Monit Assess 75:145–154. doi:10.1023/A:1014483713719

ISO/DIS 14669 (1997) Water quality—determination of acute lethal toxicity to marine copepoda (Copepoda, Crustacea). International Standard, Geneva

Kennish MJ (1997) Heavy metals. In: Practical handbook of estuarine and marine pollution. CRC Press Marine Science Series, Boca Raton, Florida, pp 253–327

Kilemade M, Hartl MGJ, Sheehan D, Mothersill C, van Pelt FNAM, O'Brien NM, O'Halloran J (2004) An Assessment of the pollutant status of surfical sediment in Cork Harbour in the South East of Ireland with particular reference to polycyclic aromatic hydrocarbons. Mar Pollut Bull 49:1084–1096. doi:10.1016/j.marpolbul.2004.08.002

Macken A, Giltrap M, Foley B, McGovern E, McHugh B, Davoren M (2008) A model compound study: the ecotoxicological evaluation of five organic contaminants with a battery of marine bioassays. Environ Pollut 153(3):627–637. doi:10.1016/j.envpol.2007.09.005

Morozzi G, Cenci G, Scardazza F, Pitzurra M (1986) Cadmium uptake by growing cells of Gram-positive and Gram-negative bacteria. Microbios 48:27–35

Nassiri Y, Ginsburger-Vogel T, Mansot JL, Wéry J (1996) Effects of heavy metals on *Tetraselmis suecica*: ultrastructural and energy-dispersive X-ray spectroscopy studies. Biol Cell 86:151–160. doi:10.1016/0248-4900(96)84779-4

Newman MC (1995) Quantitative methods in aquatic ecotoxicology. Lewis, Chelsea

Newman MC, McCloskey JT (1996) Predicting relative toxicity and interactions of divalent metal ions: microtox bioluminescence assay. Environ Toxicol Chem 15(3):275–281. doi:10.1897/1551-5028(1996)015<0275:PRTAIO>2.3.CO;2

Ní Shúilleabháin S, Mothersill C, Sheehan D, O'Brien NM, O'Hall-oran J, Van Pelt FNAM, Davoren M (2004) In vitro cytotoxicity testing of three zinc metal salts using established fish cell lines. Toxicol In Vitro 18:365–376. doi:10.1016/j.tiv.2003.10.006

Niencheski LF, Windom HL, Smith R (1994) Distribution of particulate trace metal in Patos Lagoon estuary (Brazil). Mar Pollut Bull 28:96–102. doi:10.1016/0025-326X(94)90545-2

Nriagu JO, Pacyna JM (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 333:134–139. doi:10.1038/333134a0

O'Brian P, Feldman H, Grill E, Lewis AG (1988) Copper tolerance of the life history stages of the splashpool copepod *Tigriopus californicus* (Copepoda, Harpacticoida). Mar Ecol Prog Ser 44:59–64. doi:10.3354/meps044059

Peinado MT, Mariscal A, Carnero-Varo M, Fernandez-Crehuet J (2002) Correlation of two bioluminescence and one fluorogenic bioassay for the detection of toxic chemicals. Ecotoxicol Environ Saf 53:170–177. doi:10.1006/eesa.2002.2177

Pérez-Rama M, Herrero López C, Alonso JA, Torres Vaamonde E (2001) Class III metallothioneins in response to cadmium toxicity in the marine microalga *Tetraselmis suecica* (Kylin) Butch. Environ Toxicol Chem 20:2061–2066

Pérez-Rama M, Alonso JA, Herrero López C, Torres Vaamonde E (2002) Cadmium removal by living cells of the marine microalga *Tetraselmis suecica*. Bioresource Technol 84:265–270

Pérez-Rama M, Vaamonde ET, Alonso JA (2006) Composition and production of thiol constituents induced by cadmium in the marine microalga *Tetraselmis suecica*. Environ Toxicol Chem 25(1):128–136

Rai LC, Gaur JP, Kumar HD (1981) Phycology and heavy-metal pollution. Biol Rev 56:99–151

Rainbow PS (1993) The significance of trace metal concentration in marine invertebrates. In: Dallinger R, Rainbow PS (eds) Ecotoxicology of metals in invertebrates. Lewis publishers, Boca Raton

Reid TM, Feig DI, Loeb LA (1994) Mutagenesis by metal-induced oxygen radicals. Environ Health Persp 102(3):57–61

Rice HV, Leighty DA, McLeod GC (1973) The effects of some trace metals on marine phytoplankton. CRC Criti Rev Microbiol 3:27–49

Ryan JA, Hightower LE (1994) Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. Environ Toxicol Chem 13(8):1231–1240

Saito H, Iwami S, Shigeoka T (1991) In vitro cytotoxicity of 45 pesticides to goldfish GF-scale (GFS) cells. Chemosphere 23: 525–537

Satoh A, Vudikaria LQ, Kurano N, Miyachi S (2005) Evaluation of the sensitivity of marine microalgal strains to the heavy metals, Cu, As, Pb and Cd. Environ Int 31(5):713–722

Scarano G, Morelli E (2002) Characterization of cadmium- and lead-phytochelatin complexes formed in a marine microalga in response to metal exposure. Biometals 15:145–151

Segner H, Braunbeck T (1998) Chemical response profile to chemical stress. In: Schüürmann G, Markert B (eds) Ecotoxicology: ecological fundamentals chemical exposure and biological effects. John Wiley & Sons Inc., New York, USA; Spektrum Akademisher Verlag, Germany, pp 521–557

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. Free Radic Bio Med 18:321–336

Toussaint MW, Shedd TR, Van der Schalie WH, Leather GR (1995) A comparison of standard acute toxicity tests with rapid screening toxicity tests. Environ Toxicol Chem 14:907–915

Tsiridis V, Petala M, Samaras P, Hadjispyrou S, Sakellaropoulos G, Kungolos A (2006) Interactive toxic effects of heavy metals and humic acids on *Vibrio fischeri*. Ecotox Environ Saf 63:158–167

Utgikar VP, Chaudhary N, Koeniger A, Tabak HH, Haines JR, Govind R (2004) Toxicity of metals and metal mixtures: analysis of concentration and time dependence for zinc and copper. Water Res 38:3651–3658

Viarengo A (1989) Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. CRC Criti Rev Aquat Sci 1:295–317

Warnau M, Ledent G, Temara A, Jangoux M, Dubois P (1995) Experimental cadmium contamination of the echinoid *Paracentrotus lividus*: influence of exposure mode and distribution of the metal in the organism. Mar Ecol Prog Ser 1666:117–124