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Assessing the potential of fish cell lines as tools for the cytotoxicity testing of estuarine sediment aqueous elutriates

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Abstract

In the present study, we assess the potential of fish cell lines (CHSE, EPC and RTG-2) to be used as screening tools for the ecotoxicological assessment of estuarine sediments. The processing of sediment to a form suitable for in vitro exposure is an inherent problem when using cell cultures. The approach employed in this study was to prepare aqueous elutriate extracts from whole sediments, which were subsequently used to reconstitute powdered media. This procedure allowed the exposure of cell cultures to concentrations of up to and including 100% of the original aqueous sample. Cytotoxicity was assessed using multiple endpoint measurements. Cell viability was quantified using the neutral red and alamar blue colorimetric assays, which specifically assess lysosomal and mitochondrial function, respectively. In addition, the total protein content of the cells was measured using the coomassie blue assay. Initial tests were conducted to ensure that any resultant cytotoxicity was due to sample contaminants and not osmotic stress. In addition, elutriate samples were spiked with a model toxicant to verify the ability of the cell lines to detect and respond to bioavailable contaminants. Chemical analyses were conducted on sediments from all sampling sites to assist in interpreting any observed cytotoxicity. A differential response was observed for the cytotoxicity assays following exposure treatments, which emphasises the importance of employing multiple endpoints for the determination of toxicity. Of the three cell lines utilised in this study, RTG-2 cells were the most suitable for the testing of estuarine aqueous elutriate samples on the basis of tolerance to osmolality effects. Slight toxicity was observed following exposure to the aqueous elutriates tested in this study using RTG-2 cells and the alamar blue assay. In order to fully evaluate the overall sensitivity of this cell line, further research is warranted using an extensive range of test sites incorporating more polluted sediments.

Keywords: Sediment aqueous elutriates; Fish cell lines; RTG-2; Alamar blue; Cytotoxicity

1. Introduction

Sediments, as a repository for persistent anthropogenic pollutants, have the potential to cause deleterious effects in the aquatic environment. Compliance with the EU Water Framework Directive (WFD 2000/60/EC) and the OSPAR convention (1992), obliges all signatories to ensure the protection of marine and estuarine environments. The monitoring of contaminants in sediments should therefore form an integral part of any water quality management plan.

At present, assessment and monitoring of sediment quality in Ireland is predominantly reliant on chemical analyses (Nendza, 2002). Chemical analyses alone are often inadequate for accurately characterising

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environmental samples, as it is not feasible to identify and measure the concentration of all potential toxicants. In addition, the combined effects (antagonistic or synergistic) or the bioavailability of contaminants are not addressed. Ecotoxicological assessment of sediments in tandem with chemical characterisation can therefore provide an integrated means for evaluating the environmental significance of potential toxicants. A tier-structured approach, where the significance and complexity of the toxicity tests increases with the tiers, is being increasingly employed to evaluate the potential toxicity of environmental samples (Ahlf et al., 2002; Nendza, 2002). In Tier 1 testing, the ability of in vitro tests as preliminary and relatively simple bioassays that rapidly and economically screen large numbers of samples, are of particular value (Segner, 1998; Castaño et al., 2000, 2003).

Cell cultures, in particular those derived from fish, have been successfully employed as a biological alternative to the use of whole animals for both Tier 1 screening of sediments and for assessing biomarkers of specific exposure (Table 1). The processing of sediment to a form suitable for in vitro exposure is, however, an inherent problem when using cell cultures. The predominant methodology has been to concentrate organic sediment extracts and subsequently expose them to cells in a carrier solvent. This approach however, is not a good indicator of the bioavailability of the sediment-associated contaminants. The reconstitution of powdered culture media using aqueous samples (e.g. water, effluents) provides a means of exposing cell cultures to concentrations of up to and including 100% of the original sample (Kfir and Prozesky, 1981; Mochida, 1986; Dayeh et al., 2002; Schirmer et al., 2004).

In the present study, we apply this approach to evaluate the potential of fish cell cultures to be used as a screening tool for the ecotoxicological assessment of sediment elutriates. Sediment elutriate testing was developed to evaluate the potential toxic effects of leached contaminants released from dredged material (Daniels et al., 1989; Ingersoll, 1995; Matthiessen et al., 1998). The testing of this aqueous phase, as opposed to the use of solvent extracts is therefore more analogous to in situ in vivo exposures.

The purpose of this study was to evaluate the potential of three established fish cell lines for use as Tier I screening tools in the ecotoxicological assessment of estuarine sediment. The cytotoxic effect of sediment aqueous elutriates on the cell cultures was assessed using multiple endpoint measurements, thus permitting detection of differential responses of the fish cells to contaminants. Lysosomal function was assessed using the Neutral Red (NR) assay, followed by total protein analysis using the Coomassie Blue (CB) cytotoxicity assay. In addition, Alamar Blue (AB) was used as an indicator of mitochondrial function. Finally, chemical analysis was conducted on sediment samples from all three sites to assist in interpreting any observed cytotoxicity.

2. Materials and methods

2.1. Site selection and sediment sampling

Three sampling sites from around the Irish coast were selected on the basis of previous published reports that demonstrated them to be representative of a range of contaminant burdens (Marine Institute, 1999; Byrne and O'Halloran, 2000; Kilemade et al., 2004). Ballymacoda Estuary, Co. Cork (7°55′W, 51°54′N) was chosen as a relatively uncontaminated site while the Douglas Estuary, Co. Cork (8°23′W, 51°52′N) and the East Wall site in the Liffey Estuary, Co. Dublin (06°07′W, 53°20′N) have moderate and high contaminant levels, respectively. Oxic surface sediment (top 2 cm) was sampled from random areas each measuring approximately 300 cm^2 at low tide. Sub-samples were composited and homogenised thoroughly and stored at 4 °C in polyethylene bags. Whole sediment was stored for no longer than two weeks as recommended by the American Society for Testing and Materials (ASTM, 1994).

2.2. Physical and chemical characterisation of sediments

The percentage dry weight of the sediments was determined using sub-samples of each sediment by oven-drying at 105 °C to a constant weight. Total organic carbon content was analysed by ALcontrol Laboratories (Chester, UK) using the combustion method, which conforms to ISO 17025. The accuracy of this method was ensured using a certified reference material (CRM) (Alpha Resources Inc. CSN standard AR873). The accuracy was further assessed by the inclusion of a test material (obtained from the QUASIMEME project office), which was found to be within acceptable limits. Sediments were wet sieved using the gravimetric method to obtain the fine (<63 μm) sediment fraction (Loring and Rantala, 1992). Heavy metal analyses (Pb, Cd, Cr, Cu, Ni, Zn, Al and Li) were conducted by the Marine Institute (Abbotstown, Dublin, Ireland). Lead, cadmium, chromium, copper and nickel concentrations were determined using Graphite Furnace Atomic Absorption Spectrometry with Zeeman background correction (Varian SpectrAA 220Z). Zinc, aluminium and lithium concentrations were determined using Flame Atomic Absorption Spectroscopy (Varian SpectrAA 20 Plus). A comprehensive analytical quality assurance programme involving the routine testing of quality control samples such as blanks, replicates and reference materials (including CRMs) underpinned all testing.

Table 1
Selected examples of endpoints used in sediment toxicity studies with cell cultures (adapted from Ní Shúilleabháin et al., 2003)

Endpoint	Sediment exposure phase	Cell line	Primary culture	Reference
Cytotoxicity endpoints				
Cell viability	Solvent extract fraction, solvent extract	BB, RTG-2, BF-2	Rainbow trout hepatocytes	Hollert et al. (2000), Ali et al. (1993), Kocan et al. (1985), Gagné et al. (1996), Strmac and Braunbeck (2000)
Mitotic inhibition	Solvent extract	RTG-2, BF-2	–	Kocan et al. (1985)
Protein content	Solvent extract	PLHC-1	Rainbow trout hepatocytes	Huuskonen et al. (2000), Strmac and Braunbeck (2000)
Ultrastructural changes	Solvent extract	–	Rainbow trout hepatocytes	Strmac and Braunbeck (2000)
Genotoxicity end points				
Anaphase aberration	Solvent extract	RTG-2, BF-2	–	Kocan et al. (1985)
Comet assay	Solvent extract	EPC	*Cyprius carpio* (carp)	Kammann et al. (2000, 2001)
Single-gel electrophoresis	Solvent extract	EPC	Leucocytes	Kammann et al. (2001)
Unscheduled DNA synthesis (UDS)	Solvent extract fraction	BB	–	Ali et al. (1993)
Alkaline precipitation assay	Solvent extract	–	Rainbow trout hepatocytes	Gagné et al. (1996), Gagné and Blaise (1995)
In-situ nick translation assay	Solvent extract	–	Rainbow trout hepatocytes	Gagné and Blaise (1995)
Mechanistic and biomarker end points				
CYP1A1 induction	Solvent extract, solvent extract fraction, SPMD dialysate	PLHC – 1 P450RGS Hepa-1	Rainbow trout hepatocytes	Mátlová et al. (1995), Gagné et al. (1996), Villeneuve et al. (1996), Huuskonen et al. (1998, 2000), Anderson et al. (1999), Koh et al. (2001)
Dioxin-like activity	Solvent extract, solvent xtract fraction, pore water	DR CALUX (H4IIE)	–	Hurst et al. (2004), Houtman et al. (2004), Stronkhorst et al. (2002), Vondráček et al. (2001), Murk et al. (1996), Khim et al. (2001), Machala et al. (2001), Koh et al. (2002)
Porphyrin content	Solvent extract	PLHC-1		Huuskonen et al. (2000)
Metallothionein induction	Solvent extract	–	Rainbow trout hepatocytes	Gagné et al. (1996)
Estrogenic-like activity	Solvent extract, solvent extract fraction	MVLN bioassay	–	Khim et al. (2001), Machala et al. (2001), Vondráček et al. (2001)
	Solvent extract	ER CALUX		Houtman et al. (2004), Legler et al. (2002)
Anti-estrogenic-like activity	Solvent extract	ER CALUX		Houtman et al. (2004)

BB = brown bullhead; RTG-2 = rainbow trout gonad; BF-2 = bluegill fin from sunfish; PLHC-1 = hepatocellular carcinoma of topminnow (*Poeciliopsis lucida*); EPC = *Epithelioma papulosum cyprini*; Hepa-1 = mouse hepatoma; LDH = lactate dehydrogenase; CYP1A = cytochrome P4501A; SPMD = semipermeable membrane device; ER-CALUX = estrogen responsive chemical-activated luciferase gene expression; DR-CALUX = dioxin responsive chemical-activated luciferase gene expression; MVLN = recombinant MCF-7 (mammary carcinoma fibroblast) human breast cancer cells transfected with a luciferase reporter gene under the control of estrogen response elements.

2.3. Cell culture

Three established fish cell lines were used. They were CHSE-214 cells from chinook salmon embryo (*Oncorhynchus tsawytscha*), EPC cells from carp epithelium (*Cyprinus carpio*), and RTG-2 cells from rainbow trout gonad cells (*Oncorhynchus mykiss*). Cell culture materials and subcultivation procedures were as previously described (Ní Shúilleabháin et al., 2004). Cells were plated in 96-well microplates (Nunc, Denmark) at a density of 2×10^4 per 100 μl for 24 h exposure periods. RTG-2 cells were also exposed up to and including 96 h. A seeding density of 1.8×10^4 cells per 100 μl was employed for the 48 h exposures and 1.6×10^4 cells per 100 μl were plated for both 72 and 96 h exposure periods. These seeding densities were found to be optimal

to achieve the desired confluency at the end of each exposure time period.

2.4. Elutriate extraction

Elutriate extracts were prepared with modification according to the Standard Elutriate Test (Keely and Engler, 1974; USEPA, 1977). Deionised water was added to 10 g sub-samples of sediment in a 1:4 (w/v) ratio based on the sediment dry weight. The slurry was shaken at 240 rpm for 1 h and then centrifuged at $1200 \times g$ for 30 min at 4 °C. The supernatant was collected as elutriate, sterile filtered through a 0.2 μm filter (Nalgene, NY, USA) and conductivity, salinity and pH were measured using the relevant electrodes with a Sension™ multimeter (Hach, Loveland, CO, USA). Aqueous elutriates were stored in primed glass bottles with minimal headspace at 4 °C in the dark. Cytotoxicity testing was always conducted within 24 h of elutriate preparation.

2.5. Sample preparation

Powdered media were reconstituted with elutriate extracts to allow the whole elutriate samples to be exposed to fish cultures while ensuring essential nutrients were not limiting. Powdered Minimum Essential Medium (MEM) was employed for CHSE and EPC cells and Dulbecco's Modified Medium Nutrient Mixture/F-12 Ham (DMEM: F-12) was used for RTG-2 cells (Gibco, Paisley, Scotland). Following reconstitution, the samples were sterile filtered and supplemented with 5% foetal calf serum (FCS), 25 mM HEPES, 45 IU ml^{-1} penicillin, 45 μg ml^{-1} streptomycin, and 2 mM L-Glutamine. Control media was reconstituted with deionised water and was supplemented as outlined above for elutriates. Cell cultures were exposed to a range of elutriate concentrations (20%, 40%, 60%, 80% and 100%) using control medium as diluent. Elutriate samples were also spiked with a model toxicant (zinc chloride) in a range from 0.292 to 1.467 mM to ensure the ability of the cell lines to detect and respond to bioavailable contaminants when present. The results obtained were then compared to previously established results for this chemical to ensure test validity (Ní Shúilleabháin et al., 2004).

2.6. Osmolality controls

The osmolality of aqueous elutriate samples was measured using the Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). The tolerance of the cell cultures to osmotic stress was evaluated by exposing them to increasingly saline media. These solutions were prepared by increasing the osmolality of control cell culture medium using a concentrated balanced salt solution (Machii and Wada, 1989). The osmolality range tested was approximately between 0.30 and 0.60 Osm kg^{-1}, which incorporated the measured osmolalities of the aqueous elutriates.

2.7. Cytotoxicity assays

2.7.1. Neutral red and coomassie blue assays

Viability and protein determination of the cells following exposure to test samples were investigated using the NR and CB assay, respectively. The incorporation of the NR dye by the lysosomes of living cells and the quantification of the total amount of cellular proteins were performed according to Liebsch and Spielmann (1995) with the modification of Coomassie Brilliant Blue dye being employed in place of Kenacid Blue R dye. Briefly, after 24 h of incubation with the test sample, the medium was replaced with fresh media (5% FCS) containing 40 μg/ml of NR. Following 3 h incubation to allow NR uptake, the dye was extracted with an acetic acid–ethanol solution. The absorbance of the solution in each well was measured at 540 nm (reference filter 380 nm) with a SPECTRAmax™ 250 spectrophotometer (Molecular Devices Corp., USA). Protein determinations were performed on the same plates immediately following NR determination. CB dye was added to each well and the plate agitated for 10 min. The dye was removed and the plate washed with an acetic acid–ethanol solution. The wash solution was discarded and the dye extracted with measuring solution (1 M Potassium acetate). The absorbance of the extracted dye was read at 570 nm (reference filter 380 nm) using the microplate reader.

2.7.2. Alamar blue assay

Cell viability was quantified using the Alamar Blue™ (AB) bioassay as described by Ganassin et al. (2000). The oxidised indigo blue form of this chromogenic indicator dye (Biosource, Camarillo, CA, USA) is reduced by cellular dehydrogenases, specifically targeting the mitochondrial electron transport chain, to a reduced pink form, which can be easily monitored spectrophotometrically. The bioassay was performed according to manufacturers' instructions. Briefly, test exposures were removed, cells rinsed with phosphate buffered saline (PBS) and 100 μl of a 10% (v/v) solution of AB prepared with liquid medium only (no supplements or FCS) was added to each well. As the absorption spectra of the oxidised (blue) and the reduced (pink) forms of AB overlap, the absorbance is measured at two wavelengths. These are 570 and 600 nm for the reduced and oxidised forms, respectively. Wells containing medium and AB without cells were used as blanks. A subtraction analysis (OD570-OD600) of the dual wavelength readings was conducted and the blank value was deducted from all wells. Data were expressed as percent AB reduced using the equation provided in the manufacturers' instructions. As the AB assay had not been previously em-

ployed in this laboratory, initial studies were conducted to determine the optimum incubation time required to achieve maximum reduction of the dye. This was evaluated by seeding each cell line at a density of 2×10^4 cells per 100 μl, incubating for 24 h and then measuring absorbance readings after 4, 8, 10 and 24 h incubation with AB dye.

2.8. Statistics

Experiments were conducted in at least triplicate. Raw data from cell cytotoxicity assays were collated and analysed using Microsoft Excel®. Cytotoxicity is expressed as mean percentage inhibition relative to the unexposed control ± standard deviation (SD), which was calculated using the formula [100 − ((Mean Experimental data/Mean Control data) × 100)]. Control values were set at 0% cytotoxicity. The coefficient of variation (CV) for the controls of each test was calculated to ascertain reproducibility. Statistical analyses were carried out using a one-way analyses of variance (ANOVA) followed by Dunnett's multiple comparison test. Inter- and intra-test variations were assessed using a two-way ANOVA or the appropriate Student's *t*-test. This data analysis was performed using MINITAB® release 12 (MINITAB Inc. PA, USA). Cytotoxicity data was fitted with an appropriate model (MMF or a four parameter logistic model) and IC_{50} values were calculated. These analyses was performed using Xlfit3™ a curve fitting add-in for Microsoft® Excel (ID Business Solutions, UK).

3. Results

3.1. Physico-chemical characteristics of sediment samples

Physico-chemical parameters and metal analysis results for the three sampling sites are presented in Table 2. Total organic carbon concentrations were relatively uniform between sites (1.6–2.6%). The percentage of fine particles (<63 μm) in the sediment was highest at Ballymacoda and lowest at the Douglas site. The moisture holding capacity was found to be lowest in the Ballymacoda site sample.

All the heavy metals analysed in this study were present at higher concentrations in the Douglas and East Wall samples than in the Ballymacoda sample (Table 2). The East Wall site was shown to have the highest concentrations of Pb, Cu, Cd, Ni and Zn. Levels of Cr, Li and Al were found to be relatively comparable between the sites.

Neat elutriate sample pH values ranged from 7.5 to 8.0 (Table 3) but were adjusted to a pH of 7.2 to ensure that the fish cells in culture were not unduly stressed. The osmolality (Osm kg^{-1}) of neat elutriate samples was similar to that of control media (MEM media 0.272 ± 0.013 Osm kg^{-1}; DMEM media 0.294 ± 0.004 Osm kg^{-1}). Following addition of powdered media and supplements, necessary to ensure that essential nutrients were not limiting, the osmolality of the neat samples increased to between 0.419 and 0.454 Osm kg^{-1}.

Table 2
Physico-chemical properties and metal analyses for the three sampling sites

	Ballymacoda	Douglas	East Wall
Physico-chemical parameters (%)			
Total organic carbon	1.6	2.6	2.2
<63 μm	77.5	64.0	69.0
% dry-weight	49.1	34.0	30.5
Metal analysis (μg/g d.w.)			
Lead	27.2	65.2	87.1
Copper	17.0	69.7	71.8
Cadmium	0.29	0.47	2.03
Chromium	59.8	82.3	60.7
Nickel	29.0	39.5	44.8
Zinc	94.2	227	336
Lithium	38.4	49.7	39.6
Aluminium (% d.w.)	5.17	6.79	5.20

Table 3
Physico-chemical characteristics of neat elutriate samples pre- and post-supplement addition (DMEM samples)

	Ballymacoda		Douglas		East Wall	
	Raw	Adjusted	Raw	Adjusted	Raw	Adjusted
pH	7.6	7.2	7.5	7.2	8.0	7.2
Conductivity (mS/cm)	7.9	18.4	4.8	11.7	7.5	15.5
Salinity (‰)	6.7	12.7	3.9	9.6	6.2	12.6
Osmolality (Osm kg^{-1})	0.286	0.454	0.250	0.419	0.259	0.444

3.2. Optimisation of alamar blue incubation time

The percentage reduction of AB with increasing incubation time for the three cell lines is presented in Fig. 1. Based on the time points investigated, maximal reduction of the AB dye was observed after a 24 h AB incubation period. As this was still found to be within the linear portion of the graph, a 24 h incubation time was employed for the subsequent cytotoxicity studies.

3.3. Cytotoxicity tests with elutriates and EPC cells

The potential cytotoxic effects of aqueous elutriate sample osmolality were investigated using multiple endpoint measurements following exposure of all three cell lines to an osmolality concentration range (0.341–0.594 Osm kg^{-1}). As illustrated in Fig. 2 the EPC cells were found to be the least tolerant to increasing osmolality with each of the endpoints. For example, a 40% reduction in cell viability was observed at 0.486 Osm kg^{-1}

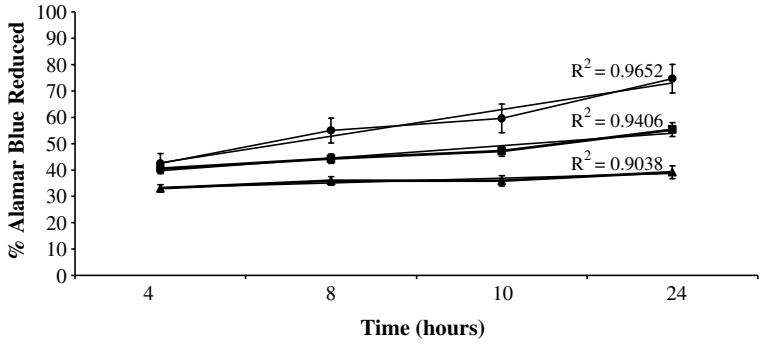

Fig. 1. Percentage Alamar Blue reduction as a function of incubation time measured spectrophotometrically for CHSE (▲) EPC (■) and RTG-2 (●) cells. Data is expressed as the mean percentage reduction of AB dye ± SD of three replicates for each incubation time point.

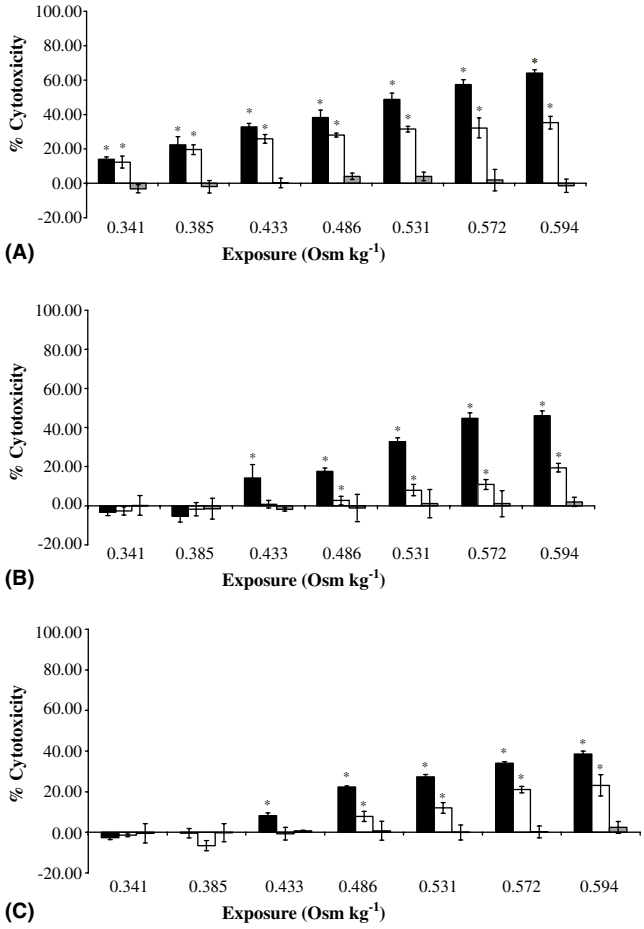

Fig. 2. The cytotoxicity effects of increasing osmolality to EPC (■), CHSE (□) and RTG-2 (▩) cells determined by the (A) NR assay, (B) CB assay and (C) AB assay. Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. Control values were set at 0% cytotoxicity. * denotes a significant difference from the control ($p \leqslant 0.01$). CV for the controls ranged from 2.6% to 14.6%.

using the NR assay (Fig. 2A). This osmolality is comparable to that of 100% supplemented elutriate (Table 3). The EPC cell line was therefore not employed for cytotoxicity screening of elutriates, as increased sample osmolality would be a confounding factor.

3.4. Cytotoxicity tests with elutriates and CHSE cells

As evident from Fig. 2 the CHSE cells were more tolerant to the effects of high osmolality than the EPC cells. A significant toxic effect was noted, however, at osmolality values comparable to supplemented 100% elutriate samples with the NR assay. Differential endpoint sensitivities are clearly demonstrated with the CB and AB assays, as these endpoints are notably more tolerant to increasing osmolality than the NR assay. The effects of elutriate toxicity were therefore investigated with this cell line as the use of multiple endpoints with varying sensitivity to increasing osmolality would discriminate whether any resultant toxicity was attributable to sample osmolality or to environmental toxicants. A significant reduction ($p \leqslant 0.01$) in cell viability with the NR assay was observed following exposure to all three sediment elutriates. This toxicity was, however, attributable to osmolality effects with all three samples employed ($p \leqslant 0.01$).

3.5. Cytotoxicity tests with elutriates and RTG-2 cells

There was no significant toxic effect of increasing osmolality on RTG-2 cells up to and including ~0.600 Osm kg^{-1} for the 24 h exposure period (Fig. 2). In addition, there was no significant effect of increasing osmolality for all three endpoints up to a 96 h exposure time period. The RTG-2 cells were therefore the most suitable for the purpose of sediment elutriate testing. The RTG-2 cells were exposed to elutriates up to and including 96 h, as these cells are known to possess a basal metabolic function. There was no significant toxicity ($p \leqslant 0.01$) observed following exposure to all three sediment elutriates with the multiple endpoints following 24–72 h time periods. A slight reproducible toxicity (<10%) was elicited with all endpoints for 100% East

Wall elutriate following 96 h exposure (results not shown).

3.6. Cytotoxicity tests with a model toxicant and spiked elutriate

Cytotoxicity data following exposure of zinc chloride ($ZnCl_2$) to CHSE cells (24 h) and RTG-2 cells (24–96 h) using the AB assay are presented in Fig. 3. A 24 h EC_{50} value of 0.137 ± 0.009 mM and 0.19 ± 0.02 mM was determined for CHSE cells and RTG-2 cells respectively following exposure to $ZnCl_2$. There was no significant difference observed in overall cytotoxicity over the 96 h exposure time period for the RTG-2 cells ($p \leqslant 0.01$). C.V. values using the AB assay ranged from 1.3% to 2.7%.

To ensure the ability of the cell lines to detect and respond to bioavailable contaminants in a predictable manner, elutriate samples were spiked with the model toxicant. The effect of spiked East Wall elutriates on CHSE and RTG-2 cells using all three endpoints are presented in Fig. 4. EC_{50} values of 0.65 ± 0.04, 1.08 ± 0.27 and 0.49 ± 0.03 mM $ZnCl_2$ derived from the NR, CB and AB data respectively were calculated for the exposed CHSE cells. Midpoint toxicity values of 0.79 ± 0.15 (NR), 1.59 ± 0.16 (CB) and 0.43 ± 0.05 (AB) mM $ZnCl_2$ were derived for the RTG-2 cells.

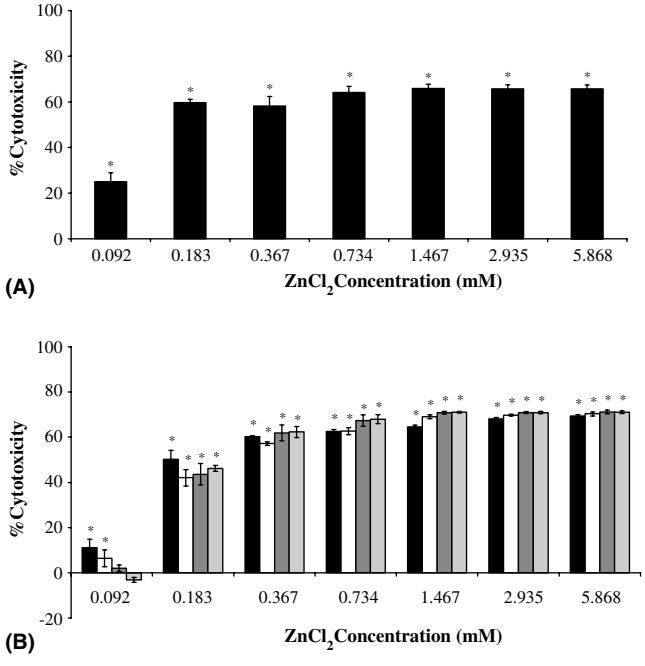

Fig. 3. (A) $ZnCl_2$ cytotoxicity to CHSE cells determined by AB assay. (B) $ZnCl_2$ cytotoxicity to RTG-2 cells determined by the AB assay for 24 h (■), 48 h (□), 72 h (▩) and 96 h (▨). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. Control values were set at 0% cytotoxicity. * denotes a significant difference from the control ($p \leqslant 0.01$). CV for the controls ranged from 1.3% to 2.7%.

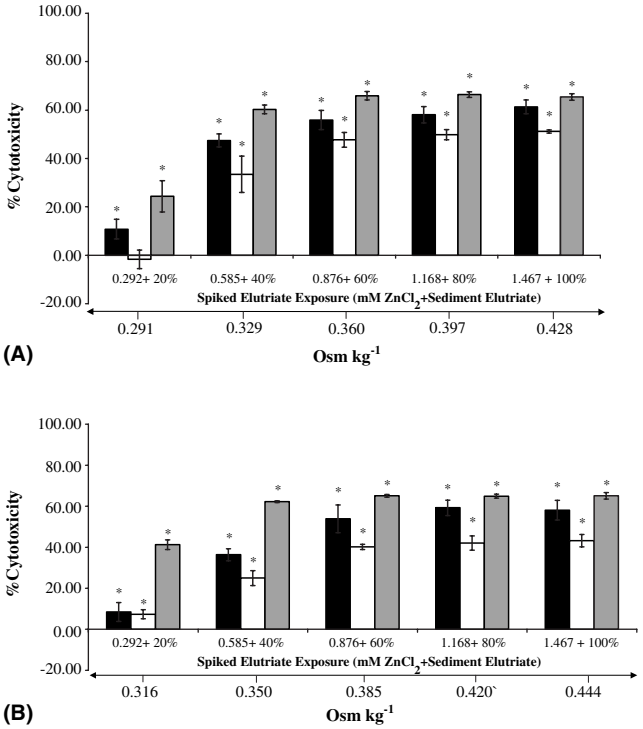

Fig. 4. Cytotoxicity of spiked East Wall elutriates with (A) CHSE cells and (B) RTG-2 cells determined by the NR assay (■), CB assay (□) and AB assay (▩). Osmolality values corresponding to each exposure concentration are shown directly below the *x*-axis. Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. Control values were set at 0% cytotoxicity. * denotes a significant difference from the control ($p \leqslant 0.01$). CV for the controls ranged from 0.13% to 14.0%.

C.V. values for the control CHSE exposures ranged from 2.3% to 10.1% while values for RTG-2 cells ranged from 0.13% to 14.0%.

4. Discussion

A conservative estimate is that about 1 million fish are killed each year in the EU Member States for research and regulatory purposes (Castaño et al., 2003). Fish lethality tests have several limitations, most notably in terms of growing economical costs and ethical concerns. For these reasons, ecotoxicologists are increasingly employing alternative experimental methods that seek to apply the principles of replacement, reduction and refinement (Hutchinson et al., 2003). The use of fish cell cultures offer relevant in vitro alternatives to in vivo tests for initial hazard evaluation of both chemicals and environmental samples (Castaño et al., 1994; Hollert et al., 2000; Dayeh et al., 2002). Good correlations have been determined based on the relative ranking order of pollutants to fish cells with their water-borne in vivo toxicity to live fish (Bols et al., 1985; Dierickx and van de Vyver, 1991; Segner and Lenz, 1993; Castaño et al.,

1996). In addition, in vitro tests with fish cell cultures can address modern requirements for standardised, cost effective and rapid throughput testing of samples. They also have the added benefit of requiring greatly reduced test volumes compared to in vivo exposures, which is particularly advantageous when screening environmental samples. Further development and validation of in vitro fish tests is therefore of great importance to fully exploit their future applications for ecotoxicological research, testing and monitoring (Castaño et al., 2003).

We have previously demonstrated the value of utilising fish cell lines to investigate the cytotoxicity of metal salts (Ní Shúilleabháin et al., 2004). The objective of this study was to evaluate the potential of these fish cell lines as screening tools for metal contaminated environmental samples. As the cell lines employed in this study were all derived from freshwater species and the sediment samples were obtained from an estuarine environment, initial tests were conducted to ensure that any observed toxicity was due to contaminants and not as a result of osmotic stress. This study clearly demonstrates that EPC cells are particularly sensitive to the effects of increasing sample osmolality ($\geqslant$0.34 Osm kg^{-1}). Hashimoto et al. (1998) have also demonstrated significant cell death in EPC cells following exposure to hypertonic solutions (>0.400 Osm kg^{-1}). In addition, adult carp (*Cyprinus carpio*), from which EPC cells are derived, are known for their strict freshwater stenohalinity (Abraham et al., 2001). Their low tolerance to increasing osmolality therefore precluded the use of EPC cells for testing estuarine environmental samples in this study. CHSE cells were found to be more tolerant to increased sample osmolality, while there was no significant effect of the osmolality range employed in this study on RTG-2 cells. This is in agreement with the findings of Sakamoto (2000), who demonstrated RTG-2 cells to be tolerant to sample osmolalities ranging from 0.200 to 0.600 Osm kg^{-1}. Of the three cell lines utilised in this study, RTG-2 cells were the most suitable for the testing of estuarine elutriate samples on the basis of their tolerance to osmolality effects.

The CHSE and RTG-2 cell lines were exposed to $ZnCl_2$ spiked elutriate samples to ensure they responded in a predictable manner to bioavailable toxicants when present. The EC_{50} values derived from the $ZnCl_2$ spiked elutriate exposure with NR and CB were in general agreement with previously published results for $ZnCl_2$ with these endpoints (Ní Shúilleabháin et al., 2004). This also demonstrated that there were no constituents present in the medium or the samples that could mask cytotoxic effects.

The differential response of the cytotoxicity assays to the various treatments emphasises the importance of employing multiple endpoints for the determination of toxicity. For example, inhibition of lysosomal function as measured by the NR assay was found to be the most susceptible to the effects of increased sample osmolality. Conversely, the AB assay, which is an indicator of mitochondrial integrity, was especially sensitive to zinc exposure. Toxicity screening of complex environmental samples therefore warrants the use of multiple endpoint measurements that can address specific modes of action of different chemical constituents.

Following exposure to the sediment elutriate samples, a significant toxic response was elicited in the CHSE cells, however, this toxicity could be attributed solely to the effects of increased sample osmolality ($p \leqslant 0.01$). Significant toxicity was not observed in the RTG-2 cells exposed to the three elutriate samples up to 72 h exposure for all endpoints. A slight toxic response (<10%) was recorded with the RTG-2 cells using the AB assay after a 96 h exposure period with the highest concentration of East Wall elutriate. This highlights the value of conducting exposures for longer time periods to address potential effects on cell division. In addition, as RTG-2 cells possess basal metabolic function, the slight toxicity observed following the longer exposure periods may indicate the presence of non-direct acting contaminants in the elutriate samples.

Metal analysis was conducted on all three sampling sites to assist in interpreting any observed cytotoxicity. The levels of heavy metals measured in the sampling sites examined in this study concur with previously published results (Byrne and O'Halloran, 2000; Kilemade et al., 2004). Previous research in this laboratory has demonstrated zinc to be cytotoxic to the tested cell lines at levels significantly lower than the levels measured at the Douglas and East Wall sites (Ní Shúilleabháin et al., 2004). As significant cytotoxic effects were not detected following exposure to sediment elutriates, this would indicate that zinc and the other heavy metals measured were not bioavailable in the elutriate samples at concentrations sufficient to cause toxicity. As noted earlier, exposure to the East Wall aqueous elutriate elicited a discernable cytotoxic effect to the RTG-2 cells following a 96 h incubation period. This site was demonstrated to be the most contaminated of the three sites investigated based on the results of metal analysis.

Corresponding in vivo toxicity assays with fish were not conducted to validate the low toxicity of these samples due to the inherent practical issues (e.g. volumes required) and costs associated with testing of sediment aqueous phases (e.g. porewater, elutriates) on vertebrate species. Comparisons were possible, however, with the Microtox® assay, a widely accepted regulatory bioassay for the screening of environmental samples. Exposure of the bacteria to these aqueous elutriates did not elicit a toxic response either (unpublished results). Toxicity was elicited however, using the Microtox® assay, following exposure to porewater extracted from East Wall sample. The effects of high sample osmolality precluded the testing of sediment porewater on fish cell cultures.

Currently, the lack of suitable commercially available cell culture models that are tolerant to high sample osmolality restricts the direct testing of estuarine/marine sediment porewaters. Results from this study suggest that RTG-2 cells may be adapted to grow in media of higher osmolality (>0.6 Osm kg^{-1}) and therefore may have future potential as screening tools for sediment porewater.

In summary, the utilisation of in vitro cell cultures as screening tools for the evaluation of sediment toxicity has been limited to the testing of solvent extracts. We have described a method that permits the testing of sediment aqueous phases, which are important exposure routes for bioavailable sediment-associated contaminants. As the current approach for the initial ecotoxicological screening of estuarine and marine sediments employs representative bacterial, algal, and invertebrate species only (Ahlf et al., 2002; Nendza, 2002), the potential risks to vertebrate species are not addressed (Hollert et al., 2003). Suitable vertebrate models are therefore required which are amenable to the routine screening of sediment aqueous phases. Previous researchers have recommended the replacement or reduction of in vivo fish tests with in vitro cell cultures and/or fish embryo bioassays (Ensenbach, 1998; Braunbeck and Strmac, 2001; Strmac et al., 2002).

While significant cytotoxicity was not detected following exposure to the aqueous elutriates tested in this study, the sensitivity of the method and the endpoints employed was demonstrated using spiked sample exposures. Furthermore, the findings of this study showed the RTG-2 cells to be the most suitable cell line for the screening of estuarine sediment aqueous phases based on their tolerance to osmotic stress. It is therefore concluded, that further studies are warranted with this cell line, which should address their overall sensitivity. This could be achieved by the screening of aqueous elutriates extracted from a greater number of sediment samples, which incorporate sites with higher contaminant burdens than tested in the present study. In addition, comparative studies with a battery of Tier I bioassays including other vertebrate models e.g. fish embryo tests, are required to provide a thorough appraisal of the potential of this fish cell line to be employed as an ecotoxicological screening tool.

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