A Model Compound Study: The Ecotoxicological evaluation of five organic contaminants employing a battery of marine bioassays

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ABSTRACT

This paper describes the ecotoxicological evaluation of five organic contaminants frequently detected in marine sediments (tributyltin, triphenyltin, benzo[a]pyrene, fluoranthene, and PCB 153) using three marine species (*Vibrio fischeri,, Tetraselmis suecica,* and *Tisbe battagliai*). The sensitivity of each species varied for all compounds. The tri-organotins were consistently the most toxic to all species.. The applicability of each test system to asses the acute toxicity of environmental contaminants and their use in Toxicity Identification Evaluation (TIE) is discussed. Suitability of the Microtox and *T. battagliai* tests for employment in TIE studies were further assessed through spiking experiments with. tributyltin Results demonstrated that the most effective treatment to remove organotin toxicity from the sample was the C_{18} resin. The results of this study have important implications for risk assessment in estuarine and coastal waters in Ireland, where, at present the monitoring of sediment and water quality is predominantly reliant on chemical analysis alone.

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1 Introduction

In recent years there has been growing concern regarding the toxic effects of chemical substances entering the aquatic environment. Sediments have been recognized as a major repository for persistent toxic substances released in the aquatic environment and frequently contain higher concentrations of pollutants than are found in the water column (Tolun et al., 2001). Persistent contaminants can ultimately enter into coastal areas and estuaries and may have short term (heavy metals, chlorophenol) or long term negative biological effects (polychlorinated biphenyls [PCBs] and polycyclic aromatic hydrocarbons [PAHs]), (Burton, 1991). Contaminants accumulate in the sediment and have the potential to cause adverse effects to indigenous biota and aquatic organisms should they become remobilised or bioavailable following chemical (e.g. changes in pH, salinity fluctuations), physical (e.g. dredging) or biological (bioturbation) processes. The monitoring of contaminants is therefore a vital part of any water quality monitoring programme.

In comparison to the extensive range of information available on the toxicity of chemical substances to freshwater organisms, there is relatively limited data on the effects of these substances on marine and estuarine species and especially on species occurring in Irish waters. Ecotoxicological activity of single chemicals can reasonably be assessed by current standardised bioassays. Although bioassays are not precise indicators of environmental effects, they are regarded as the best available method for integrating the effects of multiple contaminants.

Assessment and monitoring of the effects of pollutants in Irish marine sediments on marine organisms requires knowledge of the main toxic substances involved. Determination of acute aquatic toxicity of a chemical species is critical for

the evaluation of potential adverse environmental impacts from industrial and other point sources (Utgikar et al., 2004). Ecotoxicological data obtained may also allow for the prediction of "safe" levels in the environment. Five relevant chemical pollutants representing a diverse range of contaminants were selected for this study namely, two organotin compounds, two PAHs and a PCB compound. A summary of ecotoxicity data identified in the literature for the selected battery of bioassays and these chemicals is presented in Table 1.

Past and present usage of organotin compounds especially tributyl (TBT) and triphenyl (TPT) tins are recognised as being of great environmental concern. Tributyltin has been used in Ireland in antifouling paints on submerged marine structures such as boat hulls and salmon cages since the 1960s. An Irish bye-law was passed in April 1987 (Anon., 1987), prohibiting the use of TBT based paints on all vessels under 25 m except under special circumstances and other submerged marine structures such as salmon cages. Several marine ecotoxicological studies were initiated throughout Europe in the early 1980s after harmful effects were observed in commercial oyster-growing areas in France and Great Britain (Alzieu, 1996). Unlike inorganic tins, which do not accumulate in living organisms, tributyltin is bioconcentrated by bacteria, phytoplankton, molluscs, crustaceans and fish. Toxicity varies considerably between species and taxonomic groups (Alzieu, 1996); however, TBT is highly toxic to algae, molluscs, crustacean and fish. It has also been identified as an endocrine disrupting substance. Some neogastropods are extremely sensitive to TBT and it has been observed to causes imposex in pelagic female larval stages of gastropods at concentrations as low as 1 ng/L (Bryan et al., 1986). Tributyltin has also been observed to cause shell thickening in the Pacific Oyster (Minchin et al., 1996). Tributyltin degrades quickly in seawater where it has a residence time of days,

but it tends to adsorb onto particles and aggregates in sediments where the degradation processes are considerably slower. Despite the banning of organotin-based paints containing TBT and TPT in many European countries, and the existence of the Water Framework Directive requiring organotin levels in the marine environment to be monitored regularly from this year forward, the presence of both compounds is still of concern in the marine environment. In 2001, the U.S. EPA reviewed the use of TPT as a fungicide and determined that its benefits continued to outweigh its risks. Ten Hallers-Tjabbes et al., (2003) found that TPT had a low potential to induce imposex compared to TBT but Horiguchi et al., (1997) found the potential of the two compounds was similar.

Polycyclic aromatic hydrocarbons encompass a wide range of naturally occurring and non-naturally occurring chemicals. They may be derived from three sources: fossil fuels (petrogenic), burning of organic matter (pyrogenic) and by the transformation of natural organic matter in the environment through diagenetic processes. Due to their hydrophobicity, PAHs in the aquatic environment become associated with suspended particulate matter and are subsequently deposited in the sediments (Chiou et al., 1998). Due to their carcinogenic properties and anthropogenic origin there has been great interest in their fate and toxicity in the environment in recent years. One of the most abundant PAH pollutants in the environment is fluoranthene, a four ringed PAH. The majority of toxicity data available is concerned with acute exposure with limited information on chronic exposures. El-Alawi et al., (2002) and Barata et al., (2002) reported EC_{50} and LC_{50} for *Vibrio fischeri* and *Tisbe battagliai* respectively after exposure to fluoranthene (Table 1), but data on other marine organisms remains limited. Benzo[a]pyrene, a readily detectable PAH contaminant of marine sediments is highly persistent and is a

known carcinogen. Like fluoranthene, limited toxicity data exists in the literature for marine species.

Polychlorinated biphenyls are a group of synthetic organic chemicals that have been banned in most industrialised countries since the 1980s. While no natural sources of PCBs exist they can enter the aquatic environment through leakage, recycling, transboundary influx via major rivers and long-range atmospheric transport (Ockenden et al., 1998). PCBs are persistent in the environment and are biomagnified up the food chain and have been found in top predators in the marine environment (Leonards et al., 1997). The congener PCB 153 was selected for this study, primarily on the basis of its relative environmental persistence and as it is routinely monitored in Ireland in sediments and biota. To the best of our knowledge no toxicity data exists in the literature for PCB 153 for the test species used in this study.

Sediment porewater is defined as the water occupying the space between sediment particles (Ingersoll, 2003). It is of interest in sediment assessment as it contains the water-soluble, bioavailable fraction of contaminants and as a result, is considered to be a major route of contaminant exposure to indigenous biota. Sediment Toxicity Identification Evaluation (TIE) techniques are used in sediment assessment to identify biologically active compounds employing a bioassay directed fractionation procedure. Currently, there is no single standardized approach for conducting marine sediment TIE procedures however, there are several frequently used techniques employing marine porewaters to identify the causative agents at sites where significant toxicity is observed.

The objectives of this study were to: (1) investigate the toxicity of five OSPAR chemicals for priority action (OSPAR, 2005) with a battery of three marine bioassays comprising of bacteria (*Vibrio fischeri*), a microalgae (*Tetraselmis suecica*),

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and a harpacticoid copepod (*Tisbe battagliai*), (2) to rank the species according to sensitivity to each chemical, and (3) to identify a suitable test species and method for use in Toxicity Identification Evaluation (TIE) with sediment porewaters of suspected toxicity.

2 Materials and methods

2.1 Test substances

The organotin compounds tributyltin chloride ($C_{12}H_{27}ClSn$; CAS Registry No. 1461-22-9), triphenyltin chloride ((C_6H_5)$_3$SnCl; CAS Registry No. 639-58-7) and PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl; $C_{12}H_4Cl_6$; CAS Registry No. 35065-27-1) were obtained from LGC Promochem (UK). The PAH compounds, benzo[a]pyrene [B[a]P] ($C_{20}H_{12}$; CAS Registry No. 50-32-8) and fluoranthene [FLA] ($C_{16}H_{10}$; CAS Registry No. 206-44-0) were obtained from Sigma Aldrich (UK). The organic solvent dimethylsulphoxide [DMSO] (Sigma, Ireland) was employed as a solubilising agent in the preparation of stock solutions of the organotins and PAH compounds. Acetone was employed as the solvent in the preparation of the PCB stock solution. Analytical grade potassium dichromate ($K_2Cr_2O_7$; CAS Registry No 778-50-9) and phenol (C_6H_6O; 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. Chemical structures of all test compounds are shown in Figure 1. 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 all tests, and for each test 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. Maximum allowable concentrations (MACs) for DMSO and acetone were determined for all species before preliminary

and subsequent definitive testing. 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 ‰. Algal species and *T. battagliai* were maintained in the laboratory in accordance with standard methods (BS EN ISO 10253, 1998; ISO/DIS 14669, 1997). The culture and testing of all species was carried out in the Radiation and Environmental Science Centre (RESC). 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.

2.2.1 Microtox$^®$ assay.

Lyophilised *Vibrio 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. The 90 % test was selected as it is the method routinely employed in our laboratory for the assessment of environmental samples. 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

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 ^{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 in a final volume of 20 ml. Negative controls were incorporated for each test containing only algal growth media and algal inoculum. Solvent controls were run concurrently with each test. The cell density of each replicate was measured after 72 h using a Neubauer Improved (Bright-Line) chamber (Brand, Germany). Average specific growth rate ($\mu = \ln (N_L - \ln N_0)/ t_L$, where, t_L is the time in hours, between the last measurement of the exponential growth period and the beginning of the test, N_0 is the initial density and N_L is the measured cell density at time t_L) and percentage inhibition of average specific growth rate relative to controls were calculated for each concentration. Test validity criteria required algal growth in control flasks to increase by a factor of 16 within the 72 h test period and pH not to have varied by more than $\pm$ 1 unit during the test. 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 *Tisbe 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. The

bioassays were conducted in 12 well polystyrene plates (Corning Incorporated Costar®, New York, USA). Plates were placed in distilled water for 24 h before test initiation. Test plates 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, and solvent controls were run concurrently with all tests. 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. At test termination, dissolved oxygen concentrations were greater than or equal to 4 mg/L. Salinity and pH were controlled to within $\pm$ 2 ‰ or $\pm$ 1 unit respectively. Control species mortality of less than or equal to 10 % was required as a further validation parameter.

2.2.4 Toxicity Identification Evaluation: Preliminary evaluation of methods and suitability of test species

To determine the efficacy of the proposed Toxicity Identification Evaluation (TIE) methods for employment in marine porewater studies, several marine TIE manipulations were performed with spiked TBT-Cl samples and the Microtox® and *Tisbe battagliai* acute toxicity test systems. Starting concentrations of 0.9 µmol/L and 0.1 µmol/L for the Microtox® acute test and *T. battagliai* acute test respectively were employed for the manipulations. These concentrations of TBT-Cl were the same as those used for the initial toxicity testing. A Phase I Marine TIE characterization (Burgess et al., 1996) was conducted. The modified test procedure is outlined in Figure 2.

For the Microtox® manipulations spiked samples were divided into four 5 ml portions. The first aliquot was used to measure the toxicity of the untreated samples.

For the second and third aliquots 10 µl and 17 µl of ethylenediaminetetraacetic acid (EDTA) stock (74.68 mmol EDTA/L) and sodium thiosulphate stock (94.7 mmoles $Na_2S_2O_3$/L) were added respectively. After addition of EDTA the sample was left to chelate for three hours prior to testing. In the case of the sodium thiosulphate the sample was left for one hour before testing. The fourth aliquot was passed through a methanol-activated C_{18} resin column (SUPELCO Discovery® DSC-18, USA). The post column sample eluate was then tested for toxicity. Due to a significant toxic effect observed with the EDTA and the 90 % Microtox® test the Basic test was employed for all spiking experiments. All tests were performed in accordance with operational procedures from Azur Environmental Ltd. (Azur Environmental Ltd, 1998) and 5, 15 and 30 min readings were recorded.

For *Tisbe battagliai* manipulations the spiked sample was again divided into four aliquots each of 10 ml. The first aliquot was used to measure the toxicity of the untreated sample. The second aliquot was used to prepare a six step concentration dilution series. Twenty microlitres of EDTA stock were added to each test container and after three hours *T. battagliai* were added. For the third aliquot, another identical dilution series was prepared, 34 µl of sodium thiosulphate stock were added to each test container and after one hour the organisms were added. The final aliquot was passed through a methanol activated C_{18} resin and this post column sample eluant was used to prepare the six concentration dilution series. Organisms were then added immediately. All toxicity testing was conducted with slight modifications according to the ISO method (ISO/DIS 14669, 1997).

For both assays EDTA, sodium thiosulphate and post C_{18} column test media blanks were run concurrently. Testing was conducted on three independent occasions for both assays.

2.2.5 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 (microalgae) 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. 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 12 (MINITAB Inc. PA, USA). Statistical significance was accepted at $p \leq 0.05$.

3   Results

Testing of reference chemicals in tandem with the organic compounds was carried out to ensure the validity of each test method. Ecotoxicity data determined from each test system and its respective reference chemical is presented in Table 2. Prior to conducting any bioassays with the organic test chemicals, the maximum allowable concentration (MAC) of the organic solvents was established. The MAC is defined as the percentage concentration of solvent that does not produce a significant effect ($p \leq 0.05$) on the test organisms. Dimethylsulphoxide and acetone were used as carrier solvents for the organic chemicals employed in this study. The MACs for *Vibrio fischeri* and acetone and DMSO were both 1 %, those for *Tetraselmis suecica* were 1 % (acetone) and 0.5 % (DMSO) and for *Tisbe battagliai* the MACs were 0.1 % for both solvents. In addition, solvent controls in which acetone or DMSO was incorporated at the highest concentration present in the test solutions were run concurrently. These controls did not demonstrate any significant toxicity to the test species when compared to the media controls. The results of chemical analyses of organotin stock test solutions showed no difference between nominal and mean measurement concentrations. Stock solutions of the organotin compounds were analysed and shown to be in close agreement with nominal concentrations i.e. ± 8.9 % for TBT (SD) and ±13.2 % for TPT (SD). However percentage recoveries of concentrations determined were 102.1 % for TBT and 134.3 % for TPT (n = 3) due to method variances. Concentrations for all other chemicals are reported as nominal.

3.1 Microtox[®]

The mean EC_{50} value for the positive phenol control (fifteen analyses) was 19.93 mg/L (95 % CI = 15.81 – 25.97 mg/L) which was within the recommended EC_{50} value range of 13.00 – 26.00 mg/L (Azur Environmental Ltd., 1998). Toxic responses of the five organic pollutants employing the Microtox[®] acute test were determined and are presented in Table 3. A time dependent decrease in light levels and resulting increase in toxicity was observed with the two organotin compounds. In contrast the B[a]P and FLA elicited their most toxic response after only 5 min exposure to the test organism followed by a decrease in toxicity with increasing exposure time (Table 3).

A biostimulatory response was observed at low concentration (0.004 – 0.014 µmol/L) following exposure to TBT-Cl for 5, 15 and 30 min. This stimulatory response ranged from 1 to 33 % increase in light output when compared to the controls. However, this biostimulatory response was not statistically significant ($p \leq 0.05$) when compared to the controls. Employing calculated ecotoxicity values (EC_{50}) a sensitivity ranking order for all chemicals to the Microtox[®] system is as follows: TBT > TPT > FLA > B[a]P > PCB 153.

3.2 Microalgal toxicity test

The mean EC_{50} value for the reference chemical potassium dichromate with *T. suecica* was 14.12 mg/L (95 % CI = 11.20 – 15.13 mg/L). No equivalent data for *T. suecica* and the reference chemical were identified in the literature. *T. suecica* showed great sensitivity to the organotins, 72 h EC_{50} values were calculated to be 0.469 µmol/L and 0.013 µmol/L for TBT-Cl and TPT-Cl respectively. Toxicity testing with the PAH Benzo[a]pyrene proved more difficult. Several range finding

tests were conducted. Results were inconsistent and a reproducible dose-response curve was not obtained. Therefore no EC_{50} value was reported. Growth inhibition tests with PCB 153 also produced irregular dose-response curves with the algae and did not allow for the quantification of an accurate EC_{50} value. In the case of fluoranthene a maximum inhibition of 32 % growth was observed at the top concentration and hence it was not possible to derive an EC_{50} value.

3.3 Copepod toxicity test

The *Tisbe battagliai* bioassay was performed in triplicate and yielded a 48 h LC_{50} value of 7.42 mg/L (95 % CI = 5.73 – 10.01 mg/L) for the reference chemical potassium dichromate. Reference values of 1.6 mg/L (LC_{50} for naupliar exposure) and 5.9 mg/L (LC_{50} for adult female exposure) were reported for *T. battagliai* after 96 h exposure to $K_2Cr_2O_7$ (Hutchinson et al., 1994) The control mortality in the acute tests with *T. battagliai* was less than 5 % for all chemicals. All water quality parameters (salinity, temperature, pH and dissolved oxygen) met with International Standards Organisation guidelines (ISO/DIS, 1997). *T. battagliai* was significantly more sensitive to the organic solvents (DMSO and acetone) compared to the other test battery species. Toxicity data for tested compounds in the *T. battalgiai* test is presented in Table 4. The organotins were highly toxic to *T. battalgiai* with triphenyltin chloride (48 h LC_{50}= 0.009 µmol/L) being an order of magnitude more toxic than tributyltin chloride (48 h LC_{50}= 0.07 µmol/L). With a 48 h LC_{50} of 4.01 µmol/L, the PAH, benzo[a]pyrene appeared to be the least toxic to the benthic harpacticoid. Fluoranthene was more toxic than Benzo[a]pyrene to the copepod with a 48 h LC_{50} value of 1.19 µmol/L. The toxicity of the organic contaminants, in decreasing order was as follows: TPT > TBT > PCB 153 > FLA > B[a]P.

3.4 Toxicity Identification Evaluation: Spiking experiment results

Figure 3 presents the results of the baseline toxicity test with TBT-Cl (initial concentration of 0.9 µmol/L) and the Microtox® system and all subsequent manipulations. From this data it can be seen that the C_{18} column removed all significant toxicity. Apart from the EDTA, which showed a slight increase in toxicity, there were no significant changes in toxicity after the other manipulations. The sodium thiosulphate addition had no significant effect and the EC_{50} values generated post manipulations were nearly identical to those of the baseline test (Table 5). Absence of toxicity in the post C_{18} column sample eluate suggests that organic toxicants were active in the original sample. The Microtox® Basic test employs only 4 test concentrations, whereas the 90 % test for aqueous extracts uses 9 concentrations. It was not possible to identify an accurate NOEC value as this value was below the lowest concentration tested. Table 5 shows the EC_{50} values calculated after each manipulation. No EC_{50} value could be calculated for the post C_{18} column sample as nearly all toxicity was removed. After 15 and 30 min exposure hormesis was detected, however levels were not statistically significant.

Figure 4 shows the results of the baseline toxicity test with TBT-Cl (start concentration of 0.1 µmol/L) and *T. battagliai* with all manipulations. The addition of EDTA reduced the toxicity at the top concentrations of TBT-Cl after 24 hours but after 48 h the toxicity was reduced at all concentrations except 0.04 µmol/L compared to the baseline test. The sodium thiosulphate addition increased the observed toxicity at lower concentrations of TBT-Cl. Only at the top TBT-Cl concentration was the toxicity observed to be less than the baseline. Passage through the C_{18} column removed toxicity at all concentrations, which is indicative of organic compounds

being responsible for the observed toxicity in the initial and baseline tests. Table 6 shows the EC_{50} values and corresponding NOEC and LOEC values for all manipulations. Little information was available in the literature on the sensitivity of the test species to the reagents used in the manipulations (e.g. EDTA, $Na_2S_2O_3$) therefore blanks were run alongside all manipulations. Ethylenediaminetetraacetic acid was shown to be significantly toxic to *V. fischeri* at the top concentration in the Basic Microtox[®] test but was not at all toxic to *T. battagliai.* Sodium thiosulphate was not observed to be toxic to *V. fischeri* but significantly increased the toxicity of the TBT-Cl to *T. battalgliai* when added suggesting a possible synergistic effect (Figure 4).

4   Discussion

Persistent Organic Pollutants (POPs) are widespread contaminants in the marine environment and represent a global problem. They encompass many different groups of chemicals including PAHs, PCBs and organotins and are of the utmost concern in marine environmental evaluation and risk assessment. In environmental testing, bioassays should be employed in tandem with traditional chemical analysis to provide an integrated picture of the overall toxicity of the various exposure phases (i.e. porewater, elutriate, solvent extracts, whole sediment) from a contaminated site. There is no universally sensitive test species that can reliably predict the potential hazards associated with contaminated sediments. A multi-trophic battery of tests, which incorporates a number of test species and a variety of endpoints (e.g. lethality, growth inhibition) is therefore advocated to reduce the probability of false negative results.   In this study the sensitivities of a battery of bioassays containing an indigenous Irish alga and crustacean, to a suite of organic contaminants were investigated.   The selected tests have the advantage of being inexpensive, reproducible, easy to conduct and ecologically relevant.

The Microtox® assay with the bioluminescent bacteria *V. fischeri* represents the decomposer echelon in the multi-trophic battery of tests. It has long been considered an extremely sensitive test system and in several recent studies it has proven to be the most sensitive assay employed as part of a battery approach (Davoren and Fogarty, 2004; Jos et al., 2005). In the current study however, it was not found to be the most sensitive test system employed. The benthic harpacticoid copepod *T. battagliai* was several orders of magnitude more sensitive to the PAHs and PCBs and also more sensitive than the Microtox® system to the organotin TPT.

Tests with the Microtox® system and TBT-Cl and TPT-Cl resulted in 15 min EC_{50} values of 0.083 and 0.250 µmol/L respectively, in this research. Fifteen minute EC_{50} values of 0.029 and 0.052 µmol/L have been previously reported (Argese et al.,1998) for TBT-Cl and TPT-Cl respectively. Several other authors have reported similar EC_{50} values after 15 min exposure to TBT: 0.03 µmol/L (0.011 mg/L) [Fernández-Alba et al., 2002], 0.76 µmol/L (246.8 µg/L) [Dooley and Denis, 1987]. In this study the toxicity of both TBT-Cl and TPT-Cl were observed to increase with time. This phenomenon has previously been observed with organotins. Stasinakis et al. (2001) noted an increase in inhibition of respiration rate of activated sludge heterotrophic micro-organisms with increasing exposure time to tributyltin, dibutyltin and triphenyltin. Current work in our laboratory has also revealed increasing toxicity of TBT-Cl and TPT-Cl with increasing exposure time when examining the cytotoxic effects on the rainbow trout gonad (RTG-2) cell line (Giltrap et al. [in preparation]). A review article by Salizzato et al., (1996) indicated that a 5 min EC_{50} value is adequate to report for organic compounds. This study does not agree with this observation as toxicity for the organotins was observed to increase with lengthened exposure times. In contrast the toxicity of the PAHs to Microtox® in this study was observed to decrease with exposure time. In this study a 15 min EC_{50} value of 17.57 µmol/L was reported for fluoranthene. El-Alawi et al., (2002) reported a similar 15 min EC_{50} value of 2.16 mg/L (10.67 µmol/L). No toxicity data for PCB 153 were found in the literature for the acute Microtox® assay.

After an extensive search of the literature no toxicity data was found for *T. battagliai* and the organotins. However, Alzieu (1996) revealed that significant effects on the growth and reproduction of phytoplankton and zooplankton have been observed at concentrations of less than 0.4 µg/L of TBT (0.001 µmol/L). In this study

24 h and 48 h LC_{50} values were reported for both TBT-Cl and TPT-Cl (Table 4). Forty eight hour LC_{50} values of 0.068 µmol/L and 0.009 µmol/L were recorded for TBT-Cl and TPT-Cl respectively. Kusk and Peterson (1997) reported a 48-h LC_{50} of 0.0007 µmol/L TBT for the calanoid copepod *Acartia tonsa*. The next most toxic compound to *T. battagliai* was PCB 153 with 24 h and 48 h LC_{50} values of 0.834 µmol/L and 0.357 µmol/L respectively. Barata et al., (2002) conducted a 96 h acute toxicity test with fluoranthene and reported an LC_{50} value of 0.310 µmol/L (0.063 mg/L). In this study only 24 h and 48 h LC_{50} values of 3.211 µmol/L and 1.193 µmol/L respectively were observed for fluoranthene. Benzo[a]pyrene, a known carcinogen proved to be the least toxic of the five organics to *T. battagliai*. It is important at this stage to point out that all testing with B[a]P was conducted without metabolic activation. Benzo[a]pyrene is chemically unreactive, but during its metabolic processing by the cell, the parent compound can be activated to electrophilic derivatives, which bind covalently to nucleophilic residues in cellular macromolecules, rendering them more toxic (Miller and Whitlock, 1981).

The importance of copepods to the marine ecosystem cannot be over emphasised. The abundant calanoid and cyclopoid copepods of the plankton form the first vital link in the food chain that leads from the microscopic algal cells of the phytoplankton up to the larger fish and mammals. These phytoplankton-feeding copepods are undoubtedly the most important primary consumers in marine planktonic communities and, as such, form the base of virtually all pelagic food chains. Therefore, the sensitivity of these ubiquitous organisms to commonly occurring organic contaminants such as the organotins is of the highest consequence to the effective functioning of the marine ecosystem.

The ecotoxicology of the organotins has been of major concern in the past few decades and information available on the subject is essentially related to triorganotins (TBT, TPT). Trialkyltins are oxidative phosphorylation inhibitors and the nature of the alkyl group determines the degree of the toxic effect on different species. In this study the organotin compounds (TBT-Cl and TPT-Cl) were consistently the most toxic chemicals to all species. The phenyltin was more toxic than the butyltin in the case of *T. battagliai* and the prasinophyte *T. suecica*, while TBT was the more toxic in the Microtox[®] assay. Data available on the effects of butyltins and organotin derivatives other than TBT on aquatic marine organisms are scarce. Measurements of TBT and other organotin concentrations in waters and sediments from harbours and estuaries globally have shown a reduction in contamination compared to that observed in the early 1980s. This is due in part to the legislation and shipping restrictions that have been introduced in recent years to curtail the use of organotin containing marine antifoulant paints. The degradation of organotins in waters is short (half life is 6 – 7 days at 28 ºC for estuarine waters, up to 17 days for open ocean waters [Batley, 1996]), while oxic surface sediments are microbially mediated and persistence differs little from half lives in water. However, in deeper anoxic sediments, degradation is significantly slower with half lives of 1.9 to 3.8 years (Batley, 1996) which may lead to detrimental effects should the biota become exposed following dredging of the sediments. Therefore there is a need for further evaluation of these compounds for a large number of species to allow for probable thresholds ensuring survival and reproduction of the organisms within an ecosystem especially where dredging is likely (e.g. harbours, estuaries).

Polycyclic aromatic hydrocarbon (PAH) toxicity is structurally dependent (Kalf et al., 1997). Fluoranthene, a four-ringed PAH, is one of the most abundant

PAH contaminants in the environment. Fluoranthene, a four-ringed PAH, is one of the most abundant PAH contaminants in the environment. For this reason and because PAHs are on the OSPAR List of Substances for Priority Action, fluoranthene was chosen for inclusion in this study. Despite its abundant presence in the environment there is very little information available on its toxic effects on marine species. Most PAHs are indirect acting toxicants and require metabolic activation to elicit a toxic effect on the test organism. In this study benzo[a]pyrene was observed to be highly toxic to *T. battagliai* but was considerably less toxic to *V. fischeri*. Although it is a well known carcinogen, the metabolic derivatives of B[a]P have been observed to be far more toxic than the parent compound itself (Araújo et al., 2000). The toxicity of these metabolic products was not investigated in this study.

While a number of other PCB congeners are known to be of greater toxicity, PCB 153 was chosen for inclusion in this study as it is a persistent, routinely monitored congener in marine sediments in Ireland. The results indicate that the PCB is highly toxic to *T. battagliai*. It was found to be more toxic to the copepod than the two PAHs but not as toxic as the organotins. Despite various studies on PCBs, their mechanism of toxicity is still unknown. It was not possible to derive EC_{50} values for the Microtox$^{®}$ and algal tests in this study. Stock concentration of PCB 153 were prepared and tested on the same day with the Microtox$^{®}$ system and algae. To avoid analytical interference from plastics, glassware was used in preparation of chemicals and the use of plastics was kept to a minimum. Initial testing with *V. fischeri* yielded a dose response curve. High toxicity, in excess of 50 % effect, was observed after 5 min exposure after which toxicity was observed to decrease with time to as low as 37 % after 30 min at the top concentration (62 µmol/L). Subsequent toxicity testing with other stock solutions of PCB after storage (in a glass volumetric flask at 4 ℃ in the

dark for > 24 h) demonstrated little or no toxicity. After several definitive tests with PCB 153 and *T. suecica* the results were not found to be reproducible. Stock solutions were made up on several occasions, tested, stored and retested, and the same effect was observed. Lung et al. (2000) investigated adsorption/desorption of PCB congeners in aqueous solution onto glass surfaces. They found that adsorption loss is related to the degree of chlorination of the PCB congener. In their study PCB 180 (isomer group Cl-7) showed the greatest absorbance (55 %) onto the glass surface and PCB 52 (Cl-4) lost the least (17 %). Lung et al. (2000) also concluded that PCB congeners may adsorb more onto glass surfaces in saline water due to a 'salting out effect' i.e. hydrophobic PCBs are less soluble in more polar aqueous environments. Such effects may partly explain the reduction in toxicity observed in the Microtox[®] and algal tests after storage of the stock PCB 153 (Cl-6). The inconsistent results observed with the algal tests (conducted in glass Erlenmeyer flasks) may also be partially explained by the 'salting out effect' described by Lung et al. Recently Breitholtz et al., (2007) published a method for overcoming the difficulties of testing poorly water-soluble substances using a silica gel test system. They concluded that the system enables a more controlled exposure of hydrophobic substances that traditional water test systems since the concentration-response relationship becomes distinct and there is no carrier solvent. These methods are only in the early stages of development but may allow for a more accurate screening of hydrophobic substances for risk assessment with substances such as those employed in this study.

One of the main objectives of this research was to identify a suitable test species for employment in Toxicity Identification Evaluation (TIE) with sediment porewaters of suspected toxicity in routine assessment and monitoring of Irish sediments. Toxicity Identification Evaluation methods in marine risk assessment and

environmental evaluation have been employed in the United States for many years (Burgess et al., 1995; Burgess et al., 1996). A few studies with marine porewaters exist for European waters (Guzzella et al., 1996). Toxicity Identification Evaluation techniques, using bioassays to direct the fractionation of complex environmental samples (e.g. sediment porewater) can be applied in an attempt to identify the compounds responsible for any observed toxicity of the sample. When conducting TIE manipulations, both acute and chronic endpoints with several different species would ideally be used. This is not always feasible. The problems associated with porewater sample extraction (particularly when sandy sediments with a high proportion of > 2 mm particles are being investigated) can be prohibitive in employing tests that necessitate large volumes of test solution e.g. traditional microalgal flask methods. The Microtox® acute assay would be considered an ideal candidate for TIE based on the small sample volume required and the speed of testing (5, 15, 30 min). However, although the Microtox® assay was highly sensitive to the organotins it demonstrated poor sensitivity to the PAHs and PCB compared to *T. battaglia.* Although the *T. battagliai* test requires larger sample volumes and is of longer duration than the Microtox® test, it is considered suitable because of its greater sensitivity to a wider range of chemicals. The spiked sample manipulations conducted with the Microtox® and *T. battalgiai* acute toxicity test systems verified their suitability as test species for TIE studies. With both species it was possible to conduct all manipulations within one day. In the case of the Microtox® system all manipulations could be conducted in triplicate within three days while for *T. battagliai* it was possible to do so within one week, depending on the numbers of organisms available in culture. Results from these TIEs on simple spiked samples

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provide insight into the complexity of interpreting marine TIE data from porewater studies where mixtures of unknown substances are present.

5   Conclusion

In conclusion, all organic compounds tested elicited a toxic effect on all species employed. The concentrations tested include environmentally relevant levels previously identified in the water column (e.g. Organotins: Batley, 1996).

The Microtox® assay demonstrated high sensitivity to the organotin compounds but not the PAHs. The algal growth inhibition assays and the copepod lethality test were found to be the most sensitive of the battery tests to all chemicals. The microalgae, a primary producer and *T. battagliai,* a primary consumer can be considered essential components of the marine food web and are indigenous Irish species, lending ecological relevance to this study. Although the algae were found to be more sensitive to several of the organic contaminants assayed the copepod bioassay was selected as the most suitable species for use in future TIE studies with marine sediments. The demonstrated sensitivity of the copepods to all chemicals tested coupled with the assays' rapid and simple features make it the most suitable candidate for TIE manipulations. In general there is a paucity of ecotoxicological data for marine species and organic contaminants and especially with respect to an Irish marine context. The data presented in this report have for the most part not previously been reported in the literature. Therefore, the data described may aid in future management decisions and contribute to the new efforts being made within the EU through programmes such as REACH (Registration, Evaluation and Authorisation of Chemicals). Thus information such as that outlined in this paper can confer greater responsibility on industry in the management of the risks associated with chemical usage and disposal.

Acknowledgements

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Table 1

Trophic level	Test Species	Chemical	Exposure Time	Endpoint	Toxicity value	Reference
Bacteria	*Vibrio fisheri*	DMSO	NR	MAC	0.5 %	Kwan and Dutka, 1990
		TBT-Cl	15 min	EC_{50}	0.029 µmol/L	Argese et al.,1998
		TPT-Cl	15 min	EC_{50}	0.052 µmol/L	Argese et al.,1998
		TBT-Cl	5 min	EC_{50}	0.022 mg/L	Fernández-Alba et al., 2002
		TBT-Cl	15 min	EC_{50}	0.011 mg/L	Fernández-Alba et al., 2002
		TBT-Cl	5 min	EC_{50}	7.0 µg/L	Dooley and Denis, 1987
		TBT-Cl	15 min	EC_{50}	246.8 µg/L	Dooley and Denis, 1987
		FLA	15 min	EC_{50}	2.16 mg/L	El Alawi et al., 2002
		FLA	18 h	EC_{50}	80.99 mg/L	El Alawi et al., 2002
		TBT	30 min	EC_{50}	0.007 ± 0.0012 mg/L	Hernando et al., 2003
Copepods	*Tisbe battagliai*	Acetone	26 d	NR	25 µl/L	Bechmann, 1999
	Tisbe battagliai	FLA	4 d	LC_{50}	0.063 mg/L	Barata et al., 2002

DMSO = Dimethyl sulphoxide, NR = Not Reported, MAC = Maximum Allowable Concentration, FLA = Fluoranthene, EC_{50} = Effective Concentration of 50 % of sample population, LC_{50} = Lethal concentration of 50 % of sample population.

Table 2

Species	Reference chemical	Exposure period and endpoint	EC/LC$_{50}$[a] (mg/L)	Cited value (mg/L)	Reference
Vibrio fischeri	C_6H_6O	5 min inhibition	21.29 (14.73 – 35.26)	13 – 26	Azur Environmental (1998)
Tetraselmis suecica	$K_2Cr_2O_7$	72 h inhibition	14.12 (11.20 – 15.13)	–	
Tisbe battagliai	$K_2Cr_2O_7$	48 h lethality	7.42 (5.73 – 10.01)	1.6 [b]	Hutchinson et al. (1994)
				5.9 [c]	Hutchinson et al. (1994)

[a] EC$_{50}$ values and corresponding 95 % confidence intervals in parentheses
[b] 96 h LC$_{50}$ value for nauplii
[c] 96 h LC$_{50}$ value for adult females

Table 3.

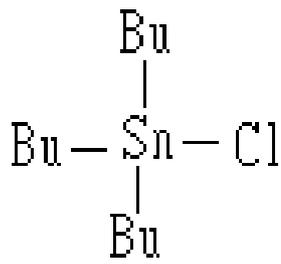| Test chemical	Exposure time (min)	Concentration range (µmol/L)	EC$_{50}$[a] (µmol/L)	NOEC (µmol/L)[b]	LOEC (µmol/L)[c]
TBT-Cl	5	0.004 – 0.900	0.129 (0.124 – 0.224)	0.056	0.113
	15	0.004 – 0.900	0.083 (0.078 – 0.132)	0.028	0.056
	30	0.004 – 0.900	0.059 (0.055 – 0.080)	0.028	0.056
TPT-Cl	5	0.004 – 0.900	0.584 (0.400 – 0.802)	0.225	0.450
	15	0.004 – 0.900	0.250 (0.153 – 0.303)	0.113	0.225
	30	0.004 – 0.900	0.184 (0.103 – 0.247)	0.113	0.225
B[a]P	5	0.588 – 150.740	70.026 (55.821 – 85.755)	9.421	18.843
	15	0.588 – 150.740	154.071 (121.721 – 202.150)[d]	18.843	37.685
	30	0.588 – 150.740	230.777 (143.996 – 832.610)[d]	75.370	150.740
FLA	5	0.730 – 186.850	6.557 (2.606 – 10.508)	1.460	2.920
	15	0.730 – 186.850	17.571 (-0.067 – 35.209)	1.460	2.920
	30	0.730 – 186.850	29.248 (-9.521 – 68.0182)	2.920	5.839

[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.
[d]EC$_{50}$ values and corresponding 95% confidence intervals calculated by extrapolation of the data by REGTOX-EV6.xls

Table 4

Test chemical	Exposure time (h)	Concentration range (µmol/L)	LC$_{50}$[a] (µmol/L)	NOEC (µmol/L)[b]	LOEC (µmol/L)[c]
TBT-Cl	24	0.01 – 0.10	0.090 (0.086 – 0.093)	0.080	0.100
	48	0.01 – 0.10	0.068 (0.056 – 0.078)	0.060	0.080
TPT-Cl	24	0.001 – 0.100	0.016 (0.012 – 0.019)	0.0075	0.01
	48	0.001 – 0.100	0.009 (0.008 – 0.010)	0.0075	0.01
B[a]P	24	0.040 – 5.945	NA	5.945	> 5.945
	48	0.040 – 5.945	4.013 (3.006 – 4.694)	1.982	3.963
FLA	24	0.124 – 7.415	3.211 (2.457 – 4.651)	1.236	4.943
	48	0.124 – 7.415	1.193 (1.013 – 1.279)	0.494	0.741
PCB 153	24	0.139 – 2.078	0.834 (0.521 – 1.119)	0.693	1.385
	48	0.139 – 2.078	0.357 (0.294 – 0.433)	0.208	0.277

[a] LC$_{50}$ 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
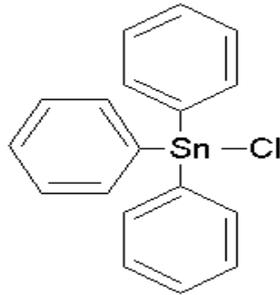[c] LOEC, lowest observed effect concentration, the lowest concentration of the tested concentration at which a significant ($p \leq 0.05$) effect was detected.
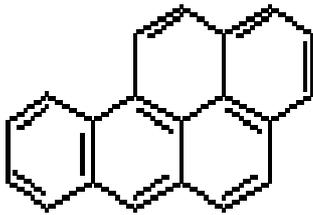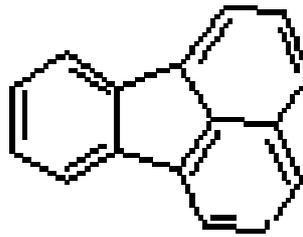NA = Not Applicable
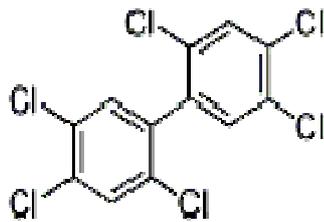
Fig 1

Tributyltin chloride

Triphenyltin chloride

Benzo[a]pyrene

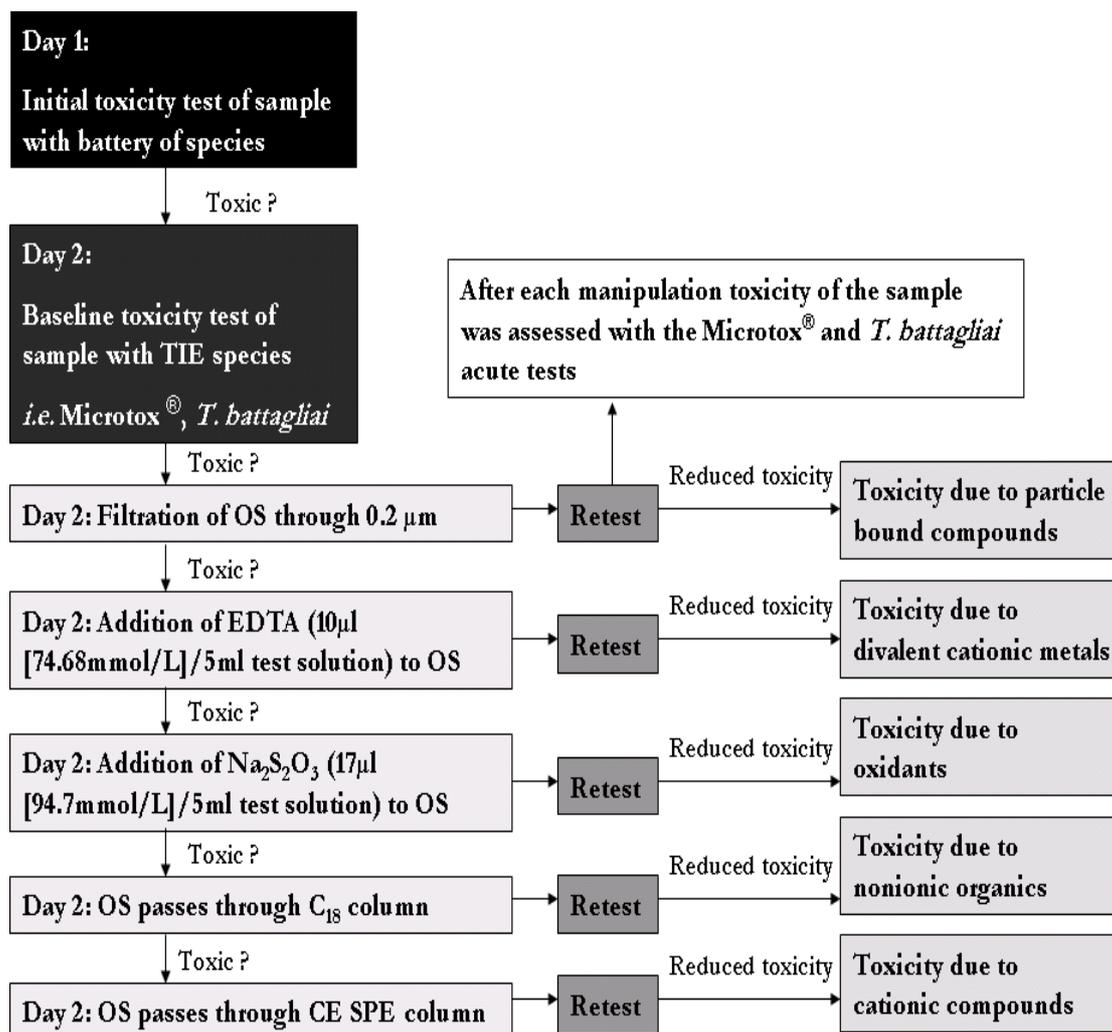Fluoranthene

2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153)

Fig. 2

Day 1:

Initial toxicity test of sample with battery of species

↓ Toxic ?

Day 2:

Baseline toxicity test of sample with TIE species

i.e. Microtox ®, *T. battagliai*

After each manipulation toxicity of the sample was assessed with the Microtox® and *T. battagliai* acute tests

↓ Toxic ?

Day 2: Filtration of OS through 0.2 μm → Retest — Reduced toxicity → Toxicity due to particle bound compounds

↓ Toxic ?

Day 2: Addition of EDTA (10μl [74.68mmol/L]/5ml test solution) to OS → Retest — Reduced toxicity → Toxicity due to divalent cationic metals

↓ Toxic ?

Day 2: Addition of $Na_2S_2O_3$ (17μl [94.7mmol/L]/5ml test solution) to OS → Retest — Reduced toxicity → Toxicity due to oxidants

↓ Toxic ?

Day 2: OS passes through C_{18} column → Retest — Reduced toxicity → Toxicity due to nonionic organics

↓ Toxic ?

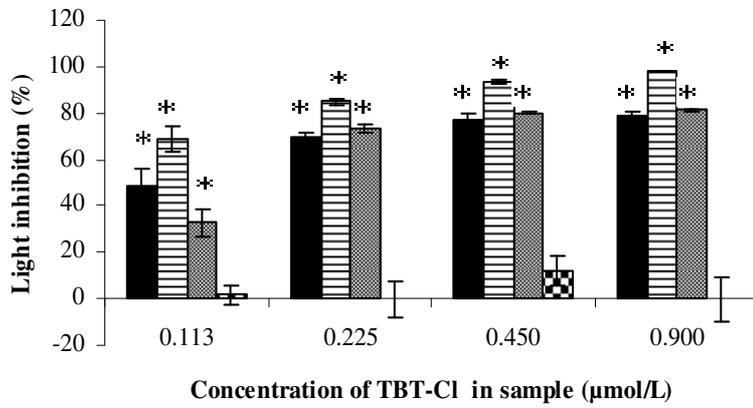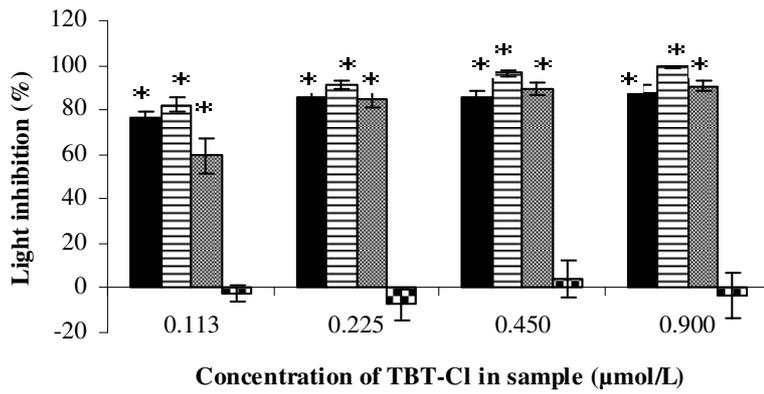Day 2: OS passes through CE SPE column → Retest — Reduced toxicity → Toxicity due to cationic compounds
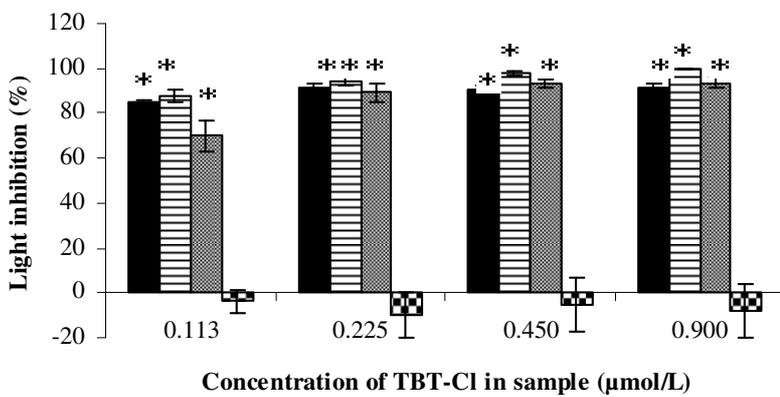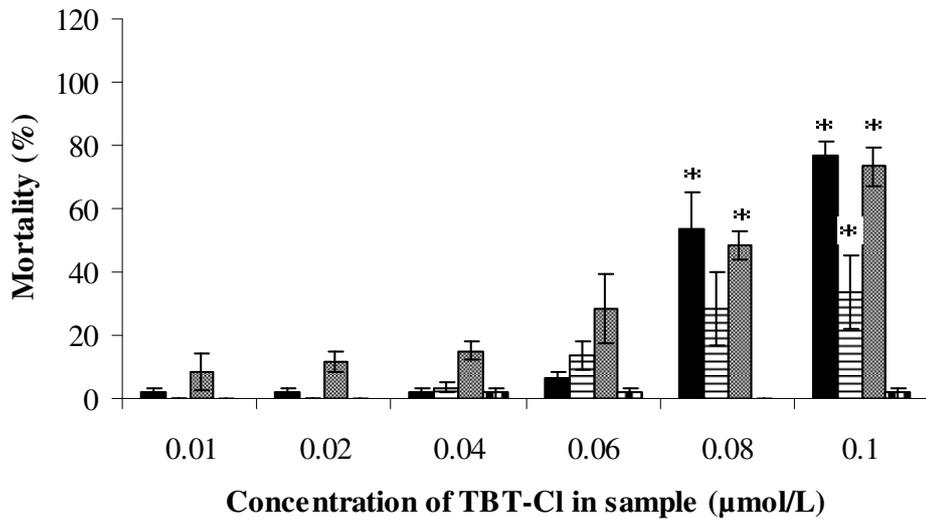
Fig. 3(a)
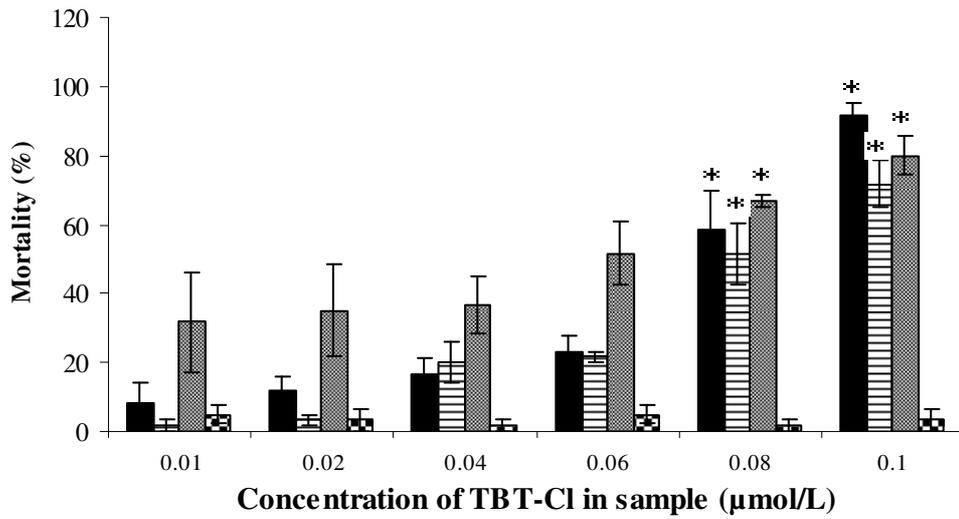

(b)

(c)

Fig. 4

(a)

(b)

Table 5

Manipulation	Exposure time (min)	Concentration of reagent stocks (mmol/L)	Volume of reagent stocks (µl)	Test type	EC_{50} (µmol/L)[a]	NOEC (µmol/L) [b]	LOEC (µmol/L) [c]
Initial toxicity test	5	NA[d]	NA	90% test	0.129 (0.124 – 0.224)	0.056	0.113
	15	NA	NA	90% test	0.083 (0.078 – 0.132)	0.028	0.056
	30	NA	NA	90% test	0.059 (0.055 – 0.080)	0.028	0.056
Baseline test	5	NA	NA	Basic test	0.152 (0.131 – 0.191)	< 0.1125	0.1125
	15	NA	NA	Basic test	0.079 (0.044 – 0.104)	< 0.1125	0.1125
	30	NA	NA	Basic test	0.057 (0.022 – 0.083)	< 0.1125	0.1125
EDTA addition	5	74.4	10	Basic test	0.070 (0.052 – 0.087)	< 0.1125	0.1125
	15	74.4	10	Basic test	0.098 (0.037 – 0.100)	< 0.1125	0.1125
	30	74.4	10	Basic test	0.027 (0.009 – 0.050)	< 0.1125	0.1125
$Na_2S_2O_3$ addition	5	94.9	17	Basic test	0.153 (0.130 – 0.192)	< 0.1125	0.1125
	15	94.9	17	Basic test	0.079 (0.042 – 0.104)	< 0.1125	0.1125
	30	94.9	17	Basic test	0.057 (0.018 – 0.084)	< 0.1125	0.1125
Post C_{18} column	5	-	-	Basic test	NST[e]	-	-
	15	-	-	Basic test	NST	-	-
	30	-	-	Basic test	NST	-	-

[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.
[d] NA, Not Applicable
[e] NST, No Significant Toxicity

Table 6

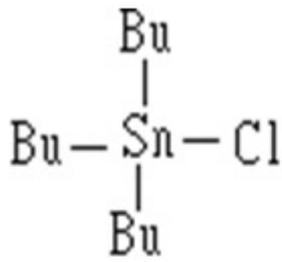| Manipulation	Exposure time (min)	Concentration of reagent stocks (mmol/L)	Volume of reagent stocks (µl)	EC$_{50}$ (µmol/L) [a]	NOEC (µmol/L) [b]	LOEC (µmol/L) [c]
Initial toxicity test	24 h	NA[d]	NA	0.090 (0.086 – 0.093)	0.080	0.100
	48 h	NA	NA	0.068 (0.056 – 0.078)	0.060	0.080
Baseline test	24 h	NA	NA	0.081 (0.077 – 0.084)	0.060	0.080
	48 h	NA	NA	0.073 (0.064 – 0.074)	0.060	0.080
EDTA addition	24 h	74.4	20	0.127 (0.104 – 0.193)	0.100	> 0.100
	48 h	74.4	20	0.079 (0.071 – 0.083)	0.060	0.080
Na$_2$S$_2$O$_3$ addition	24 h	94.4	34	0.078 (0.067 – 0.081)	0.060	0.080
	48 h	94.4	34	0.041 (0.024 – 0.058)	0.060	0.080
Post C$_{18}$ column	24 h	-	-	NST[e]	-	-
	48 h	-	-	NST	-	-

[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
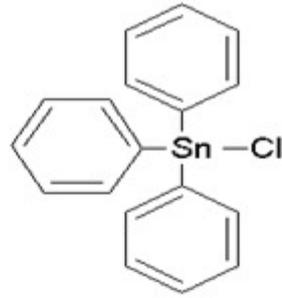[c] LOEC, lowest observed effect concentration, the lowest concentration of the tested concentration at which a significant ($p \leq 0.05$) effect was detected.
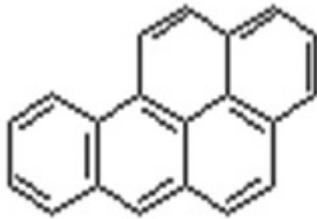[d] NA = Not Applicable
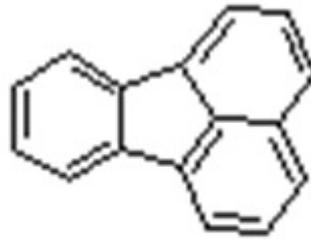[e] NST = No Significant Toxicity

Bu

Bu — Sn — Cl

Bu

Tributyltin chloride

Sn — Cl
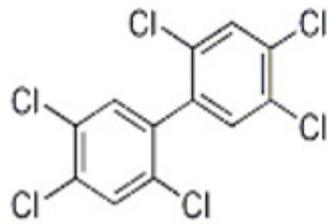
Triphenyltin chloride

Benzo[a]pyrene

Fluoranthene

2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153)

1
2   Fig. 1. Chemical structures of test compounds.
3

Day 1: Initial toxicity test of sample with battery of species

Toxic ?

Day 2: Baseline toxicity test of sample with TIE species *i.e.* Microtox®, *T. battagliai*

After each manipulation toxicity of the sample was assessed with the Microtox® and *T. battagliai* acute tests

Toxic ?

Day 2: Filtration of OS through 0.2 μm → Retest → Reduced toxicity → Toxicity due to particle bound compounds

Toxic ?

Day 2: Addition of EDTA (10μl [74.68mmol/L] /5ml test solution) to OS → Retest → Reduced toxicity → Toxicity due to divalent cationic metals

Toxic ?

Day 2: Addition of Na₂S₂O₃ (17μl [94.7mmol/L] /5ml test solution) to OS → Retest → Reduced toxicity → Toxicity due to oxidants

Toxic ?

Day 2: OS passes through C₁₈ column → Retest → Reduced toxicity → Toxicity due to nonionic organics

Toxic ?

Day 2: OS passes through CE SPE column → Retest → Reduced toxicity → Toxicity due to cationic compounds

1

2

3   Fig. 2. Overview flowchart for Toxicity Identification Evaluation procedure of spiked

4   samples of TBT-Cl. Initial toxicity testing was conducted during model compound

5   study (Day 1), while baseline toxicity testing and subsequent TIE manipulations were

6   conducted on Day 2. After each manipulation the resulting solution was tested with

7   both the Microtox® and *T. battagliai* acute tests to identify any changes in toxicity

8   post manipulation. OS = Original Sample.
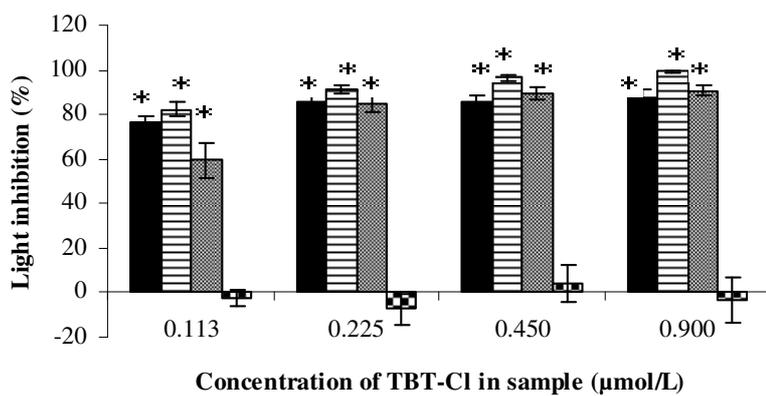
9

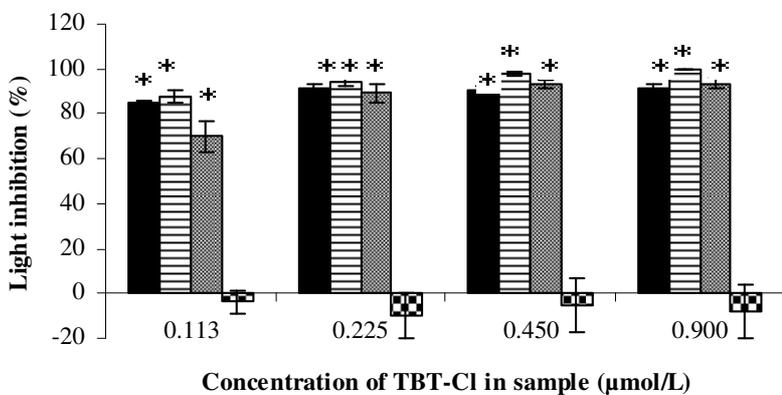10   (a)
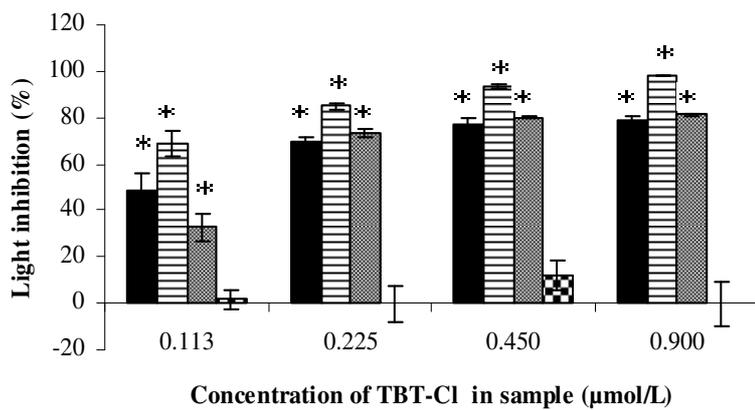
1

2   (b)

3

4   (c)

5

6

1   Fig. 3. Percentage light inhibition of *Vibrio fischeri* exposed to spiked TBT-Cl sample

2   after Baseline testing (■), post EDTA addition (▤), post $Na_2S_2O_3$ addition (▨) and

3   post C_{18} (▨) manipulation after (a) 5, (b) 15, and (c) 30 min exposure. Data are

4   expressed as a percentage of unexposed controls ± SEM of three independent

5   experiments. * denotes significance from the control ($p \leq 0.05$).
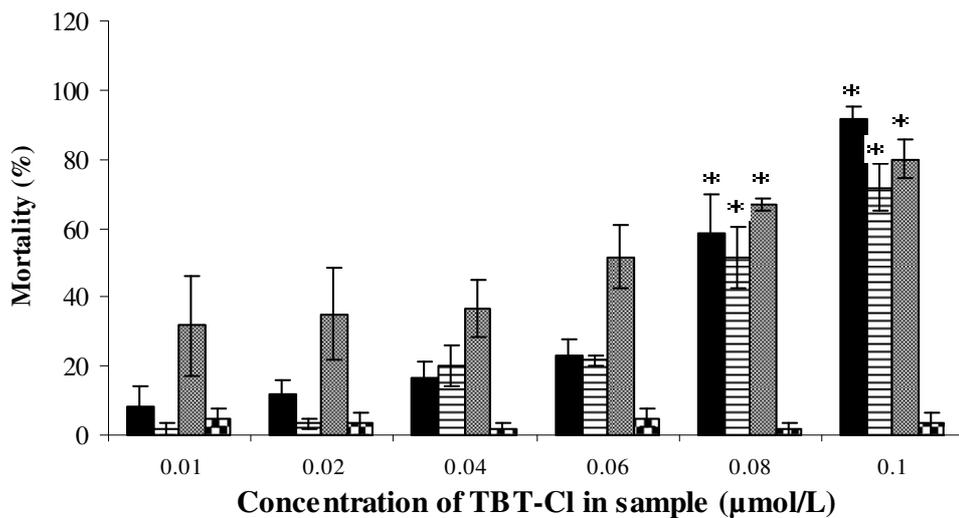
6

7

1   (a)
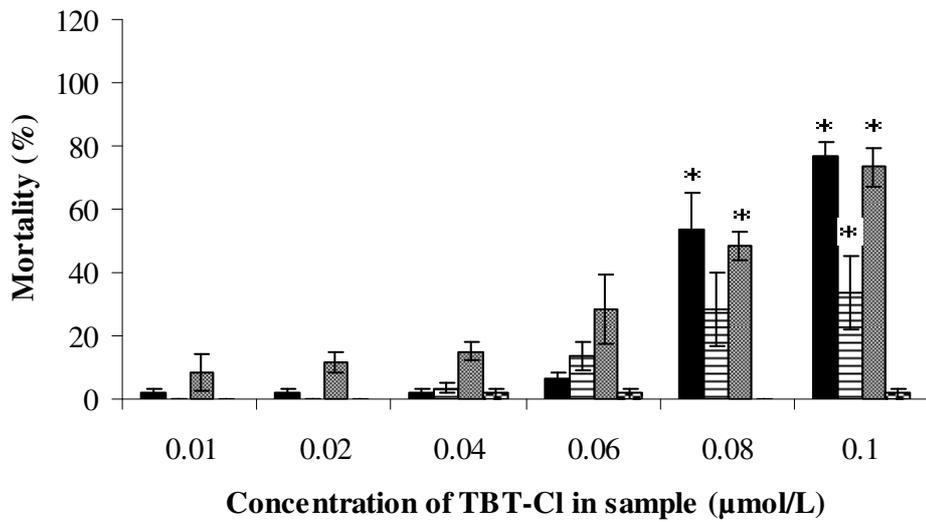
2

3   (b)

4

5

6
7

8   Fig. 4. Percentage mortality of *T. battagliai* exposure to spiked TBT-Cl sample after

9   Baseline testing (■), post EDTA addition (▤), post $Na_2S_2O_3$ addition (▨) and post

10   C_{18} (▦) manipulation after (a) 24, and (b) 48 h exposure.   Data are expressed as a

11   percentage of unexposed controls ± SEM of three independent experiments.   *

12   denotes significance from the control ($p \leq 0.05$).

13

Table 1. Ecotoxicity data from the literature on the compounds and species tested in this study

Trophic level	Test Species	Chemical	Exposure Time	Endpoint	Toxicity value	Reference
Bacteria	*Vibrio fisheri*	DMSO	NR	MAC	0.5 %	Kwan and Dutka, 1990
		TBT-Cl	15 min	EC_{50}	0.029 µmol/L	Argese *et al.,*1998
		TPT-Cl	15 min	EC_{50}	0.052 µmol/L	Argese *et al.,*1998
		TBT-Cl	5 min	EC_{50}	0.022 mg/L	Fernández-Alba *et al.*, 2002
		TBT-Cl	15 min	EC_{50}	0.011 mg/L	Fernández-Alba *et al.*, 2002
		TBT-Cl	5 min	EC_{50}	7.0 µg/L	Dooley and Denis, 1987
		TBT-Cl	15 min	EC_{50}	246.8 µg/L	Dooley and Denis, 1987
		FLA	15 min	EC_{50}	2.16 mg/L	El Alawi *et al.,* 2002
		FLA	18 h	EC_{50}	80.99 mg/L	El Alawi *et al.,* 2002
		TBT	30 min	EC_{50}	0.007 ± 0.0012 mg/L	Hernando *et al.,* 2003
Copepods	*Tisbe battagliai*	Acetone	26 d	NR	25 µl/L	Bechmann, 1999
	Tisbe battagliai	FLA	4 d	LC_{50}	0.063 mg/L	Barata *et al.,* 2002

DMSO = Dimethyl sulphoxide, NR = Not Reported, MAC = Maximum Allowable Concentration, FLA = Fluoranthene, EC_{50} = Effective Concentration of 50 % of sample population, LC_{50} = Lethal concentration of 50 % of sample population.

Table 2. Reference chemical ecotoxicity data for battery test species and endpoints.

Species	Reference chemical	Exposure period and endpoint	EC/LC$_{50}$[a] (mg/L)	Cited value (mg/L)	Reference
Vibrio fischeri	C_6H_6O	5 min inhibition	21.29 (14.73 – 35.26)	13 – 26	Azur Environmental (1998)
Tetraselmis suecica	$K_2Cr_2O_7$	72 h inhibition	14.12 (11.20 – 15.13)	–	
Tisbe battagliai	$K_2Cr_2O_7$	48 h lethality	7.42 (5.73 – 10.01)	1.6 [b]	Hutchinson *et al.* (1994)
				5.9 [c]	Hutchinson *et al.* (1994)

[a] EC$_{50}$ values and corresponding 95 % confidence intervals in parentheses
[b] 96 h LC$_{50}$ value for nauplii
[c] 96 h LC$_{50}$ value for adult females

Table 3. Ecotoxicity data for all selected chemicals with *Vibrio fischeri* (Microtox®)

Test chemical	Exposure time (min)	Concentration range (µmol/L)	$EC_{50}{}^{a}$ (µmol/L)	NOEC (µmol/L)[b]	LOEC (µmol/L)[c]
TBT-Cl	5	0.004 – 0.900	0.129 (0.124 – 0.224)	0.056	0.113
	15	0.004 – 0.900	0.083 (0.078 – 0.132)	0.028	0.056
	30	0.004 – 0.900	0.059 (0.055 – 0.080)	0.028	0.056
TPT-Cl	5	0.004 – 0.900	0.584 (0.400 – 0.802)	0.225	0.450
	15	0.004 – 0.900	0.250 (0.153 – 0.303)	0.113	0.225
	30	0.004 – 0.900	0.184 (0.103 – 0.247)	0.113	0.225
B[a]P	5	0.588 – 150.740	70.026 (55.821 – 85.755)	9.421	18.843
	15	0.588 – 150.740	154.071 (121.721 – 202.150)[d]	18.843	37.685
	30	0.588 – 150.740	230.777 (143.996 – 832.610)[d]	75.370	150.740
FLA	5	0.730 – 186.850	6.557 (2.606 – 10.508)	1.460	2.920
	15	0.730 – 186.850	17.571 (-0.067 – 35.209)	1.460	2.920
	30	0.730 – 186.850	29.248 (-9.521 – 68.0182)	2.920	5.839

[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.
[d]EC_{50} values and corresponding 95% confidence intervals calculated by extrapolation of the data by REGTOX-EV6.xls

Table 4. Ecotoxicity data for all selected chemicals with *Tisbe battagliai.*

Test chemical	Exposure time (h)	Concentration range (µmol/L)	LC_{50}[a] (µmol/L)	NOEC (µmol/L)[b]	LOEC (µmol/L)[c]
TBT-Cl	24	0.01 – 0.10	0.090 (0.086 – 0.093)	0.080	0.100
	48	0.01 – 0.10	0.068 (0.056 – 0.078)	0.060	0.080
TPT-Cl	24	0.001 – 0.100	0.016 (0.012 – 0.019)	0.0075	0.01
	48	0.001 – 0.100	0.009 (0.008 – 0.010)	0.0075	0.01
B[a]P	24	0.040 – 5.945	NA	5.945	> 5.945
	48	0.040 – 5.945	4.013 (3.006 – 4.694)	1.982	3.963
FLA	24	0.124 – 7.415	3.211 (2.457 – 4.651)	1.236	4.943
	48	0.124 – 7.415	1.193 (1.013 – 1.279)	0.494	0.741
PCB 153	24	0.139 – 2.078	0.834 (0.521 – 1.119)	0.693	1.385
	48	0.139 – 2.078	0.357 (0.294 – 0.433)	0.208	0.277

[a] LC_{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.
NA = Not Applicable

Table 5. Ecotoxicity data for all TIE manipulation on spiked sample of TBT-Cl (top concentration of 0.9 µmol/L) and the Microtox[®] acute toxicity test

Manipulation	Exposure time (min)	Concentration of reagent stocks (mmol/L)	Volume of reagent stocks (µl)	Test type	EC_{50} (µmol/L)[a]	NOEC (µmol/L)[b]	LOEC (µmol/L)[c]
Initial toxicity test	5	NA[d]	NA	90% test	0.129 (0.124 – 0.224)	0.056	0.113
	15	NA	NA	90% test	0.083 (0.078 – 0.132)	0.028	0.056
	30	NA	NA	90% test	0.059 (0.055 – 0.080)	0.028	0.056
Baseline test	5	NA	NA	Basic test	0.152 (0.131 – 0.191)	< 0.1125	0.1125
	15	NA	NA	Basic test	0.079 (0.044 – 0.104)	< 0.1125	0.1125
	30	NA	NA	Basic test	0.057 (0.022 – 0.083)	< 0.1125	0.1125
EDTA addition	5	74.4	10	Basic test	0.070 (0.052 – 0.087)	< 0.1125	0.1125
	15	74.4	10	Basic test	0.098 (0.037 – 0.100)	< 0.1125	0.1125
	30	74.4	10	Basic test	0.027 (0.009 – 0.050)	< 0.1125	0.1125
$Na_2S_2O_3$ addition	5	94.9	17	Basic test	0.153 (0.130 – 0.192)	< 0.1125	0.1125
	15	94.9	17	Basic test	0.079 (0.042 – 0.104)	< 0.1125	0.1125
	30	94.9	17	Basic test	0.057 (0.018 – 0.084)	< 0.1125	0.1125
Post C_{18} column	5	-	-	Basic test	NST[e]	-	-
	15	-	-	Basic test	NST	-	-
	30	-	-	Basic test	NST	-	-

[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.
[d] NA, Not Applicable
[e] NST, No Significant Toxicity

Table 6 Ecotoxicity data for all TIE manipulation on spiked sample of TBT-Cl (top concentration of 0.1 µmol/L) and the *T. battagliai* acute toxicity test

Manipulation	Exposure time (min)	Concentration of reagent stocks (mmol/L)	Volume of reagent stocks (µl)	EC$_{50}$ (µmol/L) [a]	NOEC (µmol/L) [b]	LOEC (µmol/L) [c]
Initial toxicity test	24 h	NA[d]	NA	0.090 (0.086 – 0.093)	0.080	0.100
	48 h	NA	NA	0.068 (0.056 – 0.078)	0.060	0.080
Baseline test	24 h	NA	NA	0.081 (0.077 – 0.084)	0.060	0.080
	48 h	NA	NA	0.073 (0.064 – 0.074)	0.060	0.080
EDTA addition	24 h	74.4	20	0.127 (0.104 – 0.193)	0.100	> 0.100
	48 h	74.4	20	0.079 (0.071 – 0.083)	0.060	0.080
Na$_2$S$_2$O$_3$ addition	24 h	94.4	34	0.078 (0.067 – 0.081)	0.060	0.080
	48 h	94.4	34	0.041 (0.024 – 0.058)	0.060	0.080
Post C$_{18}$ column	24 h	-	-	NST[e]	-	-
	48 h	-	-	NST	-	-

[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.
[d] NA = Not Applicable
[e] NST = No Significant Toxicity