Available online at www.sciencedirect.com
SCIENCE DIRECT®
The Science of the Total Environment 320 (2004) 37–50
the Science of the Total Environment
An International Journal for Scientific Research into the Environment and its Relationship with Man
www.elsevier.com/locate/scitotenv

Toxicity of the 13 priority pollutant metals to *Vibrio fisheri* in the Microtox® chronic toxicity test

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Received 15 November 2002; accepted 11 July 2003

Abstract

The Microtox® Acute Toxicity Test has been successfully used to measure the toxicity of metals and other pollutants at high concentrations (ppm) in selected environmental samples. However, metals and other toxicants are often found in much lower concentrations (ppb) in many municipal wastewaters and receiving waters. In order to assess the toxicity of these pollutants in these samples, a more sensitive toxicity assay is needed. The Microtox® chronic toxicity test has been developed to measure the sublethal effect of toxicants over multiple generations of the test species, *Vibrio fisheri*. In this study, the toxicity of the 13 priority pollutant metals [i.e. As, Se, Cd, Cr (III and VI), Cu, Pb, Sb, Ag, Tl, Zn, Be, Hg and Ni] to *V. fisheri* was evaluated using the Microtox® chronic toxicity test. In this test, the inhibitory concentration (IC), lowest observable effect concentration (LOEC), and no observable effect concentration (NOEC) were obtained after 22-h of incubation at 27 ± 1 °C, by comparing the light output of the control to that of the test sample. Among the 13 priority pollutant metals, beryllium (Be) was found to be the most toxic in the test (LOEC = 0.742–1.49 μg/l) while thallium (Tl) was the least toxic (LOEC = 3840–15 300 μg/l). The LOECs for copper (as Cu) and lead (Pb) in reagent (ASTM Type I) water were 6.78–13.6 μg/l and 626–1251 μg/l, respectively. The toxicity of copper sulfate (as Cu) in reagent water was shown and significantly reduced with the addition of natural organic matter (fulvic acid) or EDTA to the sample. The LOEC values for the 13 priority pollutant metals in this test were comparable to or lower than those reported for commonly used aquatic toxicity tests, such as the *Ceriodaphnia dubia* assay.

Keywords: Microtox® chronic toxicity test; Metals; *Vibrio fisheri*; Fulvic acid; EDTA

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doi:10.1016/S0048-9697(03)00451-0

1. Introduction

In recent years, there has been growing concern about the toxic effects of chemical substances in the aquatic environment. Many countries, including the US, are facing serious ecological and toxicological problems resulting from the discharge of complex effluents and toxic chemical substances into watersheds. Metal pollutants are among the most toxic and persistent pollutants in wastewater discharges and receiving waters (Codina et al., 1993). In Massachusetts alone, there are 65 segments in 13 watersheds (out of 30) impaired due to metal toxicity (MADEP, 1998). Therefore, it is important to know the distribution of toxic metals in pollution sources and receiving waters in order to understand the behavior of these pollutants in the aquatic environment. Chemical analysis alone can identify and quantitate trace metals in aquatic environment, but provides no direct indication of the potential effects of these metals on aquatic biota. Thus, to fully assess the impacts of trace metals on the aquatic environment, it is important to understand their biological toxicity as well as their chemical fate and transport.

Existing standard toxicity tests (USEPA, 1989a) rely on eucaryotic species, such as fathead minnow and daphnids that require long acclimatization time; these tests are labor intensive and expensive. In contrast, microbial toxicity tests rely on bacteria and other microorganisms that have similar complex biochemical pathways as higher organisms, have short life cycles, and respond rapidly to changes in the environment (Bitton, 1983); these tests are easy to perform and inexpensive. Unfortunately, however, the majority of existing microbial toxicity tests are not sensitive enough to detect metals and other toxicants in watersheds at current part-per-billion concentrations. To address this shortcoming, Strategic Diagnostic Inc. (formally known as Azur Environmental) developed the Microtox® chronic toxicity test which uses the marine bioluminescent bacterium, *Vibrio fisheri*, as the test organism. The test relies on a large bacterial population to increase its ability to measure the sublethal effects of toxicants and offers statistical advantages over the use of a small number of non-bacterial organisms. The change in the bacterial luminescent when *V. fisheri* is exposed to toxic chemicals is used as an indication of organic compound and/or metal toxicity.

Metal pollutants are among the most toxic and persistent pollutants in aquatic environment, however, researchers have argued that most of the metals are usually in the toxic forms in the laboratory tests, but are often in non-toxic forms in the field (Deaver and Rodgers, 1996). The difference in toxicity may be related to the free ion activity and not to the total metal concentration. Since the presence of chelators (such as EDTA) and natural dissolved organic matter (DOM) (such as fulvic acid) can affect the toxicity of metals, metal-DOM complexation tests and EDTA chelation tests were performed in this study to assess the effects of chelators on toxicity.

Humic substances, the dominant component of organic matter, are naturally occurring in natural waters. Humic substances consist of three fractions, humic acids, fulvic acids and humins, which have similarities but differ from one another in molecular weight and functional group content (Wetzel, 1983). The fulvic acid fraction, which can dissolve at a wide range of pHs, appears to be one of the major components of organic matter and is known for its ability to complex metal ions (EsTeves Da Silva et al., 1998). Therefore, the effect of different amounts of fulvic acid on copper toxicity was investigated using the Microtox® chronic toxicity test.

The EDTA chelation test was also evaluated in order to understand the complexation of cationic metals, such as Cu. EDTA is a powerful chelating agent that complexes many divalent cationic metals (i.e. Cu^{2+}, Ni^{2+}, Pb^{2+}, Cd^{2+} and Zn^{2+}) (USEPA, 1989b), thereby rendering them biologically unavailable (e.g. lowering the toxicity). Also, EDTA affects the permeability of the outermost layer of gram-negative bacteria, and exposure to high concentrations of this chelator decreases cell viability (Ayoub et al., 1995). EDTA was reported acutely toxic in the concentration range of 90–1300 μM to bioassay indicator organisms (USEPA, 1989b). Hence, the amount of EDTA used for the chelating test should be approximated because it can become toxic when present above a certain concentration.

The objectives of this study were to: (1) evaluate the toxicity of 13 priority pollutant metals in reagent water using the Microtox® chronic toxicity test; (2) understand the effects of EDTA and fulvic acid on copper toxicity in the Microtox® chronic toxicity test; and (3) evaluate the utility of the Microtox® chronic toxicity test in assessing metal toxicity in comparison to: (1) standard EPA short-term acute and chronic toxicity tests employing aquatic vertebrates (such as rainbow trout), daphnids and other species; (2) other microbial toxicity tests; and (3) the Microtox® Acute Toxicity Test.

2. Materials and methods

2.1. Toxicity test organism, mediums and cuvettes

The Microtox® chronic toxicity test uses the marine bioluminescent bacteria, *V. fisheri*, as the test organism. The Microtox® chronic test reagent was purchased from Strategic Diagnostic Inc. (Newark, DE, USA) as a freeze-dried bacterial form. The reagent was stored at −20 to −25 °C to preserve microbial activity. To obtain more reproducible results, the reagent was used within 2 h of reconstitution. The Microtox® chronic test medium, reconstitution solution, activation solution and test cuvettes used in the toxicity test were also purchased from Strategic Diagnostic Inc. Microtox® chronic test medium, in a freeze-dried form, contains salts and appropriate nutrients to support cell growth. Reconstitution solution is an ultrapure water used to reconstitute the chronic test medium. Activation solution is a prepared 3.5% sodium chloride solution used to rehydrate the Microtox® chronic test reagent at the appropriate osmotic pressure for the test organism. Test cuvettes used to contain the samples were made of clean borosilicate glass. The cuvettes were disposed of after use to eliminate potential carry-over of cleaning reagent to subsequent samples.

2.2. Toxicity test procedure

Individual metal samples were analyzed by the Microtox® bioassay according to the procedure in the Microtox® system operating manual (Azur Environmental, 1996). The test is based on the reduction of bioluminescence of the marine bacterium, *V. fisheri*, following direct exposure of the sample to the bacterial suspension. The vial containing the fresh culture was maintained at 5 °C between analyses using a sensitive *Model 500* Microtox photometer. Each test consisted of five different concentrations (for sample and positive control, 0.5 dilution factor) were obtained by serial dilution from the prepared stock solution. A reference toxicant, copper sulfate (as Cu), was used as positive control. The positive control was performed concurrently with the sample as a quality control. Test results were discarded if the result of the reference toxicant fell outside of the acceptability limits (i.e. LOEC values for copper from 12 to 100 μg/l). All tests were performed in three replicate sets.

2.3. Chemicals and test solutions

The following 13 ICP emission reference standard metal solutions (1000 ppm), obtained from Fisher Scientific company (Fairlawn, NJ, USA), were used: antimony (as $SbCl_3$), arsenic (as As_2O_3), beryllium (as Be in 2% HNO_3), cadmium (as Cd metal in 2% HNO_3), chromium (VI, as $K_2Cr_2O_7$), copper (as $Cu[NO_3]_2$), lead (as $Pb[NO_3]_2$), mercury (as Hg in 10% HNO_3), nickel (as Ni in 5% HNO_3), selenium (as SeO_2), silver (as $AgNO_3$), thallium (as $TlNO_3$) and zinc (as ZnO). Chromium (III) was obtained from J.T. Baker Chemicals (Phillipsburg, NJ, USA). All of the 13 priority pollutant metals were prepared by diluting reference standards using ASTM Type I reagent water (APHA, 1995). All glassware used for sample preparation, was acid-washed with 50% HCl and rinsed with Type I reagent water three times. Sample pH was measured with an Orion model 410A pH meter (Orion, Boston, MA, USA) calibrated with Fisher Scientific pH 4.00 and 7.00 buffers at the start of the test. Different aliquots of 0.1 N NaOH and 0.1 N HCl were used to adjust the pH to the acceptable range (pH 6.5–7.5) for the Microtox® chronic toxicity test. The pollutant metal samples were analyzed within 48 h of preparation.

2.4. Copper–fulvic acid complexation test

The fulvic acid solution was prepared from a Suwanee River fulvic acid standard obtained from the International Humic Substance Society (IHSS) (St. Paul, MN, USA). All of the fulvic acid samples were dissolved in Type I reagent water to a final concentration of 2, 5, 10, 15, 20 and 25 mg/l. The prepared fulvic acid solutions were mixed with a 155 μg Cu/l solution (as $CuSO_4$) followed by pH adjustment for each concentration. All tests were performed in three replicate sets with positive and negative control tests.

2.5. EDTA chelating test

Ethylenediaminetetraacetic acid ($C_{10}H_{14}N_2O_8Na_2{\cdot}2H_2O$, EDTA) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Test solutions were prepared by combining a volume of 909 μg/l EDTA solution with of 77.5 μg/l or 38.8 μg/l copper sulfate (as Cu) solution followed by pH adjustment. All tests were performed in three replicate sets with positive and negative control tests.

2.6. Data analysis

Experimental data were obtained using a *Model 500* photometer, in chronic mode, after a 22-h incubation period. These data were then analyzed with the ToxCalc™ software, a high end statistical data reduction software that compares results from exposed and control populations for reduced light emission. There are two major types of data analysis that can be performed with the ToxCalc™ software: point estimation and hypothesis testing procedures. Point estimation was used to determine the toxicant concentration that would cause an observable adverse effect in a given percent of the organisms (such as 50% Inhibitory Concentration, IC_{50}). Also, the bootstrapping procedure was used to determine interpolated inhibition-concentration point estimates and estimates of the precision (i.e. standard deviation and confidence limits) of the point estimates (USEPA, 1989a; Warren-Hicks and Parkhurst, 1992; Bruce and Versteeg, 1992).

In addition to the above point estimation testing techniques, chronic toxicity data were also used to determine the no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) by hypothesis testing procedures. Each data set was tested for normality using the Shapiro–Wilk's test. For tests with a normal distribution, Bartlett's test for homogeneity was used. The NOEC and LOEC were then calculated using Dunnett's Test for data sets with an equal number of replicates, or the Bonferroni Adjusted *t*-Test for data with unequal number of replicates. For unbalanced data sets, Wilcoxon rank sum test was performed to determine the NOEC and LOEC.

3. Results and discussion

Toxic responses of the 13 priority pollutant metals using the Microtox® chronic toxicity test were determined. The lowest pH was 6.85, and the highest was 7.15 for the 13 priority pollutant metal samples; all samples were prepared and pH adjusted just prior to the analysis. All pipettor syringes and tips were rinsed with ASTM Type I reagent water rather than with the reconstitution solution recommended by manufacturer; the reagent water was pre-tested to confirm that it was not toxic (data not shown).

3.1. Toxicity ranking of IC_{50}s

IC_{50} values are dependent on the chosen testing concentration range. Selection of appropriate testing concentration is critical to establishing the toxicity of a sample in a chronic toxicity test. To clearly identify the effect and no-effect concentrations, at the termination of the test, one or more of the highest test concentrations should have had an unequivocal adverse effect on the exposed population and one or more of the lowest concentrations should have had no effect. The average IC_{50} (i.e. the concentration causing a 50% inhibition of growth and survival compared to the control response) values for 13 priority pollutant metals in the Microtox® chronic toxicity test are shown in Table 1. The toxicity (IC_{50}) of As, Cr, Cu, Ni, Se, Ag and Zn in the Microtox® chronic toxicity test was in the same order of magnitude as the

Table 1
Toxicity of 13 priority pollutant metals in the Microtox® chronic toxicity test and comparison with Ambient water quality criteria (AWQC)

Metal	NOEC (Range-μg/l)[a]	LOEC (Range-μg/l)[a]	$IC_{50} \pm$ S.D.[b] (μg/l)[a]	CV[c] (%)[a]	AWQC (max., μg/l)
Antimony	406–811	811–1620	640 ± 43.2	6.75	NA[d]
Arsenic	578–1160	1160–2310	821 ± 187	22.7	360
Beryllium	<0.742–0.742	0.742–1.49	1.36 ± 0.178	13.1	NA
Cadmium	15.0–30.0	30.0–60.0	50.4 ± 7.61	15.1	1.79
Chromium (III)	117	234	178 ± 8.14	4.57	984
Chromium (VI)	<3.10–12.4	3.10–24.8	12.4 ± 1.77	14.3	16.0
Copper	3.39–6.78	6.78–13.6	7.08 ± 0.352	4.97	9.22
Lead	313–626	626–1250	669 ± 95.7	14.3	33.8
Mercury	12.5–25.0	25.0–50.0	33.8 ± 1.99	5.89	2.40
Nickel	<25.0–100	25.0–200	265 ± 6.17	2.33	780
Selenium	33.8–67.6	67.6–135	114 ± 21.0	18.4	20.0
Silver	3.97–7.94	7.94–15.9	7.92 ± 0.667	8.40	1.23
Thallium	1920–7670	3840–15 300	6250 ± 321	5.14	NA
Zinc	<10.0–40.2	10.0–80.4	73.8 ± 7.85	10.6	65.0

[a] Based on 3 experiments, and 5 dilutions and 4 to 5 replicates per dilution per experiment.
[b] S.D.: standard deviation.
[c] The coefficient of variation (CV%).
[d] NA: none available.

USEPA Ambient water quality criteria (AWQC) (USEPA, 1986). For the 13 priority pollutant metals, in descending order, the toxicity (in μg/l as metal element) was as follows:

$$Be > Cu > Ag > Cr(VI) > Hg > Cd > Zn > Se > Cr(III) > Ni > Sb > Pb > As > Tl$$

Beryllium (Be) was found to be the most toxic metal and thallium (Tl) was found the least toxic metal among the 13 priority pollutant metals. The results of 22-h exposures obtained using the Microtox® chronic toxicity test showed that the precision of the test is relatively high. The coefficients of variation (CV%) for IC_{50}s for the 13 metal pollutants were calculated and ranged from 2.33 to 22.7%.

3.2. Control performance

Copper sulfate (as Cu) was used to develop a quality control data base for all toxicity tests conducted in our laboratory. Quality control was performed, in quadruplicate, concurrently with each test. The data were used only when the quality control fell inside of the acceptability range. In most of the experiments, the LOEC value for the copper ranged from 25 to 50 μg/l. The precision of this test in our study was acceptable as measured by the fact that the NOECs only varied by one concentration interval at most; these data are consistent with the conclusion of Anderson and Norberg-King (1991) that the precision of a toxicity test is considered acceptable when NOECs vary by no more than one concentration interval above or below a central tendency.

3.3. Toxicity of copper to V. fisheri

Among the many metal ions present in natural waters, copper is one whose complexation by organic ligands is frequently studied because it is a biologically essential metal and forms stable complexes that heavily affect its bioavailability. In our study, copper sulfate (as Cu) was toxic in the concentration range of 2.43–19.4 μg/l (LOEC) (Tables 2 and 3). Recent data suggest that the toxic effects of copper are similar to those of silver (Erickson et al., 1996). Also, various investigators have found the toxicity based on cupric ion activity to change with different physicochemical factors (Borgmann and Ralph, 1984; Daly et al., 1990).

Table 2
Toxicity of copper (up to 38.8 μg Cu/l) in reagent water using the Microtox® chronic toxicity test

Reduced light Emission endpoint[a]	Cu concentration (μg/l)			
	NOEC	LOEC	$IC_{50} \pm$ S.D.[b]	IC_{50} (95% CL)
Experiment 1	9.72	19.4	13.9 ± 0.763	11.3–15.6
Experiment 2	9.72	19.4	14.4 ± 1.01	6.35–16.5
Experiment 3	<2.43	2.43	2.65 ± 0.483	1.72–4.32
IC_{50} Mean			10.3 ± 0.752	
IC_{50} CV%[c]			7.30	

[a] For each experiment, ran 5 dilutions and 4 replicates per dilution for samples and positive control ($CuSO_4$, as Cu), and 5 replicates for negative control.
[b] S.D.: standard deviation.
[c] The coefficient of variation (CV%).

Table 3
Toxicity of copper (up to 77.6 μg Cu/l) in reagent water using the Microtox® chronic toxicity test

Reduced light Emission endpoint[a]	Cu Concentration (μg/l)			
	NOEC	LOEC	$IC_{50} \pm$ S.D.[b]	IC_{50} (95% CL)
Experiment 1	9.70	19.4	16.8 ± 0.572	14.9–18.4
Experiment 2	9.70	19.4	16.8 ± 0.311	15.6–17.4
Experiment 3	9.70	19.4	17.2 ± 0.629	14.7–18.2
IC_{50} Mean			16.9 ± 0.504	
IC_{50} CV%[c]			2.98	

[abc]See footnote in Table 2.

Table 4
Toxicity of fulvic acid (up to 25 mg/l) in reagent water using the Microtox® chronic toxicity test

Reduced light Emission endpoint[a]	Fulvic acid Concentration (mg/l)			
	NOEC	LOEC	$IC_{50} \pm$ S.D.[b]	IC_{50} (95% CL)
Experiment 1	6.25	12.5	7.56 ± 3.32	0.0–15.7
Experiment 2	6.25	12.5	5.85 ± 2.31	0.673–13.8
Experiment 3	12.5	25	15.8 ± 1.82	9.93–19.7
IC_{50} Mean			9.74 ± 2.48	
IC_{50} CV%[c]			25.5	

[abc]See footnote in Table 2.

3.4. Toxicity of fulvic acid and EDTA to V. fisheri

Ideally, metal-binding agents, such as fulvic acid or EDTA, should have relatively low toxicity to the test organism (Ayoub et al., 1995). In this study, the toxicities of a range of fulvic acid and EDTA concentrations in reagent water were determined in the Microtox® chronic toxicity test. These two substances differed significantly in their toxicities in this test. Fulvic acid was non-toxic at least up to a concentration of 6.25 mg/l (NOEC) (Table 4). The EDTA was non-toxic only up to a concentration range of 28.4–56.9 μg/l (NOEC) (Table 5). The data obtained in our study for EDTA indicate much greater toxicity than has been reported by the USEPA (1989b) in which concentrations of disodium EDTA less than 90 μM (33 500 μg/l) were non-toxic to bioassay indicator

Table 5
Toxicity of EDTA (ethylenediaminetetraacetic acid) in reagent water using the Microtox® chronic toxicity test

EDTA Conc. Range (μg/l as EDTA)	EDTA Concentration (μg/l)[a]				IC_{50} CV[d] (%)
	NOEC	LOEC	IC_{50}[b] ± S.D.[c]	IC_{50} (95% CL)	
227–3640	<227	227	133 ± 4.83	89.6–150	3.62
114–1820	<114–114	114–227	89.6 ± 9.18	70.3–132	10.3
56.9–909	56.9–227	114–455	96.2 ± 12.0	71.1–143	12.5
28.4–455	114	227	77.2 ± 8.50	54–101	11.0
14.2–227	56.9–114	114–227	47.8 ± 6.35	32.5–70.0	13.3
7.11–114	28.4–56.9	56.9–114	96.8 ± 13.2	67.4–141	13.6

[a] For each experiment, ran 5 dilutions, and 4 replicates per dilution for samples and positive control ($CuSO_4$, as Cu), and 5 replicates for negative control.
[b] IC_{50} is the mean inhibitory concentration of three experiments.
[c] S.D.: standard deviation.
[d] The coefficient of variation (CV%).

organisms, such as *C. dubia* and fathead minnow (*P. promelas*) in water of various hardness and salinity. Also, Ayoub et al. (1995) reported that the EDTA was non-toxic at concentrations up to 30 μM (11 200 μg/l) using a microbial microplate assay (Met PAD).

3.5. Effect of fulvic acid on copper ion toxicity

Based on the amount of fulvic acid added in our study, all copper was fully complexed by fulvic acid (Saar and Weber, 1982). The addition of fulvic acid substantially reduced the copper toxicity to *V. fisheri* in the Microtox® chronic toxicity test, as expected (Table 6 and Fig. 1). Addition of 2 mg/l and 5 mg/l of fulvic acid increased the mean 22-h IC_{50} by 163% and 176%, respectively. This is similar to the effects reported by other investigators for other metals, in which complexation by organic matter also reduced bioavailability (Erickson et al., 1998). The complexation reaction observed in the presence of fulvic acids suggests the occurrence of complexation effect, with the toxicity decreasing as the fulvic acid solution concentration is increased up to 5 mg/l. Further additions of fulvic acid from 10 to 25 mg/l did not significantly affect the toxicity of copper when compared to lower fulvic acid concentrations (2–5 mg/l); this may suggest some adverse effects from fulvic acid since fulvic acid alone shows toxicity at concentrations above 6.25 mg/l. As found in our study, other investigators have also demonstrated that the presence of natural organic matter considerably reduces the toxicity of copper

Table 6
Effects of added fulvic acid (FA) on the chronic toxicity of copper (μg/l, as Cu) in reagent water using the Microtox® chronic toxicity test

Reduced light Emission endpoint	Cu Concentration (μg/l)[a]				IC_{50} CV[d] (%)
	NOEC	LOEC	IC_{50}^{b} ± S.D.[c]	IC_{50}–95% CL	
Copper alone (155 μg/l as Cu)	9.72	19.4	26.2 ± 1.27	22.0–29.3	4.85
155 μg/l Cu + 2 mg/l FA	19.4–38.8	38.8–77.5	42.6 ± 3.97	40.1–60.4	9.32
155 μg/l Cu + 5 mg/l FA	38.8	77.6	46.2 ± 11.4	16.7–72.2	24.7
155 μg/l Cu + 10 mg/l FA	38.8	77.6	46.6 ± 6.09	28.9–66.0	13.1
155 μg/l Cu + 15 mg/l FA	19.4–38.8	38.8–77.5	36.7 ± 10.2	14.3–65.8	27.8
155 μg/l Cu + 20 mg/l FA	9.72–38.8	19.4–77.5	24.3 ± 8.52	9.62–49.6	35.1
155 μg/l Cu + 25 mg/l FA	38.8	77.6	26.1 ± 7.88	7.52–50.0	30.2

[abcd] See footnote in Table 5.

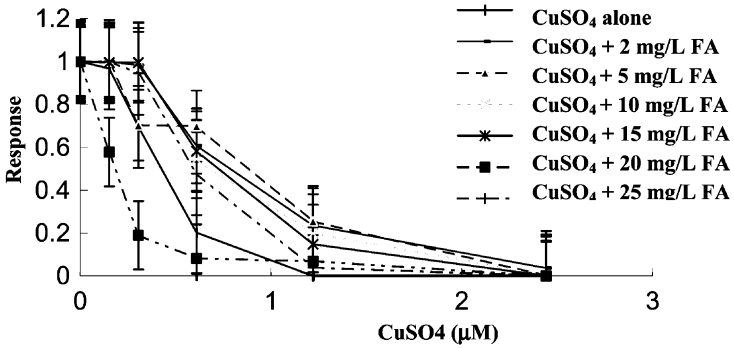

Fig. 1. Dose–response relationship of added fulvic acid (FA) on the chronic toxicity of copper in the Microtox® chronic toxicity test. Summarized data are provided in Table 6. Response measured as reduced light emission in relation to negative (blank) controls. Data points are means and bars are ±S.D. for three separate experimental runs.

to *V. fisheri* (Daly et al., 1990; Erickson et al., 1996; Srna et al., 1980).

3.6. Effect of copper ion on EDTA toxicity

Based on the amount of EDTA added in our study, all copper was fully complexed by EDTA (Morel and Hering, 1983). Experiments were carried out in the Microtox® chronic toxicity test growth medium at a constant complexing agent concentration, EDTA (909 μg/l), in the presence of various copper sulfate (as Cu) concentrations (38.8 and 77.5 μg/l). In the absence of added copper, the IC_{50} of EDTA in the Microtox® chronic toxicity test was determined to be 96.2 μg/l. The addition of copper significantly reduced EDTA toxicity in our study (Table 7 and Fig. 2). Addition of 77.5 and 38.8 μg/l of copper increased the mean 22-h IC_{50} for EDTA by 280% and 505%, respectively. Reduction in the toxicity of EDTA in the presence of copper, has also been observed by other workers using other test organisms, such as *Daphnia magna* (Pomeroy et al., 1984) and *Lemna paucicostata* (Nasu et al., 1983); and this effect is also observed with other metals, for which complexation by EDTA also reduced bioavailability.

3.7. Comparison of Microtox® chronic toxicity test data

To our knowledge, there are no previously published data on the toxicity of pure compounds in the Microtox® chronic toxicity test with the exception of one data set from the developer of the test (Bulich et al., 1995). Compared to Table 8, are the Microtox® chronic toxicity test data reported by Bulich et al. and the results obtained from our study for six metals. The data obtained in our study were consistent with the results reported by Bulich et al. The decreasing orders of toxicity for six metals in our study and in published data are as follows:

Table 7
Effect of added copper sulfate (as Cu) on the toxicity of EDTA in reagent water using the Microtox® chronic toxicity test

Test solution	EDTA Concentration (μg/l)[a]				IC_{50} CV[d] (%)
	NOEC	LOEC	IC_{50}^{b} ± S.D.[c]	IC_{50}–95%CL	
EDTA only (909 μg/l)	56.9–227	114–455	96.2 ± 12.0	71.2–142	12.5
EDTA[e] + 38.8 μg/l Cu	227–455	455–909	486 ± 27.8	391–553	5.72
EDTA[e] + 77.5 μg/l Cu	227–455	455–909	269 ± 40.1	146–381	14.9

[abcd]See footnote in Table 5.

Bulich et al. (1995) Cu > Cr (VI) > Cd > Zn > Ni > Pb

This study Cr (VI) > Cu > Cd > Zn > Ni > Pb

The toxicity ranking is the same with the exception of the rank order of Cu and Cr (VI). Therefore, for these six metals, the Microtox® chronic toxicity test results were found highly comparable between the two laboratories.

3.8. Comparisons with other toxicity tests

3.8.1. Cadmium, Chromium(VI), Copper, Mercury, Zinc, Silver, Bellium, Antimony IC_{50}s

The comparative EC_{50}, IC_{50} and LC_{50} data for these metals in different toxicity tests are shown in Table 9. The results for metals from this study were compared with fish (fathead minnow, sheephead minnow, rainbow trout and coho salmon) assay toxicity data (Bulich and Isenberg, 1981), Daphnids (*D. magna* and *C. dubia*) toxicity data (Guilhermino et al., 1997), and mean toxicity results from various sources using green algae assay (*Selenastrum capricornutum*), Mysid shrimp (*Mysidopsis bahia*), and other microbial tests, such as the *Pseudomonas fluorescens* growth inhibition test (PFNEN) (Codina et al., 1993).

Table 8
Interlaboratory comparison of the toxicity of selected metals in the Microtox® chronic toxicity test

Metal	LOEC (μg/l) as metal	
	This study (range)	Bulich et al. (1995)
Cd	30.0–60.0	60.0
Cr (VI)	3.10–24.8	35.0
Cu	6.78–13.6	19.0
Pb	626–1250	1000
Ni	25.0–200	170
Zn	10.0–80.4	100

As shown in Table 9, the Microtox® chronic toxicity test was the most sensitive assay in the detection of Cu and Cr(VI) toxicity. Regarding Cr (VI) toxicity, the data obtained from our study are in sharp contrast with the results reported by Codina et al. (1993) in which the Microtox® acute test was very sensitive in detecting metal toxicity with the exception of that of chromium. Furthermore, the data obtained from our study for cadmium are also in sharp contrast with the results reported by Kong et al. (1995) in which the Microtox® acute test failed to detect the toxicity of cadmium. In addition for Cd, Hg and Zn, *M. bahia* (shrimp), *D. magna* and *S. capricornutum* (green algae) show the highest sensitivity, respec-

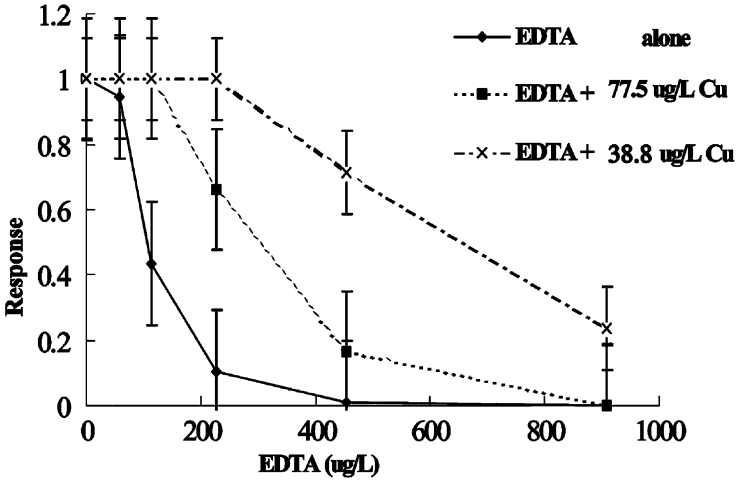

Fig. 2. Dose–response relationship of added copper on the chronic toxicity of EDTA in the Microtox® chronic toxicity test. Summarized data are provided in Table 7. Response measured as reduced light emission in relation to negative (blank) controls. Data represent means ± S.D. of three replicate sets.

Table 9
Comparison of relative toxicity of Cd, Cr (VI), Cu, Hg, Zn, Ag, Be and Sb in the Microtox® chronic toxicity test and in other toxicity tests

Metal	Concentration (μg/l as metal)					
	Microtox (22-h) IC_{50}[a]	Fish (96-h) LC_{50}[b]	Daphnids (24–48 h) EC_{50}[c]	Shrimp (96-h) LC_{50}[d]	Green Algae (96-h) EC_{50}[d]	PFNEN (20-h) EC_{50}[e]
Cd	50.4	1000–100 000	35.3–1880	30–160 000	40	2700
Cr (VI)	12.4	11200–13 3000	260–1790	–	–	3400
Cu	7.08	100–10 700	20–167	160–4900	40	51 700
Hg	33.8	10–900	5.2–30	–	–	1040
Zn	73.8	550–7200	76–8050	499–720 000	60	–
Ag	7.92	10.4–1170	0.58–10	–	–	–
Be	1.36	–	–	–	–	–
Sb	640	–	–	–	–	–

[a] This study, mean IC_{50} data for 3 experimental runs.
[b] Fish data adapted from Erickson et al. (1998), Bulich and Isenberg (1981), Dutton et al. (1986), Hogstrand and Wood (1998) and Khangarot and Ray (1987), Qureshi et al. (1982), Toussaint et al. (1995).
[c] Daphnids data adapted from Toussaint et al. (1995), Erickson et al. (1998), Guilhermino et al. (1997), Dutton et al. (1986), Khangarot and Ray (1987), Ribo and Kaiser (1987).
[d] Data adapted from Toussaint et al. (1995).
[e] *P. fluorescences* growth inhibition test (PFNEN) data adapted from Lussier et al. (1985).

tively. However, there is a large variability between the EC_{50}/LC_{50} values obtained by different workers in different tests for these metals (Dutton et al., 1986; Dutka and Kwan, 1981). Furthermore, compared to those assays most sensitive to Cd, Hg and Zn, the toxicity results for these metals in our study are all in the same order of magnitude.

The comparative IC_{50} and LC_{50}s data for Ag in different toxicity tests are also shown in Table 9. The result for silver from our study was compared with toxicity results for the seawater fish sheephead minnow (*Cyprinodon variegatus*), Rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*O. mykiss*), freshwater juvenile fathead minnow, and <1-day-old *D. magna*. The ranking of the IC_{50}/LC_{50} values clearly shows that the *D. magna* is the most sensitive toxicity testing procedure for silver, followed by fathead minnow and the Microtox® chronic toxicity test. The sheephead minnow toxicity assay was the least sensitive. In spite of the different sensitivity pattern of each test, the results show that *D. magna*, fathead minnow, and the Microtox® chronic toxicity test are relatively sensitive to silver toxicity. Also, the IC_{50} obtained from our study was in good agreement with the Hogstrand and Wood (1998) study in which the fish 96-h LC_{50} for $AgNO_3$ was reported to lie in the range of 5–70 μg Ag/l.

The mean IC_{50} obtained for Be and Sb from our study were 1.36 μg/l and 640 μg/l, respectively. Beryllium was the metal showing the highest toxic effect in the Microtox® chronic toxicity test of all the metals tested. There is very limited information on Be and Sb aquatic toxicity. Also, there are no available Ambient water quality criteria (AWQC) for these metals. No comparable aquatic toxicity data could be found for these metals.

3.8.2. Nickel, Lead, Arsenic, Chromium(III), Selenium, Thallium EC_{50}s

The comparative IC_{50} and designated endpoint data for these metals in different toxicity tests are shown in Table 10. The results for these metals from our study were compared with the results for submitochondrial particles assay (SMP), fish (fathead minnow or bluegill) and other microbial tests, involving *Spirillum volutans* and *P. fluorescens.*

The Microtox® chronic toxicity test was the most sensitive assay in the detection of Ni, Pb, As, Cr(III), Se and Tl toxicity. It was more sensitive than the fish assay up to 250-fold,

Table 10
Comparison of relative toxicity of Ni, Pb, As, Cr (III), Se and Tl in the Microtox® chronic toxicity test and in other toxicity tests

Metal	Concentration (μg/l as metal)					
	Microtox (22-h) IC_{50}[a]	Fish (96-h) LC_{50}[b]	SMP (1-h) EC_{50}[b]	Daphnids (48-h) EC_{50}[c]	S. volutans (2-h) MEC_{90}[d]	P. fluorescens (18-h) EC_{50}[e]
Ni	265	27 000–35 500	2200	7570	20 000	8700
Pb	669	1390–8000	2000	3610	40 000	14 000
As	821	43 000	44 500	5400	30 70 000	
Cr (III)	178	33 200	33 000	–	–	
Se	114	2900–40 000	229 000	–	–	
Tl	6250	–	398 000	–	–	

[a] This study, mean IC_{50} data for three experimental runs.
[b] Fathead minnow, rainbow trout and bluegill data from Cardwell et al. (1976), Read et al. (1997) and Holcombe et al. (1983).
[c] Data adapted from Dutka and Kwan (1981) and Khangarot and Ray (1987).
[d] Motility inhibition 90% (MEC_{90}) data adapted from Dutka and Kwan (1981) and Ribo and Kaiser (1987).
[e] Data adapted from reference Dutka and Kwan (1981).

although Pb toxicity was well detected by the fish test. Also, the results from our study for selenium and arsenic show that the Microtox® chronic toxicity test was more sensitive for these metals as compared to fathead minnow results reported by Cardwell et al. (1976) (selenium, 96-h LC_{50}= 2900 μg/l) and by Holcombe et al. (1983) (arsenic trioxide, 48-h LC_{50} of As^{3+} = 73 720 μg/l). Thallium is the metal showing the lowest toxic effect in the Microtox® chronic toxicity test of all the metals tested, which can also be observed in the SMP assay. Furthermore, among the microbial toxicity tests, the *S. volutans* assay showed the lowest sensitivity to both nickel and lead. Also, the EC_{50} values reported in the *P. fluorescens* assay were similar to those observed by several authors (Codina et al., 1993; Beauvien and Jolicoeur, 1984).

3.9. Comparison of the selected priority metal pollutants in the Microtox® chronic toxicity, the Microtox® acute toxicity test, EPA short-term acute and chronic toxicity tests

From the data obtained from our study and various other sources (Azur Environmental, 1996; Greene et al., 1985; Ribo and Kaiser, 1987) for the Microtox® acute toxicity test, toxicity of all metals increases with increasing exposure time. This conclusion was also reached by Vasseur et al. (1984) that reported that extending the time of exposure to 30 min led to lower EC_{50} values for metals. As shown in Table 11, the comparable data show that the Microtox® chronic toxicity test is significantly more sensitive than the Microtox® acute toxicity test for all the pollutant metals tested. For beryllium, the most sensitive metal detected among the 13 priority pollutant metals, the sensitivity of the Microtox® chronic toxicity test over the Microtox® acute toxicity test was up to 11 000-fold.

In addition, the sensitivity of the Microtox® chronic toxicity test was also in comparison to the EPA short-term acute and chronic toxicity tests using daphnia and fish as test species (USEPA, 1980USEPA, 1980a,b,c,d, 1984, 1985a,b). The Microtox® chronic toxicity test is not only more sensitive than the EPA acute toxicity tests but also fell within the approximate order-of-magnitude as the EPA short-term chronic toxicity tests for all the metal compounds. Although the accuracy and reproducibility of the Microtox® acute toxicity test was questioned by Qureshi et al. (1982) and Ribo and Kaiser (1987) and Dutka and Kwan (1981), the Microtox® chronic toxicity test has been proved capable of detecting the toxicity of metals at parts-per-billion (ppb) levels. Also, the intratest coefficient of variation (CV) generated from three replicate tests performed for each metal under the same test condition ranged from 2.33 to 22.7%

Table 11
Comparison of the relative toxicity data of selected priority pollutant metals in the Microtox® chronic toxicity test, in the Microtox® acute toxicity test, and in EPA acute and short-term chronic toxicity tests

Metal	Concentration (μg/l as metal element)				
	Microtox (5-min) EC_{50}[a]	Microtox (15-min) EC_{50}[a]	Microtox (22-h) IC_{50}[b]	EPA acute tests (24–96 h) EC_{50}/LC_{50}[c]	EPA short-term chronic tests (7 days or above) EC_{50}/LC_{50}[c]
As	73 700	43 600	821	4340–12 307	540–10440
Be	15 000	NA[d]	1.36	2500	NA
Cd	10 0000	20 000	50.4	0.1–479	2–700
Cr (VI)	22 000	18 000	12.4	59 000, 69 000	190
Cu	3500	800	7.08	286	16.5
Hg	70	50	33.8	13–1780	4.7 and 34
Ag	9500	NA	7.92	3.4–43	10

[a] Data adapted from Azur Environmental (1996) and Greene et al. (1985).
[b] This study, mean of three experimental runs.
[c] Data adapted form USEPA (1980a,b,c,d, 1984, 1985a,b) and Parkhurst et al. (1992).
[d] NA: not found in the literature.

which is comparable to the evaluation data obtained from Parkhurst et al. (1992) that C.V. ranged from 3 to 94% for single chemical tests for the acute toxicity tests and C.V. ranged from 2 to 83% for the chronic toxicity tests.

4. Conclusions

The Microtox® chronic toxicity test described is a potentially useful tool for the rapid toxicity testing of environmental samples including water and wastewater. The results of this study show that this test is capable of detecting the toxicity of metals at parts-per-billion (ppb) levels. Among the 13 priority pollutant metals, beryllium (Be) was found to be the most toxic and thallium (Tl) was found the least toxic. The intratest coefficient of variation (CV) generated from three replicate tests performed for each metal under the same test condition ranged from 2.33 to 22.7%.

The chronic toxicity of copper to *V. fisheri* was shown to be reduced substantially in our study by the addition of EDTA and by increasing the dissolved organic carbon concentration with the addition of fulvic acid. It is clear from this work that chelators can have a large effect on copper toxicity. This part of the study was only aimed at assessing the importance of EDTA and organic matter in predicting metal toxicity. The toxic effects of these metals in water and wastewater need to be further assessed under different physical, chemical and biological conditions.

In conclusion, all available evidence indicates that the Microtox® chronic toxicity test is a valuable tool for providing a rapid understanding of the relative toxic effects of metals and other inorganic pollutants. Furthermore, this rapid screening method is a comparatively inexpensive alternative to in vivo bioassays with higher organisms and can be readily used in hazard and risk assessment.

Acknowledgments

This work was funded by the Massachusetts Department of Environmental Protection. The authors gratefully acknowledge the technical and administrative assistance of the following Wall Experiment Station staff: Barbara Cusick, Deanne Daneau, Barbara Eddy, Susan Gifford, Kenneth Hulme, Maria Ruiz, Ron Stoner and James Sullivan.

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