Chemical Speciation and Toxicity of Metals Assessed by Three Bioluminescence-Based Assays Using Marine Organisms

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ABSTRACT: Metal toxicity is a function of the biology of the target organism and the chemical speciation of the metal. The toxicity of 11 metals was assessed with three cell-based bioassays based on marine organisms: the bacterium *Photobacterium phosphoreum* of the Microtox® bioassay, an environmental strain of *P. phosphoreum*, and photocytes isolated from the brittlestar *Ophiopsila californica*. Metal speciation was calculated for three commonly used media: NaCl-based Microtox® bioassay medium, artificial seawater glycerol, and artificial seawater. Decreased bioluminescence was considered a proxy for cell toxicity. In all three assays the elements Cd and Hg exhibited similar speciation as well as similar toxicity profiles. The element Cu was toxic in all three assays despite different metal speciation for the *P. phosphoreum* bioassay. The element Ag was toxic to both bacterial strains but not to photocytes despite a similar chemical speciation for all three assays. In general, the Microtox® bioassay was sensitive to all metals (except Pb), whereas the photocytes were the least sensitive to the metals. The heightened response of the Microtox® bioassay probably resulted from a combination of the limited complexing power of the medium and the greater sensitivity of the bacterial strain. © 2004 Wiley Periodicals, Inc. Environ Toxicol 19: 161–178, 2004.

Keywords: bioassay; bioluminescence; chemical speciation; free ion model; HYDRAQL; metal; Microtox®; photocyte; toxicity

INTRODUCTION

The toxicity of a substance is the measure of its incompatibility with normal biological function (Richardson, 1993). Thus, toxicity refers to the adverse impact of a substance on living organisms. It depends on various factors such as the quantity of the substance actually absorbed, the route of

contamination (ingestion, physical contact, accumulation by diffusion, or active cellular pumping), and time exposure. According to the free ion activity model, metal toxicity depends on chemical species formed by the metal and is mainly correlated to free ion concentration; however, that does not necessarily imply that the free metal ion is the only toxic species (Morel and Hering, 1993; Stumm and Morgan, 1995; Campbell and Tessier, 1996). The model merely reflects the ability of free-metal-ion concentration to be taken as an index of reactivity, even though the physiological effects of a metal can also be related to the interaction of its various chemical forms with a variety of cellular ligands. Consequently, determining the toxicity of a given

metal requires assessing its chemical speciation in the medium.

Toxicity is dependent on the species involved and the tissue and cells targeted by the toxicant (Richardson, 1993; Clark, 1997; Crosby, 1998). Overall, a toxic effect occurs as the result of a complex balance between the metal's chemical speciation and reactivity and the biology of the organism. Hence, toxicity measurements are usually performed by quantifying the alteration of given biological characteristics (e.g., gene expression, enzyme functionality, fertilization efficiency, growth, behavior) upon exposure to known added concentrations of a specific substance. Change in a biological characteristic is then considered a reflection of an adverse effect of the substance, with the observed changes possibly integrating toxicity that is occurring at various levels of biological complexity, from the genetic to the molecular, cellular, tissue, and organismal levels (Costan et al., 1993; Clark, 1997; Kennish, 1997). Biomonitoring programs use a battery of bioassays to screen such a variety of routes of contamination, involving a diversity of organisms (bacteria, crustaceans, bivalves, fishs), possibly at different stages of development (embryo, larvae, adult). Because of the complexity and cost of such an endeavor, there is strong interest in simplifying toxicity assessment. A common alternative is to use microorganisms genetically engineered to be sensitive to specific contaminants, resulting in assays that are more rapid and less costly (Wood and Gruber, 1996; Hollis et al., 2000; Dizer et al., 2002).

Bioluminescence, the biological production of visible light with an ecological function (Morin, 1983), is commonly used as an bioassay for environmental quality. Most commonly used are luminescent bacteria and dinoflagellates, as well as genetically engineered bacteria that use luminescence reporters (Danilov et al., 1985; Lapota et al., 1993; Kudryasheva et al., 1998; Heimann et al., 2002). The assumption is that decreases in bioluminescence reflect the toxicity of a given condition, because light emission is a measure of the health and metabolism of the luminescent organism.

Light production is the result of a chemical reaction involving the oxidation of a substrate, generally called luciferin, mediated by a protein called luciferase in the presence of an ionic cofactor; the intensity of produced light is proportional to the amount of reagents involved in the chemical reaction. A decrease in the intensity of the light produced therefore indicates alteration of one of the events leading to light production: either the chemical reaction (e.g., configurational inactivity of reagents), the expression of genes coding for the reagents, and/or any physiological control associated with the process.

The present study addressed metal chemical speciation and toxicity in a marine environment with three bioassays based on cellular bioluminescence, using the marine bacterium *Photobacterium phosphoreum* from the Microtox® bioassay, a wild-type strain of *P. phosphoreum*, and isolated photocytes (light-producing cells) from the brittlestar *Ophiopsila californica* (Echinodermata). This study addressed the hypothesis that toxicity assessment is different among bioassays because of differences in the physicochemical characteristics of the medium and also because of how biologically susceptible to metal toxicity the particular cells are. In addition, as the study was a test of the free ion activity model, it was expected that the toxicity would be correlated with the free-metal-ion concentrations among bioassays.

MATERIAL AND METHODS

The metals silver (Ag), aluminum (Al), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), lead (Pb), uranium (U), and zinc (Zn) were assessed for toxicity with three cell-based bioassays, each using *in vivo* bioluminescence as a reporter of physiological state. The Microtox® bioassay used strain 11177 of the bacterium *Photobacterium phosphoreum* (synonym: *Vibrio fischeri*), the *P. phosphoreum* bioassay used a wild-type strain of the same bacterium (Haygood strain 849), and the photocyte bioassay used metazoan luminescent cells (photocytes) isolated from the brittlestar *Ophiopsila californica* (Echinodermata). For each bioassay the effect of each individual metal on cellular bioluminescence was determined from three independent replicates of the experiment. Depending on metal solubility, 4–6 total metal concentrations were assayed, ranging from 10^{-8} to 10^{-3} M.

Preparation of Metal Solutions

Metal stock solutions were freshly prepared by dissolving metal chloride (Cd^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Zn^{2+}), sulfate (Al^{2+}), or nitrate (Ag^+, Pb^{2+}) salt into the medium of interest unless otherwise specified. Stock solutions for U and Cr were made from uranyl (UO_2^{2+}) acetate and sodium chromate (CrO_4^{2-}), respectively. All metals were obtained from Sigma (St. Louis, MO, USA) and were ACS certified. Stock solutions were at concentrations at which no precipitation was observed over 72 h at room temperature. The final metal concentration used in experiments was obtained by dilution of the corresponding stock solution.

For each treatment a control was prepared that was identical to the metal stock but without the metal salt. Duration of exposure to the contaminant was the same for all metals and concentrations, ranging from 0 (just before addition of the metal in the medium) to a maximum of 2, 9, or 24 h, depending on the bioassay. If a precipitate was observed in the medium over the experimental period, the assayed concentration was not considered for further investigation. All manipulations were conducted under controlled conditions, avoiding metal and microbial contamination and using disposable polystyrene or high-density polyethylene

TABLE I. Characteristics of the media used for the three bioassays

Microtox® NaCl-based medium pH: 6.7 ± 0.3; T(°C): 15 ± 0.3		*P. phosphoreum* ASG-based medium pH: 7.4 ± 0.1; T(°C): 22 ± 0.2		Photocyte ASW-based medium pH: 8.0 ± 0.1; T(°C): 8 ± 0.2	
ion	$[ion]_T$ (mM)	ion	$[ion]_T$ (mM)	ion	$[ion]_T$ (mM)
Na^+	342.0	Cl^-	341.05	Cl^-	534.4
Cl^-	342.0	K^+	303.5	Na^+	455.8
		Mg^{2+}	48.96	Mg^{2+}	52.3
		SO_4^{2-}	48.96	SO_4^{2-}	27.7
		Glycerol	31.83	Tris[c]	20.0
		NH_4^+	18.27	Ca^{2+}	9.9
		HEPES[a]	9.77	K^+	9.6
		Ca^{2+}	9.638		
		HCO_3^-	1.954		
		Na^+	1.954		
		Glycerophosphate	0.452		
		FAC[b]	0.0322		

ASG: artificial seawater glycerol; ASW: artificial seawater.

[a] HEPES: hydroxyethyl piperazine ethane sulfonic acid.

[b] FAC: ferric ammonium citrate with 15%–18% Fe ion.

[c] Tris: Tris(hydroxymethyl)-aminomethane hydrochloride.

supplies, sterile acid–washed metal-free glassware, and stainless-steel dissecting tools.

Microtox® Bioassay

The Microtox® Acute Toxicity Test kit, available from AZUR Environmental (Carlsbad, CA, USA), was used according to the Microtox® protocol guidelines for toxicity evaluation (AZUR, 1992). Briefly, lyophilized bacteria *P. phosphoreum* strain NRRL B-11177 were resuspended in 1 mL of ultrapure water (reconstitution solution; AZUR, Carlsbad, CA, USA). After 10 min, 10-μL subsamples were added into glass scintillation vials containing 900 μL of 2% NaCl solution (Diluent Solution, AZUR, Carlsbad, CA, USA), which is the only constituent of the medium for the bioassay (Table I). Metal treatment involved the addition of 100 μL of 10.1× concentrated metal stock solution to the culture. Metal stock solutions were made in 2% NaCl solution obtained from the osmotic adjusting solution (22% NaCl solution, AZUR, Carlsbad, CA, USA) diluted with the reconstitution solution. The final solution, with a total volume of 1010 μL, was incubated without agitation at 15°C for up to 2 h. The conditions employed in this experiment are known to be optimal for light to be produced in this bacterium in the absence of metals. Growth is minimal over the 3-h incubation time (Bulich and Isenberg, 1980; Bulich and Huynh, 1995). Bioluminescence was measured after various exposure times (0, 0.25, 0.5, 1, and 2 h) using a Quantalum 2000 luminometer (Zefaco Inc., Gaithersburg, MD, USA).

Photobacterium phosphoreum Bioassay

A wild-type strain of the bacterium *Photobacterium phosphoreum* was used in the *Photobacterium phosphoreum* bioassay The *P. phosphoreum* originally were collected from symbiotic bacterial light organs of the salmoniform fish *Opisthoproctus soleatus* and were kept frozen at −80°C in a 10% glycerol–seawater complete medium (Nealson, 1978).

For this bioassay, bacteria were cultured at 16°C on a solid (agar-amended) artificial seawater glycerol (ASG) medium (Table I) and checked every other day for growth and bioluminescence. An ASG medium was used rather than a rich medium to minimize bacterial growth over the duration of the bioassay and to limit metal complexation (Ramamoorthy and Kushner, 1975; Gellert et al., 1999). The effects of metal treatment on bacterial growth and bioluminescence were assessed using liquid cultures. Bacteria were inoculated from the solid ASG medium into 7 mL of liquid ASG medium using a sterile loop and were incubated overnight (ca. 10 h) under still conditions in glass culture tubes at room temperature (ca. 20°C). The culture was then subsampled, diluted 1000 times into another 7 mL of ASG medium, and gently agitated on a reciprocal shaker at room temperature. Under these culture conditions, the lag phase lasted about 20 h after inoculation, followed by a log phase of about 30 h, ending in a stationary phase approximately 55 h after inoculation. The bioluminescence cell^{-1} changed during this time, increasing in early log phase, peaking at mid–log phase, and decreasing during late log phase, as

previously reported (Hastings and Nealson, 1977). Based on this characterization, the experimental window in this study was restricted to a 9-h period (from 24 to 33 h after inoculation into liquid ASG) to ensure a constant growth rate and an increase in bioluminescence. The metal treatment involved the addition of 100 μL of the metal stock solution to the bacterial culture. Stock solutions were made either with the ASG medium (for Al, Cd, Cr, Mn, and U) or with ultrapure MilliQ-water (for Ag, Cu, Fe, Hg, Pb, and Zn) to allow complete dissolution. Beginning 24 h after the liquid ASG inoculation, cell concentration and bioluminescence were measured at times 0, 0.5, 1, 2, 3, 4, 6, and 9 h after a 3-s vortex mixing to allow homogeneous sampling. Cell concentration was determined from optical density (OD) measured with a Spectronic 20 spectrophotometer at 600 nm (Bausch & Lomb Inc., Madison, WI, USA), whereas glow luminescence was recorded from a 100-μL aliquot of the culture using the Quantalum 2000 luminometer. After each experiment the culture was tested for possible microbial contamination by inoculating the content on a solid ASG medium that was checked for growth and bioluminescence on subsequent days. In every case luminescent bacteria, assumed to be *P. phosphoreum*, were the only organisms growing on the plates.

Bacterial bioluminescence depends on cell concentration (Hastings and Nealson, 1977), so light levels measured in this bioassay were expressed per unit cell. The relationship between OD_{600} and corresponding cell concentration was established during the experimental period. Aliquots from three independent experiments were taken at various times, measured for OD, and processed for cell counting (Smith et al., 1995). Aliquots were fixed with borate-buffered formalin (2% final concentration) and stained for 10 min with 1 μg/mL $4'6$-diamidino-2-phenylindole (DAPI). They were then filtered onto 0.2 μm of black polycarbonate filters (Nucleopore), mounted on slides with low fluorescence immersion oil (Criterion, Sciences), and examined with an Olympus BH-2 epifluorescence microscope. Individual cells were counted, and the corresponding cell concentration calculated (Porter and Feig, 1980). The linear regression relationship between OD_{600} and cell abundance, $N_{cells} = 2184.202 + 224\,647.592 \times OD_{600}$, with $R^2 = 0.934$ ($p < 0.0001$), was used to calculate the number of cells from OD_{600} measurements.

Photocyte Bioassay

Photocytes were isolated from the brittlestar *Ophiopsila californica* (Ophiuroidea, Echinodermata). Brittlestars were collected by SCUBA at night in May and August 2000 at a depth of 7–10 m near the Wrigley Institute for Environmental Studies of the University of Southern California, on Catalina Island, California. Individuals collected from the sandy bottom were immediately placed in fresh circulating seawater aquariums at the Wrigley Center before being transported to the Scripps Institution of Oceanography (La Jolla, CA, USA), where they were kept in circulating seawater aquariums filled with sand from the collection area and fed once a week with plankton broth.

Photocyte isolation was based on the method of (Deheyn et al., 2000a). In summary, 3–5 brittlestars (ca. 5 g wet weight) were anesthetized for 5 min in 3.5% $MgCl_2$ in artificial seawater (Table I); arms were then separated from the disc using surgical scissors and chopped into small fragments with a scalpel blade. Cells were dissociated from arm fragments by enzymatic digestion with three treatments of pronase (35 min each at 30°C, followed by ASW rinsing). Dissociated cells from each treatment were pooled together and layered on a Percoll density gradient by ultracentrifugation (30 000 g, 30 min, 4°C). The concentrated layer of photocytes was then removed from the gradient by pipette and rinsed with ASW at 4°C. About 2×10^8 cells g^{-1} arm tissue was obtained using this procedure. Photocyte light production showed no significant changes in intensity and kinetics for up to 36 h after isolation. The photocyte bioassay was performed by exposing photocyte suspensions in ASW (25 μL) to the same volume of metal stock solution (2× concentrated), for 0, 0.5, 1, 2, 3, 4, 6, 9, and 24 h (time 0 was always < 1 h after completion of the photocyte isolation). Light emission was chemically stimulated using 200 mM potassium chloride (KCl) to exhaust total luminescence capacity (Mallefet et al., 1992), and the light emission was recorded for 40 s using a Lumat 9507 luminometer (EG&G, Perkin Elmer, Gaithersburg, MD, USA). KCl-induced light production is monophasic, consisting of a single flash of bioluminescence characterized by the maximum intensity (Imax) of light production (Deheyn et al., 2000a). Imax values were normalized by the number of photocytes in the 25-μL volume, knowing the initial mass of arm tissue used in the isolation process, the number of cells extracted per gram of tissue, and the final volume of ASW-enriched photocyte solution.

Speciation Calculations

Each bioassay required the use of a specific medium (Table I). Consequently, the chemical characteristics (pH, ionic strength, I) and metal speciation were different in each bioassay. The speciation of each metal in each medium was calculated using thermodynamic equilibrium constants obtained from a variety of sources (Martell and Smith, 1974; Martell et al., 1993; Morel and Hering, 1993) and the chemical equilibrium program HYDRAQL (Papelis et al., 1988). HYDRAQL uses the Davies activity correction, which is valid for $I \leq 0.5$. The medium used in the photocyte bioassay (ASW) was the only medium for which the ionic strength exceeded 0.5. It was necessary to invoke the MacInnes convention and the Debye–Huckel limiting law developed by Millero and coworkers (Millero and Schreiber, 1982; Millero and Hawke, 1992). It was not possible to

apply the MacInnes convention for activity correction to 7 of 183 complexes formed because the required physico-chemical data were unavailable for those complexes. However, those complexes did not contribute significantly to the metal speciation (<0.00001%) and consequently were ignored. Finally, thermodynamic data for metal complexation by glycerol and glycerolphosphate (both constituents of the medium used in the *P. phosphoreum* bioassay) were unavailable; both constituents were expected to have low complexation with metals (Atay and Robinson, 1999; Atay and Varnali, 2000).

Speciation was calculated for 25°C despite the variability in the temperature conditions of the bioassays (Table I). The formation of chemical complexes depends on temperature (Byrne et al., 1988) and can be accurately calculated if enthalpy values are known for the relevant complexes. However, enthalpy values were not available for many complexes. Therefore, bias associated with the absence of temperature correction was estimated for each medium and metal by considering exclusively the most abundant metal complexes for which enthalpy values were available (Martell and Smith, 1974; Byrne et al., 1988; Martell et al., 1993; Stumm and Morgan, 1995). Speciation at 25°C and at the temperature of the bioassay was calculated using HYDRAQL. Overall, complex concentrations at the experimental temperature were lower than that calculated for 25°C; concentration (mean ± 95% CI) was decreased by 13.5 ± 0.045% ($n = 204$) for the Microtox® bioassay (15°C), 1.4 ± 0.023% ($n = 237$) for the *P. phosphoreum* bioassay (22°C), and 17.5 ± 0.043% ($n = 240$) for the photocyte bioassay (8°C). Thus, the speciation presented here may overestimate the concentration of some complexes by up to 17.5%.

Statistical Analysis

The original bioluminescence values or cell culture concentration (OD_{600}) were $\log(x + 1)$ transformed before statistical analysis, as suggested when homoscedasticity is not fulfilled in a data set (Motulsky, 1995; Zar, 1996). Decreases in bioluminescence or cell concentration were tested for significance using the upper-tailed paired "ratio" *t*-test analysis (H_0: mean difference ≤ 0), which is appropriate when the relative difference between treatment and control is a more consistent measure than the absolute value of the treatment (Motulsky, 1995; Zar, 1996). In addition, any occasional increase in bioluminescence or cell concentration (negative MD_t) was tested using a lower-paired ratio *t* test (H_0: mean difference > 0).

Multifactorial analysis of variance (ANOVA) was performed on primary data of the *P. phosphoreum* bioassay to identify the percentage of variation in bioluminescence because of changes in cell concentration (OD_{600}). The percentage was determined by calculating the ratio between the sum of squares associated with the factor and the total sum of squares (all possible factors of variation + residual; Zar, 1996).

The common toxicity indices EC_{50} and LT_{50} could not be used in this study because bioluminescence and cell concentration often did not decrease by more than 50% after metal exposure. Therefore, the parameter MD (mean difference = control − treatment) was used to compare metal effects among bioassays. Thus, positive MD values indicated a decrease in treatment compared to the control, whereas negative MD values described an increase in the treatment compared to the control. MD_t values represented the mean difference calculated at every experimental time point. The test used to determine significance of the MD_t values was based on the upper-tailed paired *t*-test analysis of each metal, and for each concentration the change in MD_t values with time exposure was described using a single-component first-order kinetics exponential model: $MD_t = MD_{eq} (1 - \exp^{-k*t})$, where MD_t and MD_{eq} are the mean differences at time t (h) and at equilibrium, respectively, and k is the rate constant (h^{-1}) (Kuroshima et al., 1993; Slob, 2002). For each metal parameters of the model together with metal chemical speciation were used for comparative analysis of bioassays. All statistical analyses were performed with 0.05 designated as the significance level (α) and using Statview 5.0, Statistica, and SuperAnova 1.11 software (SAS Institute, Inc.).

RESULTS

For each bioassay, bioluminescence of the control (no metal) sample changed with time. For the Microtox® bioassay, bioluminescence of the control decreased over the 2-h incubation time, with the greatest decrease occurring within the first 0.5 h (Fig. 1). For the *P. phosphoreum* bioassay, bioluminescence increased steadily concomitantly with cell concentration between the first and ninth hours of the experiment. For the photocyte bioassay, bioluminescence usually increased over the first 6 h of the experiment, then decreased to the original level. These trends were reproduced for all replicates, but the bioluminescence level varied among replicates (data not shown).

In general, with increasing time and/or concentration of metal exposure, the bioluminescence of the three bioassays decreased compared to the control(Fig. 1). Changes in cell concentration followed the same trend upon metal exposure in the *P. phosphoreum* bioassay. In this bioassay the growth phase of the culture affected the bioluminescence capacity of the bacteria. Therefore, the percentage of the observed variation in bioluminescence was related to change in cell concentration with time. The exposure concentrations of the metals, calculated using multifactor ANOVA, were Ag, 13.1; Al, 25.9; Cd, 44.2; Cr, 14.6; Cu, 16.5; Fe, 27.1; Hg, 7.2; Mn, 32.7; Pb, 25.7; U, 25.5; and Zn, 17.6. This indi-
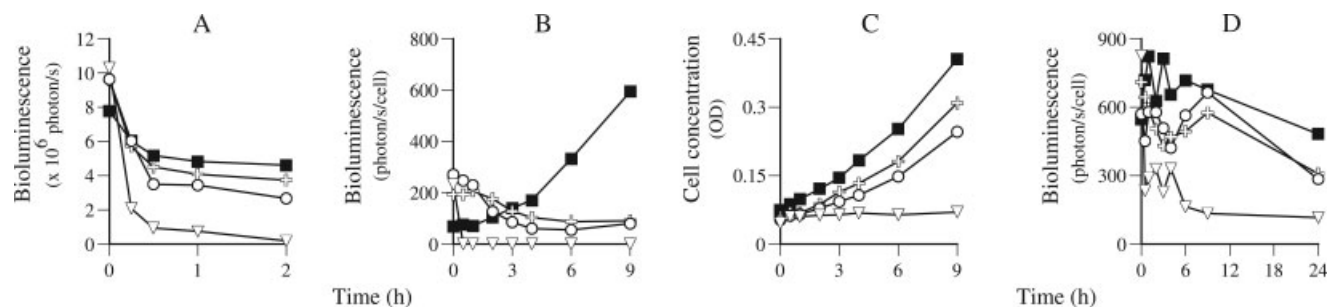
Fig. 1. Effect of Cd concentration as a function of time on (A) the Microtox® bioassay, (B, C) the *P. phoshoreum* bioassay, and (D) the photocyte bioassay (symbols legend: ■, control 0 M; ✥, 10^{-7} M; ○, 10^{-5} M; △, 10^{-3} M).

cates that for metals like Hg most of the observed change in bioluminescence was a direct effect of the metal on light production, whereas for metals like Cd, changes in bioluminescence were also resulted from changes in cell concentration.

Modeling Changes in Bioluminescence

The effect of metal exposure on bioluminescence was modeled for each metal concentration and bioassay using an exponential model of the variation of the MD_t values over time. As shown for Cd for the three bioassays (Fig. 2), MD_t reached a plateau for the highest and intermediate metal concentrations before the end of the exposure. At the lowest metal concentrations, a plateau was not always reached during the experiment, indicating that at those concentrations, the effects on bioluminescence would be greater for longer exposure times of the bioassay (Fig. 2).

Effect of Exposure Time

The effect of exposure time was tested for the highest metal concentration considered (Table II). For the Microtox® assays, bioluminescence MD_t values significantly decreased (positive MD_t values) over the entire exposure period (0.25–2 h, as shown for Cd in Fig. 1), except for Hg and Mn, for which the decrease was significant only after exposures of 1 and 0.5 h, respectively.

For the *P. phosphoreum* bioassay, bioluminescence MD_t values significantly decreased over the entire exposure period (0.5–9 h, as shown for Cd in Fig. 1) for Ag, Cd, Cu, and Hg. A similar effect was observed for Zn, but the decrease was not always significant (Table II). Bioluminescence MD_t values did not change significantly on exposure to Al, Fe, Pb, Cr, Mn, and U, other than increasing significantly after 6 h for Pb (Table II). The observed effects of the metals on
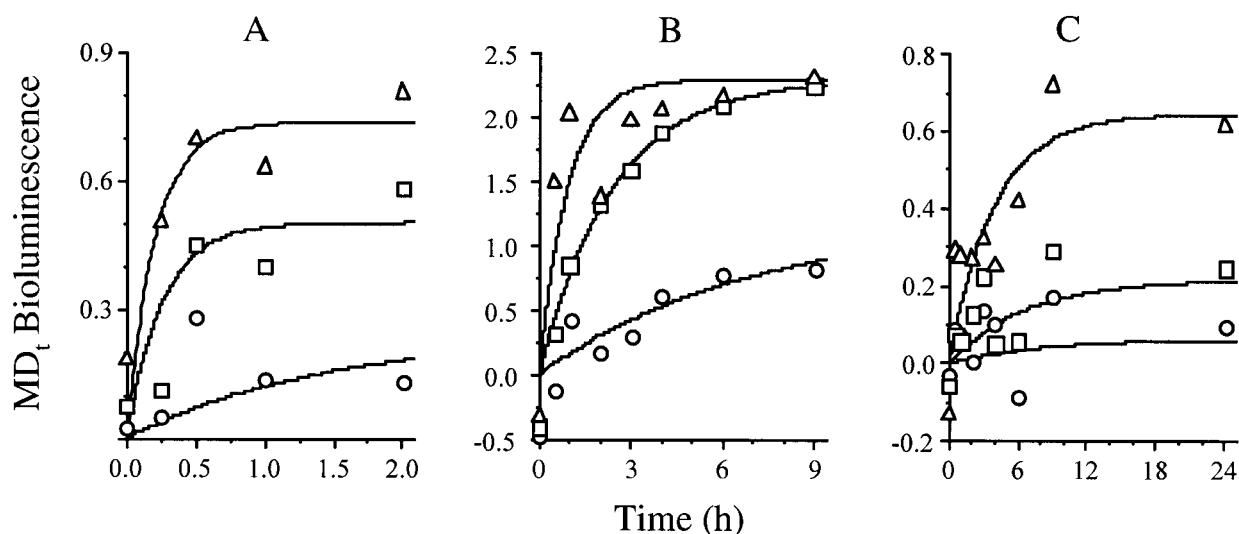
Fig. 2. Exponential models describing values of bioluminescence mean difference from the control (MD_t) as a function of time exposure to the three highest total concentrations of Cd for (A) the Microtox® bioassay, (B) the *P. phoshoreum* bioassay, and (C) the photocyte bioassay (symbols legend: △, 10^{-3} M; ○, 10^{-4} M; □, 10^{-5} M).

the bioluminescence of *P. phosphoreum* were not always related to the effects on bacterial growth. Cell concentration MD_t decreased significantly for Ag, Cd, Cu, and Hg (Fig. 1), with the decrease generally becoming significant after 6 h of exposure, except for Ag (1 h) and Hg (3 h), as shown in Table II. There was no significant decrease in the cell concentration MD_t values for Al, Cr, Fe, Mn, Pb, U, and Zn, yet, except for Mn and Cr, MD_t values were markedly increased on metal exposure; this effect was significant for Al (negative MD_t values; Table III).

For the photocyte bioassay, bioluminescence MD_t values decreased significantly for Cd (2, 4, 9, and 24 h), Cu (9 and 24 h), and Hg, whereas it was significantly increased for Cr (6 and 9 h), Mn (0.5–4 h), Pb (0.5 h), and U (4 h), as shown in Table II.

Effect of Concentration

The effect of total metal concentration on bioluminescence was tested at the longest time exposure for each bioassay (2 h for the Microtox® bioassay, 9 h for the *P. phosphoreum* bioassay, 24 h for the photocyte bioassay). For the Microtox® bioassay, typically observed MD_2 was significantly decreased at 10^{-3} and 10^{-4} M for all metals and at as low as 10^{-5} M for Ag, Al, U, and Zn and 10^{-6} M for Hg (Table III).

For the *P. phosphoreum* bioassay, the observed bioluminescence MD_9 significantly decreased for concentrations $> 10^{-6}$ M for Ag and Hg, and $> 10^{-5}$ M for Cd and Cu (Table III). Similarly, the cell concentration observed MD_9 of was significantly decreased for the same metals and concentrations (data not shown). The observed bioluminescence MD_9 consistently decreased, although the change was not significant upon exposure to Cr and U at concentrations $> 10^{-7}$ M, whereas there was no trend in the variation of growth. The observed bioluminescence MD_9 significantly increased on exposure to Al (except 10^{-5} M), Pb, Mn (except 10^{-3} M), and Zn (except 10^{-4} M), as shown in Table III. Similarly, the observed cell concentration MD_9 was enhanced for the same metals, expect for Al. The case of Fe exposure was unusual; the observed bioluminescence and cell concentration MD_9 values both decreased at concentrations $< 10^{-6}$ M and increased at higher concentrations (Table III).

For the photocyte bioassay, the observed MD_{24} significantly decreased for Cd at 10^{-3} M, for Cu at 10^{-3} M, and for Hg at 10^{-6} to 10^{-4} M. The observed MD_{24} significantly increased for Al at 10^{-7} M and for Mn from 10^{-7}–10^{-4} M (Table III).

The effect of metal exposure over time was modeled for each metal concentration of each bioassay. In general, MD_t values observed at the longest time exposure and MD_{eq} values calculated by the model were similar (Table III), indicating that the maximum effects of the metals were reached from the longest time exposure. Exceptions were at lower metal concentrations, meaning that the observed metal effect was not the greatest possible for those concentrations because of the time limit of the bioassay. The kinetic parameter, k, increased with metal concentration, suggesting a faster decrease of MD_{eq} bioluminescence values for increasing metal concentrations (Table III). For the same metal and metal concentration the kinetics of metal effect were different among bioassays. For Cd, Cu, and Hg metals in all three bioassays that had a significant effect on MD_{eq} bioluminescence, k was greatest for the Microtox® bioassay and lowest for the photocyte bioassay (Table III).

Metal Speciation

The highest soluble metal concentration varied among the three media, ranging from 10^{-3} M for the Microtox® bioassay to as low as 10^{-5} M for some metals in the *P. phosphoreum* and photocyte bioassays (Table IV). Ionic strength of the medium varied among the bioassays; it was 0.34 for the Microtox® bioassay, 0.43 for the *P. phosphoreum* bioassay, and 0.61 for the photocyte bioassay. The pH was similar between the Microtox® and photocyte bioassays for a given metal, ranging from 3.5 to 6.4 depending on the element, whereas it was 7.7 for the *P. phosphoreum* bioassay (Table IV). Each bioassay was characterized by the specific chemical speciation of the metal in the corresponding medium; the concentration of free metal ions as well as that of various metal complexes varied depending on the metal, the pH, and the ligands present in the medium (Table IV).

Based on chemical equilibrium computations, metal speciation and corresponding concentrations were similar among the three media for Ag, Cd, Cr, Hg, Mn, and Zn (Table IV). Dominant complexes were chlorides occurring in concentrations $\geq$ the free-ion concentration, except for Cr, which formed oxide complexes in concentrations $\leq$ the free-ion concentration.

For Al, Cu, and Pb, metal speciation and corresponding concentrations were similar between the Microtox® and photocyte bioassays (Table IV). Dominant complexes were hydroxides (Al only), chlorides, and sulfates occurring in concentrations $\leq$ the free ion concentration. According to the thermodynamics calculations, $Al(OH)_3$ was predicted to precipitate, but only in the Microtox® bioassay medium. In the *P. phosphoreum* bioassay medium, Al occurred as a soluble or precipitated hydroxide complex in greater concentrations than as Al^{3+}. Consequently, Al^{3+} had a lower concentration in this essay than in the other two. The element Cu formed a soluble hydroxide-citrate or ammonium complex or precipitated as a hydroxide complex in concentrations usually $>$ the free ion form. The Cu^{2+} concentration in the *P. phosphoreum* bioassay medium was less than that for the other bioassays. The element Pb occurred in soluble chloride and sulfates complexes or precipitated as carbonate hydroxide in concentrations sim-

TABLE II. Time course of mean difference from the control (MD$_t$: mean control value−mean treatment value at time t (exposure) for each metal for the three bioassays

Metal	Time (h)	Microtox®		P. phosphoreum			Photocyte	
		Conc. (M)	MD$_t$ Biolum.	Conc. (M)	MD$_t$ Biolum.	MD$_t$ OD	Conc. (M)	MD$_t$ Biolum.
Ag	0	10^{-3}	−0.051	10^{-5}	0.056	0.043	10^{-4}	0.040
	0.25		**0.263**		NA	NA		NA
	0.5		**0.438**		**2.165**	0.075		−0.034
	1		**0.616**		**2.207**	**0.107**		−0.012
	2		**0.722**		**2.178**	**0.154**		−0.003
	3		NA		**2.140**	**0.221**		−0.004
	4		NA		**2.216**	**0.310**		0.003
	6		NA		**2.370**	**0.447**		0.047
	9		NA		**2.579**	**0.642**		0.061
	24		NA		NA	NA		0.394
A1	0	10^{-3}	0.080	10^{-3}	0.041	0.044	10^{-4}	−0.001
	0.25		**0.888**		NA	NA		NA
	0.5		**1.049**		0.362	***−0.081***		−0.049
	1		**0.992**		0.210	−0.063		0.018
	2		**0.940**		0.069	***−0.048***		0.026
	3		NA		−0.081	−0.016		0.095
	4		NA		−0.150	0.004		−0.063
	6		NA		−0.308	**0.055**		−0.103
	9		NA		−0.468	0.086		0.049
	24		NA		NA	NA		0.004
Cd	0	10^{-3}	0.188	10^{-3}	−0.308	0.040	10^{-3}	−0.128
	0.25		**0.506**		NA	NA		NA
	0.5		**0.699**		**1.497**	0.012		0.294
	1		**0.634**		**2.033**	0.045		0.280
	2		**0.811**		1.388	0.084		**0.274**
	3		NA		**1.985**	0.115		0.325
	4		NA		**2.065**	0.168		**0.255**
	6		NA		**2.167**	**0.279**		0.424
	9		NA		**2.311**	**0.380**		**0.723**
	24		NA		NA	NA		**0.620**
Cr	0	10^{-3}	0.142	10^{-4}	0.162	0.050	10^{-3}	−0.033
	0.25		**0.283**		NA	NA		NA
	0.5		**0.422**		0.085	0.056		−0.012
	1		**0.639**		−0.042	0.060		***−0.099***
	2		**0.598**		0.132	0.034		−0.016
	3		NA		0.063	0.053		−0.031
	4		NA		0.088	0.054		0.061
	6		NA		0.113	0.097		***−0.091***
	9		NA		0.210	0.081		0.039
	24		NA		NA	NA		−0.015
Cu	0	10^{-3}	0.065	10^{-4}	−0.175	−0.027	10^{-3}	−0.006
	0.25		**0.594**		NA	NA		NA
	0.5		**0.591**		**1.850**	0.033		0.118
	1		**0.544**		**1.784**	0.060		0.148
	2		**0.501**		**1.628**	0.081		0.124
	3		NA		**1.669**	0.146		0.194
	4		NA		**1.473**	0.190		**0.265**
	6		NA		**1.484**	**0.295**		0.471
	9		NA		**1.087**	**0.430**		**0.750**
	24		NA		NA	NA		**1.607**
Fe	0	10^{-3}	0.059	10^{-4}	−0.105	−0.026	10^{-4}	−0.029
	0.25		**0.872**		NA	NA		NA
	0.5		**0.788**		−0.041	−0.087		0.016
	1		**0.848**		−0.098	−0.081		−0.001
	2		**0.787**		−0.012	−0.079		−0.013

TABLE II. (Continued)

Metal	Time (h)	Microtox® Conc. (M)	MD$_t$ Biolum.	P. phosphoreum OD Conc. (M)	MD$_t$ Biolum.	MD$_t$ OD	Photocyte Conc. (M)	MD$_t$ Biolum.
	3		NA		−0.044	−0.076		−0.020
	4		NA		−0.166	−0.063		0.014
	6		NA		−0.156	−0.059		0.011
	9		NA		−0.214	−0.069		0.038
	24		NA		NA	NA		0.037
Hg	0	10^{-3}	−0.009	10^{-4}	0.003	−0.079	10^{-4}	0.010
	0.25		0.785		NA	NA		NA
	0.5		0.812		**2.071**	−0.008		**3.443**
	1		**0.903**		**2.135**	0.006		**3.490**
	2		**0.947**		**2.069**	0.079		**3.476**
	3		NA		**2.051**	**0.157**		**3.471**
	4		NA		**2.038**	**0.238**		**3.392**
	6		NA		**1.935**	**0.384**		**3.405**
	9		NA		**1.619**	**0.545**		**3.509**
	24		NA		NA	NA		**3.371**
Mn	0	10^{-3}	0.132	10^{-3}	0.002	0.032	10^{-3}	−0.049
	0.25		0.642		NA	NA		NA
	0.5		**0.550**		−0.024	0.024		***−0.057***
	1		**0.739**		−0.032	0.018		***−0.044***
	2		**0.683**		0.087	0.031		***−0.073***
	3		NA		0.153	0.061		***−0.050***
	4		NA		0.221	0.110		***−0.047***
	6		NA		0.274	0.205		−0.059
	9		NA		0.284	0.236		−0.065
	24		NA		NA	NA		−0.033
Pb	0	10^{-3}	−0.104	10^{-5}	−0.086	0.043	10^{-5}	−0.022
	0.25		**0.936**		NA	NA		NA
	0.5		**0.878**		−0.140	0.039		***−0.054***
	1		**0.942**		−0.081	0.047		−0.028
	2		**0.947**		−0.103	0.023		0.004
	3		NA		−0.124	0.028		−0.033
	4		NA		−0.228	0.013		−0.034
	6		NA		***−0.407***	−0.010		−0.035
	9		NA		***−0.707***	−0.054		0.060
	24		NA		NA	NA		−0.008
U	0	10^{-3}	−0.037	10^{-4}	−0.043	−0.059	10^{-4}	−0.012
	0.25		**0.922**		NA	NA		NA
	0.5		**0.847**		0.033	−0.114		−0.029
	1		**0.906**		0.055	−0.103		−0.025
	2		**0.856**		0.115	−0.113		0.000
	3		NA		0.140	−0.098		−0.031
	4		NA		0.116	−0.065		***−0.031***
	6		NA		0.014	0.010		0.037
	9		NA		0.221	0.100		−0.016
	24		NA			NA		−0.024
Zn	0	10^{-3}	0.056	10^{-4}	0.041	−0.079	10^{-4}	0.022
	0.25		**0.638**		NA	NA		NA
	0.5		**0.602**		**0.586**	−0.093		−0.017
	1		**0.648**		0.648	−0.082		−0.017
	2		**0.573**		0.563	−0.050		0.006
	3		NA		**0.563**	−0.007		0.001
	4		NA		0.584	0.027		0.022
	6		NA		**0.725**	**0.101**		0.046
	9		NA		0.725	**0.226**		0.021
	24		NA		NA	NA		0.045

Only highest metal total concentration is shown. Mean difference values in bold (positive MD$_t$) and bold italics (negative MD$_t$) are significant ($P \leq$ 0.05). NA: not analyzed.

TABLE III. Mean difference of bioluminescence for the control (MD: mean control value−mean treatment value) observed at longest time exposure, and parameters (MD$_{eq}$, k, and R^2) of the exponential model describing change in the mean difference between the control and the treatment

Metal	[Metal]$_{Tot}$ (M)	Microtox®				P. phosphoreum				Photocyte			
		Observed MD$_2$	Model MD$_{eq}$	k	R^2	Observed MD$_9$	Model MD$_{eq}$	k	R^2	Observed MD$_{24}$	Model MD$_{eq}$	k	R^2
Ag	10^{-8}	0.001	−0.06	1.50	0.41	0.945	1.30	0.10	0.83	−0.047	−0.07	0.02	0.70
	10^{-7}	0.038	0.04	1.72	0.60	0.700	1.20	0.07	0.51	−0.032	−0.03	0.10	0.02
	10^{-6}	0.031	0.04	0.75	0.72	**1.833**	1.85	0.48	0.99	−0.019	−0.03	0.10	0.02
	10^{-5}	**0.583**	0.67	0.89	0.95	**2.579**	2.27	3.46	0.95	−0.006	−0.02	0.20	0.04
	10^{-4}	0.558	0.56	2.84	0.99	NA	NA	NA	NA	0.394	0.25	0.07	0.77
	10^{-3}	**0.722**	0.74	1.77	1.00	NA	NA	NA	NA	NA	NA	NA	NA
Al	10^{-8}	0.133	0.18	0.90	0.02	0.030	0.08	0.15	0.07	0.001	0.01	0.10	0.002
	10^{-7}	0.120	0.18	2.00	0.02	−0.034	0.07	0.40	0.28	***−0.051***	−0.07	0.09	0.27
	10^{-6}	**0.186**	0.20	1.80	0.12	−0.158	0.05	0.30	0.18	−0.022	0.002	0.04	0.01
	10^{-5}	**0.423**	0.39	5.17	0.74	0.023	0.15	0.30	0.14	0.065	0.06	0.08	0.08
	10^{-4}	**0.753**	0.74	3.57	0.92	−0.127	0.15	0.10	0.69	0.004	0.01	0.09	0.002
	10^{-3}	**0.940**	0.99	9.49	0.99	−0.468	−0.44	0.11	0.77	NA	NA	NA	NA
Cd	10^{-8}	0.192	0.22	0.70	0.42	0.172	−0.05	0.16	0.35	0.162	0.21	0.07	0.25
	10^{-7}	0.055	0.15	0.90	0.01	0.305	0.37	0.22	0.74	0.043	0.16	0.04	0.09
	10^{-6}	0.047	0.18	0.80	0.08	0.414	0.42	0.30	0.84	0.094	0.10	0.09	0.49
	10^{-5}	0.131	0.20	0.90	0.13	**0.825**	1.20	0.14	0.78	0.094	0.12	0.15	0.07
	10^{-4}	**0.583**	0.58	1.66	0.84	**2.245**	2.29	0.41	0.98	0.244	0.25	0.22	0.52
	10^{-3}	**0.811**	0.74	4.83	0.89	**2.311**	2.29	1.07	0.79	**0.620**	0.64	0.25	0.72
Cr	10^{-8}	0.127	0.18	0.90	0.05	−0.082	−0.17	0.67	0.08	−0.042	−0.05	0.40	0.001
	10^{-7}	0.119	0.12	4.56	0.93	0.253	0.17	0.40	0.74	−0.017	−0.03	0.40	0.01
	10^{-6}	0.278	0.30	10.00	0.92	0.310	0.13	0.40	0.56	0.009	−0.03	0.40	0.04
	10^{-5}	0.230	0.23	13.68	0.48	0.280	−0.01	0.04	0.31	−0.065	−0.04	0.40	0.01
	10^{-4}	**0.417**	0.43	1.49	0.91	0.210	0.18	0.40	0.06	−0.037	−0.01	0.40	0.13
	10^{-3}	**0.598**	0.64	2.43	0.92	NA	NA	NA	NA	−0.015	0.01	0.42	0.06
Cu	10^{-8}	0.020	−0.01	0.40	0.64	−0.414	−0.31	0.89	0.01	−0.069	0.01	0.04	0.12
	10^{-7}	0.133	0.18	0.90	0.67	0.010	−0.05	0.40	0.21	1.347	0.17	0.31	0.25
	10^{-6}	0.175	0.25	0.80	0.64	−0.106	−0.29	0.63	0.15	−0.016	−0.01	0.34	0.07
	10^{-5}	0.333	0.30	4.63	0.86	**1.106**	1.42	1.21	0.91	0.021	0.05	0.40	0.49
	10^{-4}	**0.477**	0.51	23.45	0.95	**1.087**	1.50	13.72	0.87	0.186	0.18	0.13	0.73
	10^{-3}	**0.501**	0.54	20.31	0.95	NA	NA	NA	NA	**1.607**	2.95	0.03	0.99
Fe	10^{-8}	0.114	0.18	1.50	0.00	0.374	0.36	1.06	0.84	0.011	−0.01	0.40	0.005
	10^{-7}	−0.052	−0.10	0.80	0.03	**0.403**	0.32	0.23	0.93	−0.013	−0.01	0.40	0.42
	10^{-6}	0.037	0.02	0.40	0.62	0.290	0.10	0.27	0.66	0.002	−0.01	0.40	0.14
	10^{-5}	0.022	0.05	4.00	0.41	−0.082	−0.05	0.40	0.42	−0.003	0.02	0.25	0.21
	10^{-4}	**0.824**	0.76	11.48	0.98	−0.214	−0.18	0.40	0.30	0.037	0.04	0.15	0.47
	10^{-3}	**0.787**	0.80	17.77	0.98	NA	NA	NA	NA	NA	NA	NA	NA
Hg	10^{-8}	0.012	−0.10	0.90	0.24	−0.115	−0.05	0.01	0.14	−0.083	0.01	0.10	0.01
	10^{-7}	0.041	−0.02	0.70	0.43	−0.362	−0.40	0.08	0.23	−0.001	−0.01	0.04	0.05
	10^{-6}	**0.977**	0.93	9.97	0.98	**1.359**	1.76	6.76	0.94	**1.615**	3.29	0.02	0.99
	10^{-5}	**0.989**	0.93	15.33	0.98	**1.619**	1.97	9.56	0.95	**3.357**	3.28	0.53	0.96
	10^{-4}	**0.942**	0.88	12.05	0.97	**1.619**	1.97	9.56	0.95	**3.371**	3.44	5.98	1.00
	10^{-3}	**0.947**	0.90	6.28	0.99	NA	NA	NA	NA	NA	NA	NA	NA
Mn	10^{-8}	0.022	−0.02	0.80	0.69	***−0.368***	−0.38	0.33	0.80	0.017	−0.02	0.10	0.09
	10^{-7}	0.062	0.07	0.70	0.43	−0.042	−0.10	0.40	0.51	***−0.076***	−0.05	0.06	0.01
	10^{-6}	0.179	0.17	4.21	0.95	−0.411	−0.49	0.11	0.85	***−0.068***	−0.06	0.06	0.01
	10^{-5}	0.155	0.10	1.00	0.88	−0.092	−0.13	0.62	0.62	−0.077	−0.06	0.07	0.03
	10^{-4}	**0.547**	0.57	1.77	0.98	***−0.546***	−0.50	0.40	0.81	***−0.070***	−0.06	0.07	0.02
	10^{-3}	**0.683**	0.67	9.76	0.91	0.284	0.30	0.30	0.88	−0.033	−0.05	0.08	0.01
Pb	10^{-8}	−0.052	−0.10	1.00	0.06	***−0.380***	−0.19	0.28	0.66	−0.030	−0.04	0.10	0.03
	10^{-7}	−0.091	−0.10	1.00	0.09	−0.507	−0.37	0.11	0.74	−0.043	−0.03	0.21	0.03
	10^{-6}	0.156	0.20	9.72	0.92	−0.568	−0.44	0.19	0.70	−0.032	−0.04	0.08	0.00
	10^{-5}	0.477	0.54	3.61	0.94	***−0.707***	−0.56	0.23	0.68	−0.008	−0.001	0.05	0.13
	10^{-4}	**0.866**	0.86	8.32	1.00	NA	NA	NA	NA	NA	NA	NA	NA
	10^{-3}	**0.947**	0.92	12.68	0.99	NA	NA	NA	NA	NA	NA	NA	NA
U	10^{-8}	0.034	−0.05	4.00	0.20	−0.025	0.05	0.60	0.03	0.015	0.01	0.08	0.23
	10^{-7}	−0.033	−0.08	3.00	0.02	0.124	0.18	0.37	0.64	−0.021	−0.02	0.08	0.002
	10^{-6}	0.106	0.12	3.20	0.94	**0.479**	0.29	0.16	0.70	−0.018	−0.02	0.08	0.002
	10^{-5}	**0.862**	0.90	3.84	0.99	−0.015	0.03	0.60	0.03	0.027	0.02	0.10	0.34
	10^{-4}	**0.883**	0.88	5.36	0.99	0.221	0.14	0.49	0.50	−0.024	−0.02	0.14	0.02
	10^{-3}	**0.856**	0.87	15.59	0.99	NA	NA	NA	NA	NA	NA	NA	NA
Zn	10^{-8}	0.030	0.03	2.28	0.68	***−0.587***	−0.30	0.34	0.66	−0.051	−0.05	0.10	0.28
	10^{-7}	0.152	0.14	2.64	0.90	***−0.959***	−0.49	0.38	0.59	−0.033	−0.03	0.13	0.09
	10^{-6}	0.228	0.23	3.68	0.86	***−0.944***	−0.59	0.16	0.66	−0.041	−0.03	0.20	0.23
	10^{-5}	**0.399**	0.40	1.99	0.98	−0.676	−0.52	0.16	0.66	0.001	0.002	0.20	0.001
	10^{-4}	**0.466**	0.55	4.94	0.93	0.725	0.63	3.64	0.44	0.045	0.05	0.10	0.51
	10^{-3}	**0.573**	0.60	15.85	0.97	NA	NA	NA	NA	NA	NA	NA	NA

See Methods section for model description. Mean difference values in bold (positive MD) and bold italics (negative MD) are significant ($P \leq 0.05$). NA: not analyzed.

TABLE IV. Concentrations of free metal ions and dominant forms of metal complexes for each element assessed with the three bioassays, and pH of each medium after addition of metal salt, as calculated by the HYDRAQL program

Metal salt	$[\text{Metal}]_{\text{Tot}}$ (M)	Microtox®			P. phosphoreum			Photocyte		
		pH	Chemical form	Conc. (M)	pH	Chemical form	Conc. (M)	pH	Chemical form	Conc. (M)
$AgNO_3$ (Ag)	10^{-5}	5.5	Ag^+	5.8×10^{-11}	7.7	Ag^+	7.4×10^{-11}	5.5	Ag^+	2.2×10^{-11}
			$AgCl_3^{2-}$	5.8×10^{-6}		$AgCl_3^{2-}$	6.0×10^{-6}		$AgCl_3^{2-}$	6.7×10^{-6}
			$AgCl_4^{3-}$	3.5×10^{-6}		$AgCl_4^{3-}$	3.3×10^{-6}		$AgCl_4^{3-}$	3.0×10^{-6}
			$AgCl_2^-$	6.5×10^{-7}		$AgCl_2^-$	7.1×10^{-7}		$AgCl_2^-$	2.6×10^{-7}
$Al_2(SO_4)_3$ (Al)	10^{-4}	4.4	Al^{3+}	1.7×10^{-4}	7.7	Al^{3+}	1.4×10^{-14}	4.8	Al^{3+}	1.3×10^{-4}
			$Al(OH)^{2+}$	1.2×10^{-5}		$Al(OH)_4^-$	6.6×10^{-7}		$Al(SO_4)^+$	6.2×10^{-5}
			$Al(OH)_2^+$	6.9×10^{-6}		$Al(OH)_3^0$	3.2×10^{-7}		$Al(OH)_2^+)$	6.0×10^{-6}
			$Al(SO_4)^+$	4.4×10^{-6}		$Al(OH)_3$ (s)	2.0×10^{-4}		$Al(OH)^{2+}$	2.8×10^{-6}
			$Al(OH)_3$ (s)	5.5×10^{-6}						
$CdCl_2$ (Cd)	10^{-3}	5.5	Cd^{2+}	5.2×10^{-5}	7.7	Cd^{2+}	5.7×10^{-5}	5.5	Cd^{2+}	2.4×10^{-5}
			$CdCl^+$	4.9×10^{-4}		$CdCl^+$	4.9×10^{-4}		$CdCl^+$	5.2×10^{-4}
			$CdCl_2$	3.8×10^{-4}		$CdCl_2$	3.5×10^{-4}		$CdCl_2$	3.7×10^{-4}
			$CdCl_3^-$	8.1×10^{-5}		$CdCl_3^-$	7.1×10^{-5}		$CdCl_3^-$	8.0×10^{-5}
						$CdSO_4$	2.4×10^{-5}		$CdSO_4$	1.5×10^{-6}
Na_2CrO_4 (Cr)	10^{-4}	6.4	CrO_4^{2-}	5.3×10^{-5}	7.7	CrO_4^{2-}	7.6×10^{-5}	6.2	CrO_4^{2-}	7.6×10^{-5}
			$NaCrO_4^-$	2.6×10^{-5}		$KCrO_4^-$	2.2×10^{-5}		$HCrO_4^-$	1.3×10^{-5}
			$HCrO_4^-$	2.1×10^{-5}		$HCrO_4^-$	1.3×10^{-6}		$NaCrO_4^-$	1.1×10^{-5}
						$NaCrO_4^-$	3.2×10^{-7}		$KCrO_4^-$	1.4×10^{-7}
$CuCl_2$ (Cu)	10^{-4}	5.5	Cu^{2+}	7.4×10^{-5}	7.7	Cu^{2+}	2.7×10^{-8}	5.5	Cu^{2+}	7.4×10^{-5}
			$CuCl^+$	2.3×10^{-5}		$Cu_2(OH)_2Citr_2$	3.8×10^{-7}		$CuCl^+$	2.1×10^{-5}
			$CuCl_2$	3.3×10^{-6}		$Cu(NH_3)_2$	8.2×10^{-8}		$CuCl_2$	2.6×10^{-6}
						$Cu(OH)_2$ (s)	9.9×10^{-5}		$CuSO_4$	2.4×10^{-6}
$FeCl_3$ (Fe)	10^{-4}	3.5	Fe^{3+}	3.5×10^{-11}	7.7	Fe^{3+}	8.6×10^{-24}	3.6	Fe^{3+}	9.1×10^{-10a}
			$Fe(OH)^+$	2.2×10^{-10}		$Fe(OH)_3$ (s)	1.0×10^{-4}		$Fe_3(OH)_4^{5+}$	2.5×10^{-7}
			$Fe(OH)_3$ (s)	1.0×10^{-4}					$Fe(OH)_3$ (s)	9.9×10^{-5}
$HgCl_2$ (Hg)	10^{-4}	5.5	Hg^{2+}	5.2×10^{-18}	7.7	Hg^{2+}	6.7×10^{-18}	5.5	Hg^{2+}	1.9×10^{-18}
			$HgCl_4^{2-}$	5.1×10^{-5}		$HgCl_4^{2-}$	4.8×10^{-5}		$HgCl_4^{2-}$	7.6×10^{-5}
			$HgCl_3^-$	4.0×10^{-5}		$HgCl_3^-$	4.1×10^{-5}		$HgCl_3^-$	1.9×10^{-5}
			$HgCl_2$	9.3×10^{-6}		$HgCl_2$	1.0×10^{-5}		$HgCl_2$	5.1×10^{-6}
$MnCl_2$ (Mn)	10^{-3}	5.5	Mn^{2+}	6.1×10^{-4}	7.7	Mn^{2+}	5.1×10^{-4}	5.5	Mn^{2+}	6.1×10^{-4}
			$MnCl^+$	2.4×10^{-4}		$MnSO_4$	1.9×10^{-4}		$MnCl^+$	2.5×10^{-4}
			$MnCl_2$	1.4×10^{-4}		$MnCl^+$	1.8×10^{-4}		$MnCl_2$	1.2×10^{-4}
						$MnCl_2$	9.9×10^{-5}		$MnSO_4$	1.7×10^{-5}
$Pb(NO_3)_2$ (Pb)	10^{-5}	5.5	Pb^{2+}	1.7×10^{-6}	7.7	Pb^{2+}	1.0×10^{-7}	5.5	Pb^{2+}	1.4×10^{-6}
			$PbCl^+$	5.9×10^{-6}		$PbCl^+$	3.2×10^{-7}		$PbCl^+$	6.5×10^{-6}
			$PbCl_2$	1.9×10^{-6}		$PbSO_4$	1.3×10^{-7}		$PbCl_2$	1.6×10^{-6}
			$PbCl_3^+$	5.2×10^{-7}		$Pb_3(CO_3)_2OH_2$ (s)	3.1×10^{-6}		$PbCl_3^+$	3.7×10^{-7}
									$PbSO_4$	1.5×10^{-7}
$UO_2(CH_3O_2)_2$ (U)	10^{-4}	4.2	UO_2^{2+}	1.3×10^{-7}	7.7	UO_2^{2+}	5.6×10^{-11}	4.9	UO_2^{2+}	4.2×10^{-5}
			$UO_2(OH)_2^0$	1.0×10^{-4}		$(UO_2)(CO_3)_3^{4-}$	1.5×10^{-5}		UO_2SO_4	6.5×10^{-6}
						$(UO_2)_2CO_3(OH)_3^-$	1.5×10^{-5}		UO_2Cl^+	8.3×10^{-6}
						$(UO_2)(CO_3)_2^{2-}$	6.6×10^{-6}		$(UO_2)_4(OH)_7^+$	6.3×10^{-6}
						$UO_2(OH)_2$ (s)	4.7×10^{-7}		UO_2OH^+	5.2×10^{-6}
$ZnCl_2$ (Zn)	10^{-4}	5.5	Zn^{2+}	7.7×10^{-5}	7.7	Zn^{2+}	5.2×10^{-5}	5.5	Zn^{2+}	7.7×10^{-5}
			$ZnCl^+$	1.9×10^{-5}		$Zn(SO_4)$	1.4×10^{-5}		$ZnCl^+$	1.8×10^{-5}
			$ZnCl_2$	2.2×10^{-6}		$ZnCl^+$	1.2×10^{-5}		$ZnCl_2$	1.7×10^{-6}
			$ZnCl_3^-$	1.6×10^{-6}		$ZnOHCl$	8.3×10^{-6}		$Zn(SO_4)$	1.3×10^{-6}
						$Zn(SO_4)_2^{2-}$	3.5×10^{-6}		$ZnCl_3^-$	1.0×10^{-6}

Total concentration is based on the amount of metal salt added to the medium. Only the highest total concentration common to the three bioassays is represented. Species that precipitated as solids according to the HYDRAQL calculation are marked (s).

ilar to that for Pb^{2+}. The Pb^{2+} concentration was lower in the *P. phosphoreum* bioassay medium than it was in the two other bioassays (Table IV).

Each bioassay had unique metal speciation characteristics for Fe and U (Table IV). For the Microtox® bioassay, Fe occurred as soluble or precipitated hydroxide complexes in concentrations greater than that for Fe^{3+}. Speciation was similar, but the species concentrations were greater for the photocyte bioassay However, for the *P. phosphoreum* bioassay most Fe precipitated as hydroxide complexes, the free

ion form occurring only at a low concentration. The element U occurred as a hydroxide complex in the Microtox® bioassay medium in concentrations greater than that for the free ion form. In the *P. phosphoreum* bioassay medium, U formed soluble carbonate and precipitate hydroxide complexes, in concentrations greater than that for the free ion form. In the photocyte bioassay U occurred as dissolved sulfate, chloride, or hydroxide complexes in concentrations similar to that for the free ion form. Overall, UO_2^{2+} concentration followed the ranking of *P. phosphoreum* bioassay > Microtox® bioassay > photocyte bioassay (Table IV).

Bioassay Sensitivity

MD_t values were different among bioassays and followed the ranking Microtox® < *P. phosphoreum* < photocyte bioassay for Cr and Mn, photocyte < *P. phosphoreum* < Microtox® for Hg, and Microtox® < photocyte < *P. phosphoreum* for Ag and Cu. The three bioassays had similar concentrations of free ions in the medium at the different MD_t values, a pattern usually present for the metal complexes (Fig. 3A–C). The element Ag was the exception; Ag^+ concentration in the medium was not similar among bioassays but increased with increasing MD_t values, a pattern also observed for $AgCl_2^-$ but not for the other complexes (Fig. 3D–G).

MD_t values were similar for two bioassays and greater for the third bioassay for Al, Cd, Fe, Pb, and U, or lower for the third bioassay for Zn. The Microtox® bioassay had MD_t values similar to those of the *P. phosphoreum* bioassay for Al, Fe, Pb, and U; the *P. phosphoreum* bioassay had MD_t values similar to those of the photocyte bioassay for Zn, and the Microtox® and photocyte bioassays had similar MD_t values for Cd. Usually, bioassays with similar MD_t values had different free-ion concentrations in the medium, whereas bioassays with different MD_t values had concentrations of free ions similar to that of one of the other bioassays (Fig. 3H–I). This pattern was not necessarily observed for the complexes (Fig. 3J–K).

DISCUSSION

The toxicity of metals was assessed using three cell-based bioluminescence assays in which a decrease of light production was used as a proxy for toxicity. In addition, chemical speciation of the metals was determined for the three common culture media used in the assays. Toxicity was observed for Cd, Cu, and Hg in all three bioassays, in Ag for the Microtox® and *P. phosphoreum* bioassays, and in Al, Cr, Fe, Mn, Pb, U, and Zn for only the Microtox® bioassay (Fig. 4). The differences observed between the bioassays reflected variations in chemical speciation and/or the biological nature of the bioassay. The relative importance of chemical speciation and biological susceptibility determines the toxicity of metals.

Catalytic Versus Noncatalytic Bioassays. Bioassays can be categorized into different groups based on how toxicants alter expression of the biological character measured by the bioassays. Catalytic bioassays respond to the alteration of enzyme activity and protein integrity, whereas noncatalytic bioassays measure alteration of antibody production and nucleic acid transcription and/or translation (Van Der Lelie et al., 1994). Capacity for long-term adaptation to the presence of a toxicant therefore indicates a noncatalytic bioassay. In this study adaptation to metals would result from the ability of cells to replicate over the course of the bioassay. The photocyte and Microtox® bioassays belong to the catalytic bioassay group. The photocyte bioassay involves eukaryotic cells that do not replicate and thus have limited adaptability to metal exposure. Similarly, the Microtox® bioassay measures acute toxicity originating from a physicochemical change because the bacteria have a slow generation time (> 2 h) that does not allow adaptation to changes in the surrounding environment over the time of the bioassay (Ross, 1993; Bulich and Bailey, 1995; Froehner et al., 2002). The *P. phosphoreum* bioassay belongs to the noncatalytic group; the bacteria replicate and possibly adapt to changes in the surrounding environment by changes in gene and/or protein expression (Thomulka et al., 1996).

Toxicity Depends on the Metal Speciation and Biological Nature of the Cell. For Cd and Hg the chemical speciation and the decrease in light production were similar for all three bioassays despite differences in pH and ionic strength (I) among the media (Fig. 4). This result suggests that the chemical speciation and toxicity of those metals do not vary greatly with physicochemical conditions. Toxicity of a metal species would then depend on a combination of factors that includes the biological nature of the cells. This study showed, for instance, that for similar Cd^{2+} concentrations, the bacteria bioassays showed different levels of toxicity, thus indicating that a biological factor that was different between the two bioassays was involved in determining the level of toxicity. The same conclusion is reached by comparing the photocyte and Microtox® bioassays that showed different free-ion concentrations yet similar toxicity levels. The most abundant metal species present in the three media considered were chloro-complexes, which suggests they might also be toxic species. However, chloro-complexes were usually found in higher concentrations in the photocyte and Microtox® bioassays than in the *P. phosphoreum* bioassay that showed greater toxicity. Thus, if chloro-complexes are toxic (Bienvenue et al., 1984; Sarin et al., 2000), the cells in the three bioassays have distinct biological tolerances to these chloro-complexes.

The chemical speciation of Al and U was different among the three bioassays, as was the observed difference in toxicity (Fig. 4). For U, the hydroxide complex was the most dominant complex in the Microtox® bioassay medium,
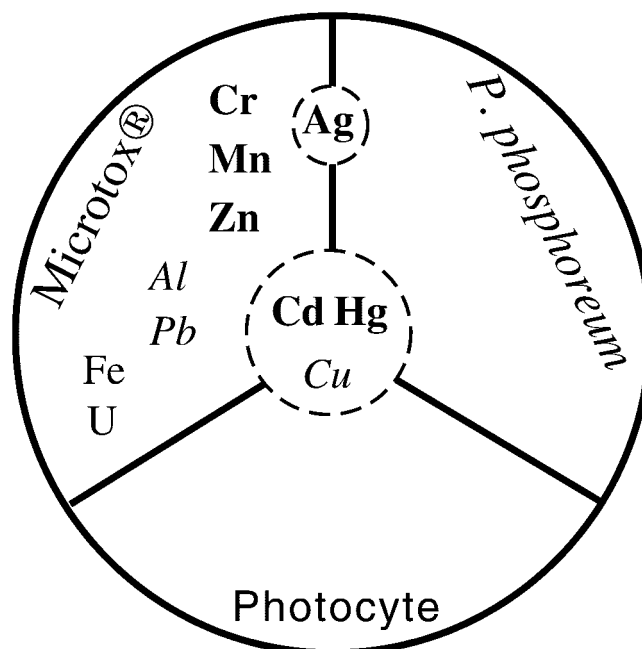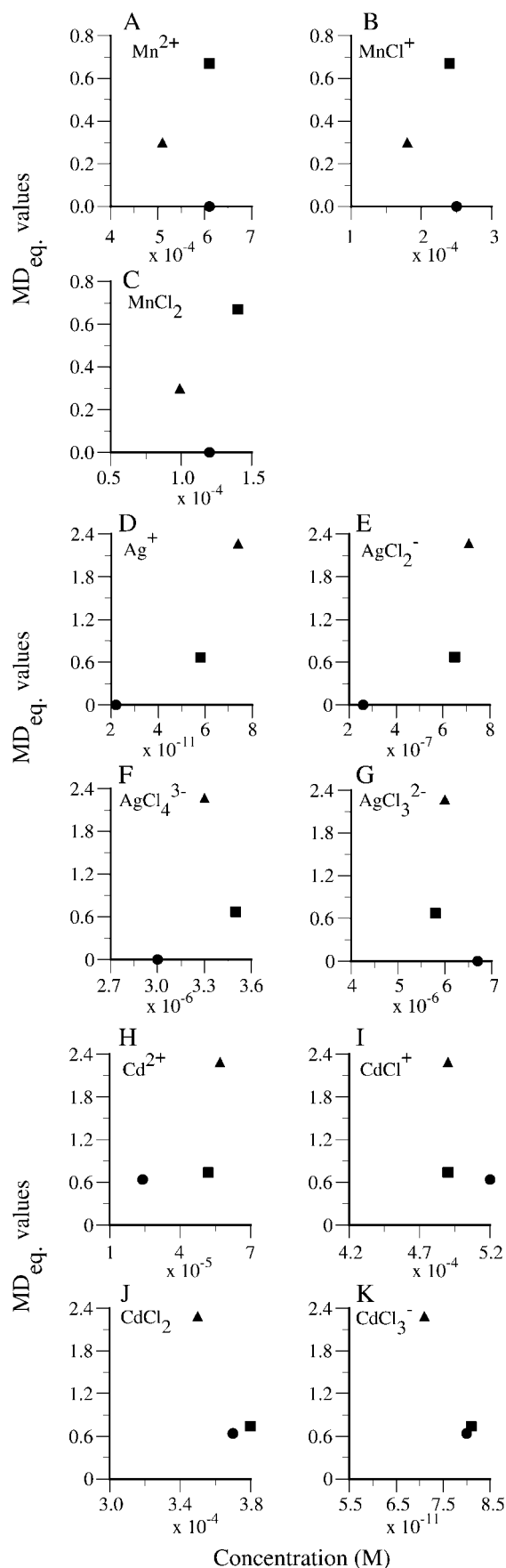
Fig. 4. Summary of metal toxicity among the three bioassays. Metals listed in bold shared similar chemical speciation among the bioassays, whereas for those in italics speciation was similar between the Microtox® and photocyte bioassays. Metals listed in regular font showed different speciation among the bioassays.

in which toxicity was observed and the least concentrated in the media of the other bioassays, in which toxicity was not observed. This suggests that U toxicity may result from hydroxide complexes. Similarly, Al toxicity was detected only in the Microtox® bioassay medium, even though a similar free-ion concentration was found in the photocyte bioassay medium. The concentration of hydroxide complexes was more than 10 times higher in the Microtox® bioassay medium, suggesting that this may be the toxic form of this metal, a finding in agreement with those in previous studies (Parent et al., 1996; Simkiss and Taylor, 2001).

The element Ag was toxic in the Microtox® and *P. phosphoreum* bioassays but not in the photocyte bioassay, despite similar metal speciation for all three media. The lack of toxicity in the photocyte bioassay may be related to differences in susceptibility of the cells to metals as well as to differences in ionic strength among the media. Toxicity in the bioassays was correlated with the concentrations of Ag^+ and $AgCl_2^-$, suggesting that these metal species are toxic. The concentration of the metal complex $AgCl_3^{2-}$ in the three assays was highest in the photocyte bioassay, supporting that this species is the least toxic. In addition, Ag elicited a

Fig. 3. Toxicity (MD_t values) of (A–C) Mn, (D–G) Ag, and (H–K) Cd for the three bioassays as a function of concentration of each metal species (symbols legend: ■, Microtox® assay; ▲, *P. phosphoreum* assay; ●: photocyte assay).

toxic response only in bioassays using bacteria, suggesting that with this metal there are different mechanisms of toxicity for prokaryotic and metazoan cells.

The elements Cr, Mn, and Zn showed similar chemical speciation for the three media, yet toxicity was observed only with the Microtox® bioassay. This was not an artifactual result, because in this study the greatest free-ion concentration found to induce toxicity in the Microtox® bioassay was on the same order of magnitude as the 15-min EC_{50} of the bioassay (McCloskey et al., 1996). This result thus emphasizes that the biological nature of the cell and/or physicochemical parameters of the medium should also be considered to properly assess the toxic potential of metal species.

The element Cu was toxic at 10^{-4} M with both bacterial-based bioassays but not with the photocyte bioassay. The Microtox® and photocyte bioassays had the same metal speciation for Cu. Thus, the difference in toxicity response may result from differences in biological processes leading to toxicity or from the difference between the two media in ionic strength, assuming that biological processes can be affected by the load of salt in the medium (Hall and Anderson, 1995; Hall et al., 1995a; 1995b; Cook et al., 2000). The *P. phosphoreum* bioassay also exhibited toxicity despite a different chemical speciation than the Microtox® bioassay and a low concentration of free Cu. Yet in this case, thermodynamic calculations predicted Cu precipitation under a hydroxide form. As precipitate was not observed over the time frame of the experiment, it is difficult to establish whether toxicity observed in this bioassay was from free ions and/or cupric hydroxides.

Thermodynamic calculations for equilibrium conditions at 25°C predicted Fe precipitation as a ferric hydroxide in all three bioassays. However, no precipitation was observed during the experiment, and therefore, in the absence of precipitate, Fe hydroxide complexes dominated the speciation in all three assays. Only the Microtox bioassay showed evidence of toxicity, suggesting that Fe hydroxides may be toxic species.

Toxicity Assessment Using the Microtox® Bioassay Has Poor Metal Specificity. The Microtox® and *P. phosphoreum* bioassays use two strains of the same bacterial species, *Photobacterium phosphoreum*. Strain 11177, used in the Microtox® bioassay, is highly sensitive to contaminants (http://www.azurenv.com) including metals (Codina et al., 1993; Ghosh et al., 1996). Indeed, the toxicity response of the *P. phosphoreum* Microtox® bioassay to Cr, Mn, and Zn contrasted with the lack of effect of the same metals with similar speciation on the wild strain of *P. phosphoreum*. In the present study, the Microtox® bioassay exhibited poor specificity to different metals, consistent with previous findings (Ross, 1993). According to the Microtox® bioassay results, all metals were toxic at high concentrations (10^{-4} and 10^{-3} M), whereas only Ag, Al, Hg, U, and Zn were toxic at lower concentrations. In contrast, for the *P. phos-*

phoreum bioassay, the wild strain showed decreased bioluminescence only to Ag, Cd, Cu, and Hg. Fe plays an important role in the regulation of growth and bioluminescence in *P. phosphoreum* (Haygood and Nealson, 1984, 1985a, 1985b). This element maintains a delicate balance between promoting growth, which has a stimulatory effect on light production, and repressing bioluminescence. Accordingly, the *P. phosphoreum* bioassay showed that bioluminescence and cell concentration decreased for lower Fe concentrations (10^{-8}–10^{-6} M) and increased for higher concentrations (10^{-5}–10^{-4} M). This pattern was not observed for the Microtox® bioassay strain. In fact, the effect of Fe was the opposite of that on the wild-type strain, with bioluminescence decreasing for higher concentrations of the metal.

Toxicity Depends on the Cell Concentration of the Bacteria. The *P. phosphoreum* bioassay showed that metals affected bacterial bioluminescence as well as growth. For Ag, the decrease in bioluminescence was simultaneous with the decrease in cell concentration. However, in general, bioluminescence was a more sensitive indicator than growth and decreased prior to the decrease in cell concentration, as observed for Cd, Cu, Hg, and Zn. The relation between cell concentration and bioluminescence in *P. phosphoreum* is supported by an elaborate physiological coupling (Hastings and Nealson, 1977; Haygood and Nealson, 1985b) that suggests consideration of both parameters simultaneously. Bacteria could indeed reach optimal bioluminescence and growth in responses to changes in the medium if allowed sufficient time.

This study showed that on exposure to Al, bioluminescence decreased throughout the 2-h duration of the Microtox® bioassay, corresponding to the cell generation time (Bulich and Bailey, 1995; Bulich and Huynh, 1995). For the *P. phosphoreum* bioassay, bioluminescence decreased and cell concentration increased for the first 2 h of metal exposure; however, the trend was reversed after 3 h of exposure, with an increase in bioluminescence and decrease in cell concentration, suggesting reduced Al toxicity for chronic exposure. Such variation of effects with time of exposure (viz., cell division rate) was also observed for Pb, U, and Zn. The use of long-term exposure to contaminants has been suggested as a way to better assess toxicity when using bacteria (Dillon, 1993; Thomulka et al., 1993; Newman and McCloskey, 1996; Thomulka et al., 1996; Backhaus et al., 1997; Froehner et al., 2000). Such a long-term exposure Microtox®-based bioassay has been developed, the Microtox® Chronic Toxicity test (Bulich and Bailey, 1995; Bulich and Huynh, 1995; Johnson, 1998; see also http://www.azurenv.com); the disadvantage of this bioassay is that it involves more time, expense, and specific skills for microbial work than the original Microtox® Acute Toxicity test.

The Microtox® Bioassay Can Overestimate Toxicity of Marine Samples. Free-metal-ion concentration can be compared for various media, including a freshwater medium

(McCloskey et al., 1996), the Microtox® bioassay medium (Newman and McCloskey, 1996), and an artificial seawater medium (the latter two used in this study). The proportion of free ions in the medium (ratio of free-ion concentration to total unspeciated concentration) followed the sequence: freshwater > Microtox® > artificial seawater. For most metals free-metal-ion concentration was about 1–2 times greater in freshwater than in the Microtox® bioassay medium; but it was, 10 times greater for Cd and 10^8 times greater for Hg. The free-metal-ion concentration of most metals was about 4 times greater in the Microtox® bioassay medium than in artificial seawater, except it was 40 times greater for Fe, 100 times greater for U, and 200 times greater for Al. Free-metal-ion concentration values calculated in this study for the Microtox® bioassay were similar to (only about 1.2 times higher) previous values for the same metals and bioassay (Newman and McCloskey, 1996), indicating that the speciation calculations were accurate. Chemical speciation of metals in solution is affected by ionic strength and other physicochemical parameters like salinity, pH, and calcium concentration of the medium (Brezonik et al., 1991; Ho et al., 1999; Cook et al., 2000). Therefore, assuming that toxicity is related to metal speciation, a toxicity assessment of a freshwater or marine sample might be biased when using the Microtox® bioassay, which has a NaCl-based medium (Villaescusa et al., 1996). This bioassay is known to overestimate the toxicity of metals more than do seawater-based nonmicrobial bioassays (Becerro et al., 1995; Ghosh et al., 1996). This could be because of the difference in speciation (i.e., fewer complexes in the Microtox® bioassay medium), but it also could be a result of the inherent sensitivity of Microtox® cells to metals. In addition, the Microtox® bioassay underestimates the toxicity of metals in freshwater relative to freshwater-based nonmicrobial bioassays (Ankley et al., 1990; George et al., 1995; Pardos et al., 1999; Radix et al., 2000). This also could be because of the difference in speciation (i.e., fewer complexes in freshwater).

Metal toxicity depends on multiple factors that cannot be integrated into a single bioassay, so it is generally accepted that a battery of assays should be used to better assess metal effects and cover the array of toxicity factors (Codina et al., 1993; Cronin and Dearden, 1993; Ross, 1993; Thomulka et al., 1996). Because of speciation issues as well as the inherent sensitivity of the Microtox® bioassay strain, it appears that it is not appropriate to use the Microtox® bioassay for assessing natural conditions of contamination and toxicity for metazoans (Dutka and Kwan, 1981; Radix et al., 2000).

The Photocyte Bioassay, Which Belongs to the Catalytic Group, Allows Assessment of the Physiological Effect of Metals Under Realistic Marine Conditions. The photocyte bioassay uses isolated light-producing cells from an osmoconforming invertebrate. Seawater is therefore the best medium in which photocytes can be maintained (Deheyn et al.,

2000a). The artificial seawater medium used in this study approaches the salt composition and physicochemical properties of natural seawater, which has an average ionic strength of 0.611 (0.511–0.650 range; 0.61 in the bioassay; Turner et al., 1981; Atkinson and Bingman, 1997).

Of the 11 metals tested, the photocyte bioassay showed toxicity for Cd, Cu, and Hg. These metals are also known to affect sea urchin fertilization and embryonic development at comparable concentrations (Kennish, 1997). The elements Cr, Zn, Al, and Fe inhibit sea urchin embryonic development (Pagano et al., 1996; Kennish, 1997) at concentrations that had no effect on photocytes. As the echinoderm embryonic development bioassay belongs to the noncatalytic group of toxicity assays, the photocyte–embryonic development toxicity discrepancy does not necessarily indicate the photocyte bioassay is the least sensitive; rather, it implies a difference in sensitivity for those metals between the pre- and post-metamorphic stages of development in echinoderms. Unfortunately, there are no published metal toxicity data for postmetamorphic echinoderms to allow appropriate comparison with the photocyte bioassay.

Metals are known to affect bioluminescence capacity in eukaryotic organisms (Okamoto et al., 1999; Deheyn et al., 2000b; Sudhaharan and Reddy, 2000), yet bioluminescence has been used as a proxy for metal toxicity only for dinoflagellates (Lapota et al., 1993; Heimann et al., 2002) and brittlestars (present study). The photocyte bioassay was more than 30 times less sensitive to Cd and Cu than the bioluminescence bioassay using the dinoflagellate *Pyrocystis lunula*, yet the two bioassays showed similar sensitivity to Pb (Heimann et al., 2002). In contrast, the photocyte bioassay showed sensitivity to Cu and Zn similar to that of the bioluminescence bioassay when using the dinoflagellate *Lingulodinium polyedrum* (formerly *Gonyaulax polyedra*; Lapota et al., 1993). Therefore, the photocyte bioassay has a sensitivity range comparable to that of other bioluminescence-based bioassays using eukaryotic cells.

CONCLUSIONS

The metal speciation in the original environment of the sample must be preserved in the bioassay to avoid over- or underestimation of toxicity. The use of specific strains of microorganisms is useful to detect low levels of a given element potentially bioavailable in the environment, but toxicity established by a strain may not extrapolate to toxicity for other biological systems.

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