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A DNA-BASED ASSAY FOR TOXIC CHEMICALS IN WASTEWATER

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Abstract—Chemical toxicants, particularly metal ions, are a major contaminant in global waterways. Live-organism bioassays used to monitor chemical toxicants commonly involve measurements of activity or survival of a freshwater cladoceran (*Ceriodaphnia dubia*) or light emitted by the marine bacterium *Vibrio fischeri*, used in the commercial Microtox® bioassay. Here we describe a novel molecule-based assay system employing DNA as the chemical biosensor. Metals bind to DNA, causing structural changes that expel a bound (intercalated) fluorescent reporter dye. Analyses of test data using 48 wastewater samples potentially contaminated by metal ions show that the DNA-dye assay results correlate with those from *C. dubia* and Microtox bioassays. All three assays exhibit additive, antagonistic, and synergistic responses that cannot be predicted by knowing individual metal concentrations. Analyses of metals in these samples imply the presence of chemical toxicants other than metal ions. The DNA-dye assay is robust, has a 12-month shelf life, and is only slightly affected by sample pH in the range 4 to 9. The assay is completed in a matter of minutes, and its portability makes it well suited as a screening assay for use in the field. We conclude that the DNA-dye test is a surrogate bioassay suitable for screening chemical toxicity. Environ. Toxicol. Chem. 2011;30:1810–1818. © 2011 SETAC

Keywords—Bioassays Chemical toxicants Biosensor *Ceriodaphnia dubia* Microtox®

INTRODUCTION

Bioassays for toxic metal ions and organic molecules commonly involve small live organisms that sense chemical toxicants. Survival and motility of the freshwater cladoceran *Ceriodaphnia dubia* [1–4] can be monitored using serial dilutions of a test water sample. However, this assay requires a continual supply of juvenile (<24 h old) animals, is labor intensive, and is relatively slow (e.g., 1–3 d to yield a result). Another widely used assay (Microtox®; JW Industrial Instruments) monitors changes in bioluminescence of a marine bacterium (*Vibrio fischeri)* [5] in response to chemical toxicants. Here, a reduction in luminescence provides the measure of toxicity.

The molecular basis of the *C. dubia* assay is unknown, but the mechanism regulating *V. fischeri* luminescence appears to involve several proteins, transcription regulators, and cAMP [6]. These bioassays do not identify individual toxic chemicals but sense the total toxicity resulting from all toxicants present in a sample.

Surrogate bioassays for toxic chemicals have been reported using ubiquitous structural proteins [7] and enzymes [8], but they are not ideal biosensors for several reasons. They normally must be stored refrigerated, frozen, or freeze dried; they are not robust (are very sensitive to variations in temperature and pH); and they have short shelf lives even when refrigerated (4°C).

Ideally, bioassays should be simple to perform. They should be very sensitive to toxic metals (in the parts per billion range) but be largely unaffected by nontoxic elements (e.g., Na, K, Ca), or by the normal range of pH and temperature. They should give a virtually instant result, and if possible the assay should be easily portable (pocket-sized, with reasonable power demands) for use at a potentially toxic site.

This report is the first to describe the use of DNA as a chemical sensor. A fluorescent reporter dye bound to DNA senses changes in DNA conformation in the presence of complex toxicants, particularly heavy metal ions. This DNA-dye assay is designed to be technically simple (add test water sample to 2 ml diluted DNA-dye, mix, and measure the fluorescence intensity using a hand-held fluorimeter; see *Materials and Methods*) and has many of the above-mentioned characteristics. We characterize the sensitivity of the assay against 16 common toxic metal ions as well as their toxicities when measured in paired combinations.

MATERIALS AND METHODS

DNA-dye assay

The assay is performed as follows: 2.0 ml of MilliQ® (Millipore) water is dispensed into a disposable polystyrene fluorescence cuvette (Starstedt), and 20 µl of HazardScreen® (www.hazardscreen.com) test mix containing DNA-dye is added. We used two variants of the DNA-dye assay (HAZ-1 and HAZ-2) that contained different sources of genomic DNA but used the same fluorescent reporter dye. The cuvettes were capped (Starna), mixed, and used within one week. Progressive

All Supplemental Data may be found in the online version of this article.

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serial additions up to 1.0 ml of potentially toxic environmental test sample cause a decrease in sample fluorescence in proportion to its toxicity. Fluorescence intensity was measured using either a Turner Designs Picofluor or a HazardScreen fluorimeter. These instruments were calibrated according to the manufacturer's instructions. Briefly, a DNA-dye calibrant was prepared by adding 2 ml MilliQ water to a cuvette; then, 20 μl DNA-dye was added, and the cuvette was capped and mixed by inversion. A blank cuvette was made using 2 to 3 ml MilliQ water only (no DNA-dye) to set the minimum fluorescence intensity to zero. The hand-held fluorimeter was switched on, and the CAL button was pressed. At the prompt, the water (blank) was inserted and read. This set the blank to zero arbitrary units (AU). Then, the diluted DNA-dye calibrant was read, which set the maximum fluorescence intensity to 1,000 AU. The calibration step is needed only once per day.

Titration of wastewater samples involved progressive addition of up to 1.0 ml sample to 2.0 ml MilliQ water containing 20 μl DNA-dye. Fluorescence intensities were corrected for the volume of added test sample. Sample measurements were monitored five or six times over 60 s, and toxicity determinations were repeated three to five times with typical errors of ±2%.

Toxicity levels were set as follows: nontoxic (NT) samples fall within the range 1,000 to 800 AU (determine by the range of sample intensities in the pH range 4–9; see Supplemental Data, Fig. S1); extremely toxic (ET) samples fall in the range 200 to 0 AU (achieved using standardized 1 mg/L Hg). Fluorescence intensities between 800 and 200 AU are divided into three 20 percentiles: 800 to 600 AU is slightly toxic (ST), 600 to 400 AU is toxic (T), and 400 to 200 AU is very toxic (VT).

When the HazardScreen assay is used in the field, it is usually inconvenient progressively to add up to 1.0 ml of test sample to the 2.0 ml of the above-mentioned calibrant. In this case, the fluorimeter was calibrated to 1,000 AU using 3.0 ml MilliQ water and 20 μl DNA-dye. Later that day, in the field, 1 ml of water sample was added, and the fluorescence was read directly without having to allow for the volume change. Readings that declined from 1,000 to below 800 AU were repeated and treated as potentially toxic.

Levels of toxicity for the DNA-dye assays were assessed against standardized solution of Hg(II), in which 1 mg/L achieves a fall in fluorescence intensity from 1,000 to approximately 200 AU with concentrations measured by inductively coupled plasma-mass spectrometry (ICP-MS). The concentrations of other metal ions were measured by ICP-MS or inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

Collection of wastewater samples

In total, 48 samples were collected by field inspectors from the New South Wales Department of Environment Climate Change and Water (DECCW) in the course of DECCW's legislative activities and stored in the dark at 4°C. These were supplied in two batches (39 + 9) deidentified samples. They were sourced from mining and industrial effluent sites, chemical spills, and other sites that were suspected sources of toxicants.

Ceriodaphnia dubia and Microtox assays

Samples were first tested using the *C. dubia* assay [2] recommended by DECCW and then subsequently using the Microtox acute toxicity test [4]. They were then tested by the DNA method at The University of Sydney without prior knowledge of the *C. dubia* or Microtox results. Both assays were performed in quadruplicate using hexavalent chromium as a reference toxicant.

Samples with intrinsic fluorescence

Some environmental wastewater samples do exhibit intrinsic fluorescence, which can potentially interfere with the method described here. Among the 48 samples provided by DECCW, 30 were intrinsically fluorescent (autofluorescent). This was tested by adding 2 to 3 ml of sample to a new cuvette (no DNA-dye was added) and measuring the fluorescence intensity in a calibrated fluorimeter. None of the authors was involved in the selection or collection of the water samples used in the present study. Field inspectors of the DECCW collected samples. The autofluorescence emission spectrum overlapped the DNA-dye fluorescence emission.

The toxicity of weakly autofluorescent samples can be assessed from the rapid decline in DNA-dye fluorescence (see Supplemental Data, Fig. S1), followed by an increase in intensity resulting from the autofluorescence. However, other samples were so intensely fluorescent that no initial decrease could be observed. Accordingly, all autofluorescent samples were treated with 35% H_2O_2 as an oxidative quencher. H_2O_2 was subsequently hydrolyzed to H_2O by boiling the sample, and then any residual peroxide was hydrolyzed using a minimum amount of catalase, because we observed that this enzyme binds some metal ions. Final sample fluorescence intensities were adjusted for volume changes caused by adding H_2O_2 and catalase.

Nuclear magnetic resonance spectroscopy

Calf thymus DNA type 1 (Aldrich) was dissolved in D_2O (12.0 mg/ml). Mercuric acetate was added to half of the DNA sample to achieve concentrations of 13.5 mM. Samples were stored in nuclear magnetic resonance (NMR) tubes at 45°C for 6 h and then at 4°C until required; NMR spectra (512 increments) were acquired at 25°C on a Bruker Avance III 600. The residual HDO signal was suppressed.

Analysis of metals

The mass concentrations of metals in all samples were measured by ICP-MS or ICP-AES at ANSTO and/or at the School of Chemistry, University of New South Wales. Metals listed in Supplemental Data, Table S1, were measured using three replicates for each sample. A set of standards was analyzed at the beginning, after every tenth sample, and at the end, as controls to monitor for instrument drift. Standards for instrument calibration and checks were prepared by dilution with 5% v/v sub-boiled HNO_3 from 1,000 mg/L (ppm) certified standards, which is directly traceable to the National Institute for Standards and Technology. Environmental water samples as well as stock solutions of metals used for bioassays were diluted and rerun if concentration of the metal ions was outside the calibration range. Samples were analyzed by inductively coupled plasma techniques, mass spectrometry (ICP-MS; Agilent 4500 Quadrupole) or atomic emission spectroscopy (ICP-AES, Varian VISTA AX CCD). The ICP-MS was calibrated using standards from 1 to 500 mg/L spiked with Li, Sc, Y, Rh, In, Lu, Bi, and As internal standards. The ICP-AES was calibrated using a series of standards from 0.01 to 100 mg/L. A set of standards was analyzed after every tenth sample to allow for instrument drift. Metal ion solutions used in this report were standardized using the above methods.

RESULTS

Testing environmental water samples

As mentioned above, none of the authors was involved in the selection or collection of the water samples used in the present study. The results of the DNA-dye assays are shown in Figure 1. Here, small volumes of a test sample were progressively added to 2 ml diluted DNA-dye test mix (HAZ-1 or HAZ-2; see *Materials and Methods*) and the fluorescence intensities were measured. These curves were corrected for volume changes resulting from the added test sample and, when necessary, for changes introduced by the removal of intrinsic fluorescence (see *Materials and Methods*). Sample codes were assigned by the DECCW, and the results were classified in 20 percentiles according to the toxicities determined by a standardized Hg solution. Each curve is the mean of at least two titrations. The average median effective concentration (EC50) value for HAZ-1 for Hg (described in Table 1) was 0.12 μM (50 μg/L).

The DNA-dye test solution was stored at 4°C and protected from light to avoid photobleaching as recommended by the manufacturer and used within 12 months of the expiry date provided with each DNA-dye batch. We found that diluted samples of DNA-dye (20 μl in 2 ml MilliQ water) are stable at room temperature for at least 24 h and for 5 to 7 d at 4°C if stored in the black storage tube provided in the HazardScreen kit. Samples that were tested for toxicity can be retained for remeasurement for up to 24 h. The concentrated DNA-dye samples remained stable over 12 to 15 months within a narrow range (±10%) provided that they were protected from light and refrigerated (data not shown).

The DNA-dye assay results were stable over the range of pH conditions normally encountered in environmental samples. Fluorescence intensity (AU) decreased from 1,000 AU to not less than 800 AU over the pH range 4.8 to 9.2 (Supplemental Data, Fig. S1). The top figure shows triplicate titrations commencing at pH 8 using HAZ-1. The lower curve illustrates high- and low-pH titrations commencing at pH 5 using HAZ-2. Thus, both DNA-dye samples exhibited a wide range of pH stability. With the exception of extreme events such as acid spills, it is unusual for environmental samples to fall outside of this pH range, and those that do were toxic to the bioassays reported here (e.g., see column 3, Table 1).

The test samples exhibited biotoxicities (Table 1) ranging from NT through ST to T, VT, or ET. Three samples (7019, 7025, and 7026; see Fig. 1) were so toxic that they had to be diluted $>10^6$-fold with MilliQ water before fluorescence intensities returned to the nontoxic toxic range. Data from these samples are virtually coincident with the ordinate of Figure 1.

All of the autofluorescent samples were titrated into 2 ml MilliQ water containing 20 μl DNA-dye (Supplemental Data, Fig. S2), and the total fluorescence intensity was measured. Some samples exhibited a strong autofluorescence that effectively swamped the DNA-dye signal (e.g., 7005 and 7035 labeled in Supplemental Data Fig. S2) without exhibiting an initial decrease. Others produced a large initial intensity fall resulting from the effects of toxicants on the DNA-dye followed by a rise (7004 and 7006 in Supplemental Data Fig. S2) resulting from the autofluorescence. When this autofluorescence was quenched using H_2O_2 (see *Materials and Methods* and Fig. 1), most samples (indicated with an asterisk in Table 1) tested as T or worse. Thus, for most autofluorescent samples, toxicities can be estimated from the initial slopes of their titration curves. Given that all but six of the 31 autofluorescent water samples tested as toxic, it is probably wise to screen water samples routinely for autofluorescence in the absence of DNA-dye using a calibrated fluorimeter.

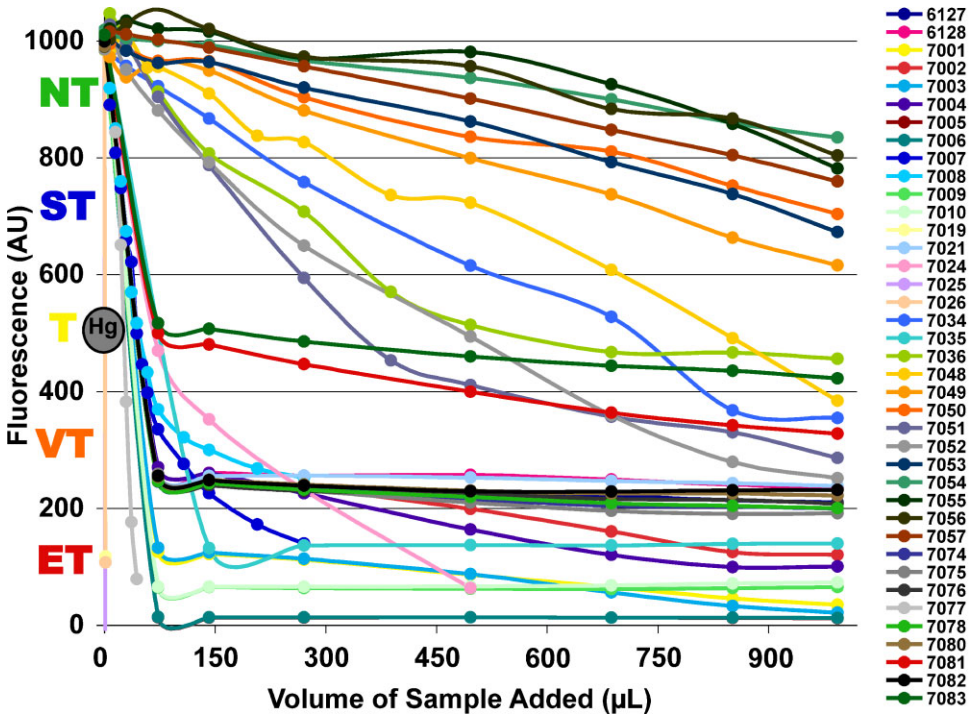

Fig. 1. Effects of the test samples on the fluorescence intensity of DNA-dye (HAZ-1) titrated as a function of the volume fraction of the sample. Intensities are corrected for volume changes from sample addition or the quenching of autofluorescence (see *Materials and Methods*). Sample toxicities are defined as nontoxic (NT), slightly toxic (ST), toxic (T), very toxic (VT), or extremely toxic (ET) based on reductions in DNA-dye fluorescence intensities. Hg in the circle indicates the fluorescence obtained with 0.05 ppm Hg.

Table 1. Toxicity data for 48 water samples determined by the HAZ-1 DNA-dye assay, *Ceriodaphnia dubia* assay, and Microtox® assays (JW Industrial Instruments)[a]

DECCW sample code	pH (toxic pH italicized)	ICP-MS/ICP-AES data identified toxic metals (toxicity is based on EC50 data [see Supp. Data, Fig. S3 (ET > VT > T > ST > NT)]	DNA-dye (<1 min) assay		*Ceriodaphnia dubia* (48 h) acute assay		Microtox (15 min) acute assay	
			Toxicities: ET > VT > T > ST > NT EC50 (dilution for nontoxic result)	Rank order of toxicity	Toxicities: ET > VT > T > ST > NT EC50 (undiluted toxicity %)	Rank order of toxicity	Toxicities: ET > VT > T > ST > NT EC50 (% sample)	Rank order of toxicity
6127*	6.1	NT	VT 3.5	18	NT (0)	30	ST (HE 8)	26
6128*	7.2	NT	VT 3.5	18	NT (0)	30	T (HE 30)	25
7001*	5	NT	ET 1.5	5	NT (0)	30	NT (0)	31
7002*	8.4	NT	VT 3.0	11	NT (0)	30	ST (HE 8)	26
7003*	5	NT	ET 1.5	5	NT (5)	30	NT (0)	31
7004*	7.1	NT	VT 3.0	11	ST (30)	28	T (HE 33)	24
7005*	7.5	NT	ET 1.5	5	VT 6.5 (100)	8	T 102(HE46)	16
7006*	8.2	ST (Cr)	ET 1.5	5	VT 3.0 (100)	6	T 38	23
7007	7.6	NT	VT 3.0 ($\times 10^3$)	11	NT (0)	21	NT (0)	31
7008	8.1	NT	VT 3.0 ($\times 10^3$)	11	NT (0)	21	NT (0)	31
7009*	6.8	T (Cu)	ET 1.5	5	ET 0.03 (100)	4	ET 1.3	4
7010*	7.7	T (Cu)	ET 1.5	5	ET 0.04 (100)	5	ET 0.8	3
7019	*3.7*	ET (Al > Cu > Cr > Hg > Mn)	ET $<5 \times 10^{-6}$	1	ET 1.3×10^{-5} (100)	1	ET 1.6	5
7021*	9.2	ST (Ba)	VT 3.5	18	T 84 (95)	24	ST 421 (HE25)	21
7024*	8.4	NT	VT 3.0	11	T 94 (100)	25	ST 892 (HE14)	22
7025	*0.1*	ET (Al > Fe > Cr > Cu > Mn > La)	ET $<5 \times 10^{-3}$ ($>10^5$)	1	ET 4.7×10^{-3} (100)	2	ET 0.05	1
7026	*2.6*	ET (Cu > Mn > Cr > Fe)	ET $<5 \times 10^{-4}$ ($>10^4$)	1	ET (100)	3	ET 2.6	6
7034	7.3	NT	ST 26 (×10)	34	NT (0)	21	NT (0)	31
7035*	6	NT	VT 3.0	11	VT 5.5 (100)	7	T 209 (HE32)	19
7036	7.1	NT	T 18 (×10)	30	NT (0)	30	ST 231 (HE24)	20
7048	7.3	NT	ST 28 (×10)	35	T 50 (90)	17	ST 207 (HE28)	18
7049	7.4	NT	NT > 33 (nil)	36	T 57 (95)	19	NT (0)	31
7050	7.7	NT	NT > 33 (nil)	36	T 57 (95)	19	NT (0)	31
7051	6.8	T (La)	T 12 (×100)	28	T 17 (95)	9	NT (0)	31
7052	6.7	VT (La)	T 16 (×100)	29	T 17 (95)	9	NT (0)	31
7053	8.2	NT	NT > 33 (nil)	36	NT (5)	30	ST (HE 6)	28
7054	8.1	NT	NT > 33 (nil)	36	T (70)	26	NT (0)	31
7055	7.9	NT	NT > 33 (nil)	36	ST (30)	28	ST (HE 6)	28
7056	7.9	NT	NT > 33 (nil)	36	T (0)	27	NT (0)	31
7057	7.8	NT	NT > 33 (nil)	36	NT (0)	30	ST (HE 5)	30
7074*	8.1	NT	NT 3.5	18	T 19	12	T 47	12
7075*	8.5	ST (Mn > Cu)	VT 3.5	18	T 19	12	T 53	13
7076*	8.3	ST (Cu)	VT 3.5	18	T	30	T 89	15
7077	5.9	ET (Mn > Fe > Ba)	ET 1.0 ($\times 10^3$)	4	T 17	9	VT 3.8	7
7078*	7.5	NT	VT 3.5	18	T 35	14	VT 6.1	8
7080*	8	NT	VT 3.3	17	T 37	15	VT 8.4	9
7081*	7.3	NT	T 10	27	NT	30	T 25	11
7082*	6.8	NT	VT 3.5	18	T 37	15	VT 8.4	9
7083*	7.4	NT	T 18	30	NT (0)	30	T 108 (HE50)	17
10010*	7.8	NT	NT > 33 (nil)	36	NT <50 (×5)	30	NT NC	31
10012*	8.3	NT	NT > 33 (nil)	36	NT	30	T 79	
10013*	8.1	NT	T 9.5	26	T <50 (×5 < 50)	30	NT NC	31
10014*	8.0	NT	T 18	30	NT	30	ET 0.28	2
10016*	8.1	NT	NT > 33	36	NT <50 (×5)	30	NT NC	31
10017*	8.0	NT	NT > 33	36	NT	30	NT NC	31
10018*	8.0	NT	NT > 33	36	NT	30	NT NC	31
10019*	8.2	NT	T 24	33	T 55	18	NT NC	31
10020*	8.1	NT	NT > 33	36	NT	30	T 87	14

[a] Column 1 shows sample codes provided by the New South Wales Department of Environment Climate Change and Water (DECCW); asterisk indicates that the water sample was intrinsically fluorescent. Column 2 shows that pH values outside of pH 4 to 9 were toxic. Column 3 lists ICP-MS and ICP-AES metal levels that exceed the DECCW-permitted levels in wastewater (see Fig. 1 legend). Column 4 shows EC50 values for the DNA-dye assays (see Fig. 1). Parenthetical numbers show dilutions needed for nontoxic levels. Columns 5, 7, and 9 show rank samples in increasing toxicity by the DNA-dye method, *Ceriodaphnia dubia*, and Microtox, respectively. Column 6 shows the *C. dubia*-determined toxicities (NT through ET) based the EC50 values of undiluted samples. Dilution factors for VT samples are in parentheses. Column 8 shows the Microtox (NT to ET) EC50 data; parenthetical numbers indicate percentage immobilization in undiluted samples. NC = luminescence decrease was <50%; ICP-MS = inductively coupled plasma-mass spectrometry; ICP-AES = inductively coupled plasma-atomic emission spectroscopy; NT = nontoxic; ST = slightly toxic; T = toxic; VT = very toxic; ET = extremely toxic.

The ICP-MS and/or ICP-AES analyses (see *Materials and Methods*) were used to determine the total concentrations of 16 elements (Ag, Al, Ba, Cd, Co, Cr, Cu, Fe, La, Hg, Mn, Ni, Pb, Sn, U, and Zn) in all 48 samples. Only those metals that exceeded the Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ) and the Australian and New Zealand Environment and Conservation Council (ANZECC) [9] are listed in column 3 of Table 1 (complete ICP analyses are provided in Supplemental Data, Table S1). All three assays correctly identified as toxic the 12 samples that contained toxic levels of identified metal ions (note that Microtox did not detect Ba in sample 7021). In addition, the DNA-dye

Table 2. Summary of EC50 values for DNA-dye (HAZ-1, HAZ-2), Microtox® (JW Industrial Instruments) and *Ceriodaphnia dubia* assays for 19 metal ions for drinking and wastewater[a]

Metal	HAZ-1 EC50 (ppm)	HAZ-2 EC50 (ppm)	Microtox 15 min EC50 (ppm)	*Ceriodaphnia dubia* (24 hr) EC50 (ppm)	Drinking water levels[b] (ppm)	Wastewater levels[c] (ppm)
Hg(II)	0.050	0.0126	0.034 [11]	0.010	0.001	0.010
Cr(III)	0.261	0.0135	0.178 [11]	NA	0.05	80
Co(II)	0.921	0.0136	141	3.7	0.05	4.6
Cu(II)	0.106	0.0137	0.007 [11]	0.07	2.0	65
Al(III)	0.733	0.0163	NA	NA	0.05–0.2	100
La(III)	0.366	0.0418	550	0.043[d]	0.02	0.08
Ag(I)	0.055	0.104	0.008 [11]	0.003	0.10	1–3.7
Sn(II)	ND	0.207	NA	NA	0.7	700
U(VI)	0.86	0.324	NA	0.07	0.02	1.43 [30]
As(III)	NS	0.347	0.82 [29]	NA	0.007	20
Ni(II)	7.67	0.409	0.265 [11]	0.07	0.02–0.1	21
Mn(II)	40.7	0.702	274	42	0.5[e]	100
Cr(VI)	95.7	0.758	0.012 [11]	NA	NA[e]	80
Zn(II)	61.8	1.12	0.074 [11]	0.21	5.0	200
Cd(II)	33.4	1.37	0.504 [11]	0.18	0.002[c]	1.5–10
Fe(II)	2.02	1.4	NA	33	0.3	100
Pb(II)	45.9	1.7	0.669 [11]	0.14	0.05[d]	50
Ba(II)	200	12.8	24,946	NA	0.7c–1.0[d]	1.0[d]
V(V)	ND	69.5	NA	NA	0.02	32.5 [31]

[a] NA = not available; ND = not determined; NS = not sensitive.
[b] http://www.nhmrc.gov.au/publications/synopses/eh19syn.htm; Australian Drinking Water Guidelines, 8–15 and 10–22.
[c] http://www.mincos.gov.au/publications/australian_and_new_zealand_guidelines_for_fresh_and_marine_water_quality.
[d] No health-based guideline is considered necessary for this metal.
[e] http://www.epa.gov/safewater/contaminants/index.html#inorganic.

assay detected toxicity in 19 of the 39 samples in the initial sample batch (nine more were subsequently supplied and examined) that did not contain toxic levels of metal ions (as assessed by ICP-MS or ICP-AES Table 1, column 3). These samples are identified in Table 1 (column 4) as slightly toxic (ST; 7034, 7048), toxic (T; 7036, 7081, 7083, 10013, 10014, 10019), very toxic (VT; 6127, 6128, 7002, 7004, 7007, 7008, 7035, 7078, 7080, 7082), or extremely toxic (ET; 7001, 7003, 7005) by the DNA-dye assay (Table 1, column 3, see also Table 2). All but four (7007, 7034, 7036, 7048) of these had significant intrinsic fluorescence. The *C. dubia* and/or Microtox assessments agree with all but six of the 19 nonmetal toxic samples. Five of 48 samples uniquely tested as toxic (ST to ET) with the DNA-dye assay, four uniquely tested ST or worse using *C. dubia,* and four tested ST using Microtox. Almost half (18 of 48) of the samples were reported as toxic by all three assays. The data are summarized in Figure 2.

Although all of the assays correctly identified toxic metal ions or acidic pH (<4.0), only the DNA-dye produced no false-negative data. These are defined as a nontoxic (NT) result by one assay in the presence of toxic (ST-ET) results by both other assays. Seven and five false-negative results were produced by *C. dubia* and Microtox, respectively (Table 1). Thus, although there is broad agreement between the assays, the variable nature of bioassays suggests that more than one biological assay should be used. Given these data, the DNA-dye test should be combined with at least one of the other two bioassays. The present investigation is concerned primarily with metal ion toxicities, but it is clear from Table 1, column 3, that many of the wastewater samples are toxic as a result of toxicants other than metal ions.

Comparisons of DNA-dye, C. dubia, and Microtox data

Table 1 lists the results for the three assays (DNA-dye, *C. dubia*, and Microtox) using identical water samples. Toxic metal ions are identified in column 3. The assay data in columns 4, 6, and 8 do not reveal the concentrations of toxicants; rather, they list the EC50 values (the volume of test sample required to produce a 50% decrease in fluorescence [DNA-dye], activity [*C. dubia*], or luminescence [Microtox]). Columns 5, 7, and 9 rank the toxicities for each assay. Toxicity is coded (NT, ST, T, VT, and ET) and reflects the 20-percentile bands used in Figure 1. Note that, the lower the EC50 value, the greater the toxicity. Where an EC50 value could not be determined for the Microtox assays, the highest percentage effects (HE) was used (large HE values are highly toxic). Toxicity levels determined by the DNA-dye assay agree well with data from

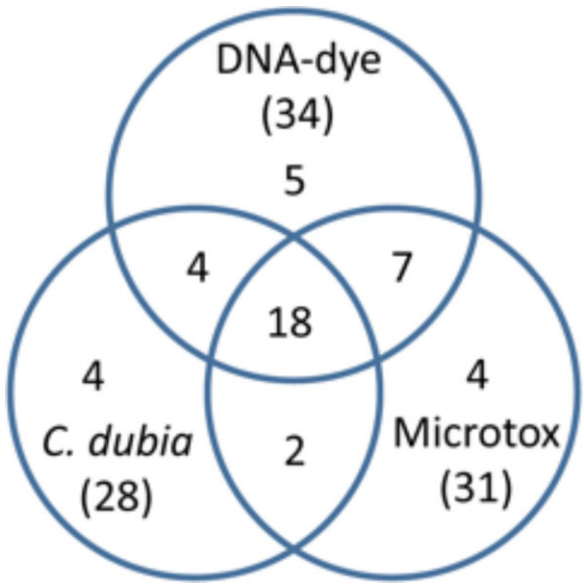

Fig. 2. Venn diagram illustrating the numbers of samples recorded as toxic (everything from slightly toxic to extremely toxic) for the HAZ-1 assay, the *Ceriodaphnia dubia* assay, and the Microtox® assay (JW Industrial Instruments). Four samples tested nontoxic by all three methods. The total number of toxic samples identified by each of the assays is shown in parentheses. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

Table 3. Values for the linear correlation coefficients (log [EC50]) for DNA-dye (HAZ-1), Microtox® (JW Industrial Instruments) and *Ceriodaphnia dubia assays* (linear correlations are illustrated in Figure 3)

	DNA-dye	*C. dubia*	Microtox
DNA-dye	1	0.87	0.75
C. dubia	0.87	1	0.70
Microtox	0.75	0.70	1

C. dubia (22 of 40 samples or 55%) and Microtox (25 of 40 samples or 63%). For water samples screened by all three methods, the Pearson's linear correlation coefficients (r) between log (EC50) values are given in Table 3. The correlation between the DNA-dye and *C. dubia* data was significant ($\alpha = 10^{-7}$). Different screening methods tend to interact differently with complex toxic samples [10], but the stronger correlation between DNA-dye and *C. dubia* implies that the mechanisms of metal disruption of DNA structure and acute toxic effects of metals on *C. dubia* produce very similar data.

Two hypotheses were tested. First, the log (EC50) results for DNA-dye correlate with those of Microtox and *C. dubia*, and, second, the log(EC50) results for DNA-dye can be modeled by measured concentrations of metals in the test samples. Determination of the Pearson's linear correlation coefficient (r) between log(EC50) values for the three data sets (Table 3) shows that the correlation between the values for the DNA-dye assay and the *C. dubia* data was significantly better ($a = 10^{-7}$) than that for the DNA-dye and Microtox data.

Different screening methods when presented with complex toxic samples produced different EC50 values even when they involved related animals such as *Ceriodaphnia dubia* and *Daphnia magna* [10]. However, the agreement between the data from DNA-dye and *C. dubia* assays implies that the mechanisms of disruption by a metal ion of the dye binding in DNA and acute toxic effects of metals in the test organism may be similar.

Because some of the metal concentrations correlated with the bioassay data, a principal-components model was built with 16 metal concentrations as variables. Seven principal components explained 98% of the variance of the log (EC50) values. The scores of these seven principal components were regressed against log (EC50) values for each method. Figure 3 shows the modeled data for the three assay sets. Both DNA-dye (r^2 between calculated and measured log [EC50] = 0.92) and *C. dubia* ($r^2 = 0.96$) data fitted well, but Microtox modeled less well ($r^2 = 0.66$). However, this conclusion might not hold true if the sample size is significantly increased.

Metal ion titrations

Environmental samples, particularly those associated with mining operations, commonly contain several metal ions, but each may exert a different toxicity. Supplemental Data, Figure S3, illustrates the means of triplicate titration curves for 16 metals (chosen because they are common contaminants of wastewater) using the DNA-dye preparation HAZ-1. Increasing metal toxicities were assessed by their EC50 values provided in Table 3 for two DNA-dye preparations (HAZ-1 and HAZ-2). The DNA-dye assays yielded the following order of decreasing toxicities: HAZ-1: Hg(II) $\sim$ Ag(I) > Cu(II) > Cr(III) > La(III) > Al(III) > U(II) > Co(II) > Fe(II) > Ni(II) > Cd(II) > Mn(II) > Pb(II) > Zn(II) > C r(VI) > Ba(II). HAZ-1: Hg(II) $\sim$ Cr(III) $\sim$ Co(II) $\sim$ Cu(II) $\sim$ Al(III) > La(III) > Ag(I) > Sn(II) > U(VI) $\sim$ As(III) $\sim$ Mg(II) $\sim$ Ni(II) > Ca(II) > Mn(II) $\sim$ Cr(VI) > Sr(II) $\sim$ Zn(II) $\sim$ Cd(II) $\sim$ Fe(II) $\sim$ Pb(II) >> Ba(II) >> V(V).

The three most toxic metals for HAZ-1 (Hg $\sim$ Ag > Cu) are also the most toxic for Microtox [11], *C. dubia* [12], and other organisms (*Hyalella azteca* [13], *Oncorhynchus mykiss* [14]) although the order of less toxic metals varies substantially between organisms, as does the order for *C. dubia* and Microtox. Incident Screen, the company that produces the HazardScreen® DNA-dye samples, did not allow us to release the precise differences between HAZ-1 and HAZ-2 formulations other than to state that they contained different sources of DNA. These DNA-dye preparations display similar toxicity rankings. HAZ-2 is from four- to 120-fold more sensitive than HAZ-1, except for La(III) and Ag(I) (Table 3). HAZ-1 was not sensitive to As, whereas the EC50 for As using HAZ-2 was 0.347 mg/L. Three metal ions (Sn, As, and V) were not tested using HAZ-1. The higher sensitivity of HAZ-2 to metal ions (Table 3) suggests that this preparation may be useful for testing drinking-quality water. These and other differences are of considerable interest and will be the subject of a separate report.

DNA has been known for nearly 50 years to bind metal ions [15], and Hg has also been known to separate the strands of DNA [16], alter its structure [17], and hence cause the release an intercalating dye. Nearly 30 years ago, Richardson et al. [18] used fluorescence polarization to demonstrate that metal ions displaced acridine orange (a carcinogenic fluorescent reporter

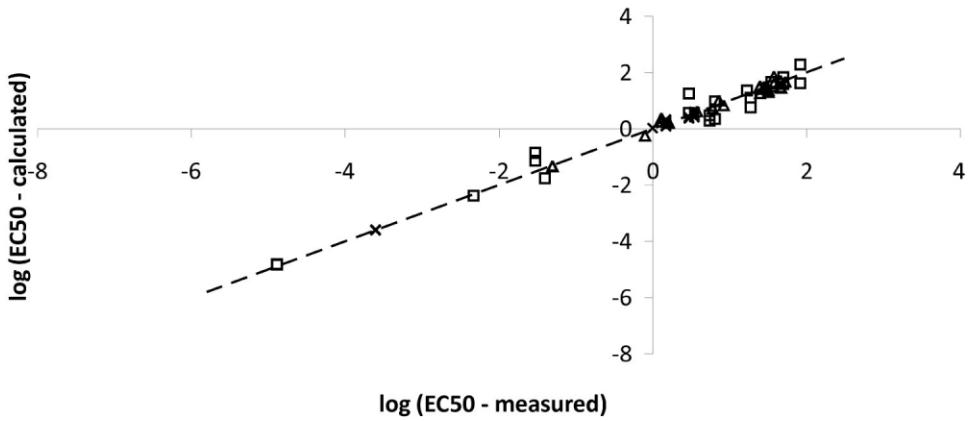

Fig. 3. Plot of calculated log(median effective concentration [EC50]) against measured log (EC50) for regression models with seven principal components. Squares = DNA-dye; crosses = *Ceriodaphnia dubia*; circles = Microtox® (JW Industrial Instruments, Australia). The solid line represents calculated versus observed log (EC50) values.

dye) bound to calf thymus genomic DNA, but they failed to recognize its potential as a sensor of toxic metal ions. This was probably because their DNA-dye (acridine orange) system was much less sensitive to metal ions than the HazardScreen system. For example, their EC50 for Cu(II) was 20 μM (1268 ppb) compared with the DNA-dye values of 1.67 μM (105 ppb) and 0.216 μM (13.7 ppb) for HAZ-1 or HAZ-2, respectively. Furthermore, although the order of metal toxicities reported by Richardson et al. [18; Cu(II) > Fe(II) > Ni(II) > Cd(II) > Zn(II) > Co(II) > Mn(II) > Pb(II)] was similar to that of HAZ-1, the calf thymus DNA-acridine orange was so insensitive that it would not have been useful as a sensor for heavy metal ions even by wastewater standards.

Binary combinations of heavy metal ions

An important difference between bioassays and mass spectrometric analyses of metal ions is that the former can exhibit synergistic or antagonistic effects that are not predicted by the latter. In environmental water, a metal ion rarely occurs [19] alone (Table 1), so we, like others [20–25], examined the toxicities of pairwise combinations of 16 metal ions (Fig. 4). When two toxic metals simply have an additive effect, they probably have common targets and similar modes of action. When the EC25 concentrations (metal concentrations needed to produce a 25% decrease in fluorescence) of two metals are combined, they might produce a total toxicity equal to the sum of their EC25 values (±2σ). For example, mixtures of Hg with Cr, Hg with Zn, Cu with Co, Cu with Al, and Cr with Zn were all simply additive [22].

However, some metal ion combinations appear to be less toxic than predicted from the sum of their average EC25 values (±2σ). For example, Co with Pb, Co with Zn, Cr with Co, and Cr with Cu all exhibited this antagonistic feature. However, some metal combinations are more toxic than predicted from their sum; i.e., they are synergistic. For example, the combinations of Cu with Hg or Cd with Al were more toxic than the sum of their average EC25 values, and a few combinations had more than twice (±2σ) their predicted toxicities. Figure 4 summarizes these findings. Nearly half the combinations (44%) were additive, 35% antagonistic, and 21% synergistic, including 9% that were highly synergistic.

Hg(II) binding to DNA

Mercury is one of the most toxic metal ions [26]. It affects the conformation or structure, or both, of DNA depending on its concentration. Mercury binds preferentially to DNA bases with the relative affinities T > G >> A, C [26], significantly reducing DNA viscosity [17], which can be explained by induced changes in the DNA helix [15]. This leads to a fourfold increase in the NMR signal, presumably because of the spin–spin T_2 relaxation being inversely related to the solution viscosity [27].

Adding Hg(II) to calf thymus genomic DNA produces a very broad ^{1}H NMR signal, but it is still possible to observe shifts in the spectrum. Variations in the aromatic or heterocyclic region of the spectrum (see Supplemental Data, Fig. S4, chemical shift in the 5.5–8.5 ppm region) are consistent with mercury binding to DNA bases. The broadening (up per curve) is associated with a reduction in motion at these sites compared with the region below 5 ppm. This supports the suggestion that mercury binds to DNA base pairs.

DISCUSSION

Live animal bioassays such as the *C. dubia* motility assay require expensive infrastructure to ensure a continuous supply of the sensitive immature organisms, involve lengthy (1–3 d) assay times, and are labor intensive. This assay does not lend itself to use in the field.

The Microtox assay kit is based on batches of freeze-dried marine bacteria. Reconstitution is done in 2% NaCl and takes 15 min, but, once the bacteria are reconstituted, the assay should be performed within 1 to 2 h [11], which may not always be convenient. Furthermore, water samples must be supplemented with 0.35 M NaCl, and, because chlorine is toxic to *V. fischeri*, water samples must also be dechlorinated before they are tested.

In contrast, the DNA-dye assay requires 20 μl of a DNA-dye mix that has a long shelf life and, once diluted, remains stable for up to one week. We have shown that the DNA-dye assay provides reproducible results using only 1 ml of environmental sample per assay. A battery-powered fluorimeter is used to assess biotoxicity in a matter of minutes (an instructional video of the assay is available at http://www.hazardscreen.com/). The responses to different metal ions probably reflect their different affinities and binding sites on DNA. The HazardScreen DNA-dye products have sensitivities and specificities similar to those opf the *C. dubia* and the Microtox assays. The absence of false negatives with the DNA-dye compared with *C. dubia* (seven false negatives) and Microtox (five false negatives) suggests that it is more sensitive than the other two assays. Therefore, we tentatively conclude that the DNA-dye assay is suitable as a

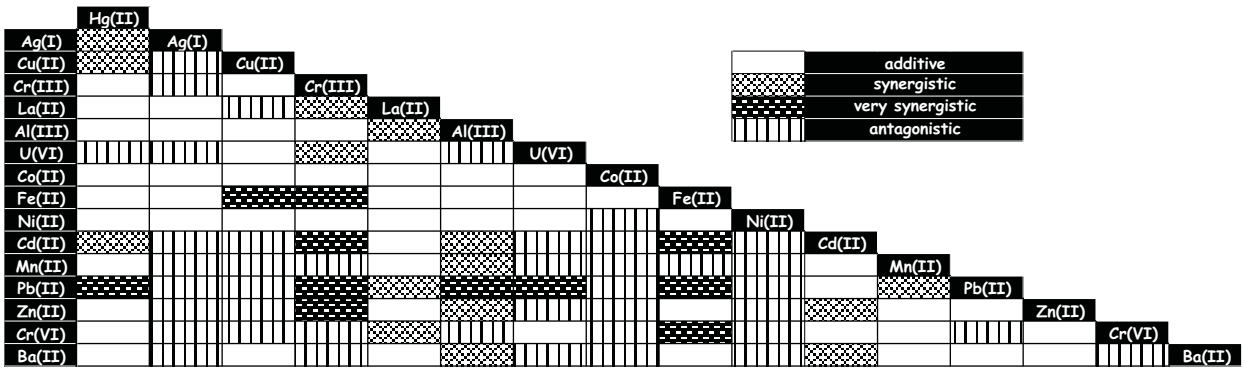

Fig. 4. Observed toxicities for binary combinations of metals ions using HAZ-1 DNA-dye. Open boxes indicate metals that have additive toxicities (44%). Boxes with vertical bars represent metals that, when combined, are less toxic than their separate additive effects; i.e., their effects are antagonistic (35%). Lightly shaded boxes represent metals with observed toxicities that are 50% greater than their separate additive effects, that is, synergistic (21%). The more darkly shaded boxes indicate metal combinations for which the observed toxicities are more than twice the toxicity predicted from their separate (independent) effects, that is, very synergistic (9%).

screening assay of samples in the field. However, this conclusion must be justified with a larger sample size.

The behavior of the DNA-dye to binary combinations of metals resembles the responses of *C. dubia* and Microtox (*V. fischeri*) assays. Microtox exhibits combination effects that agree with DNA-dye data for six (Cu with Cr, Cu with Pb, Cu with Zn, Cd, with Co, Co with Pb, and Co with Cr) of 13 published combinations [21,22]. No information is available describing the effects of heavy metal combinations on *C. dubia*, but three metal pairs were tested (Cd + Hg, Cd + Zn, and Hg + Zn) using a closely related species (*D. magna*), and the results agree with the DNA-dye test data for the first two pairs [23]. Cadmium with Al is also synergistic in barley [25].

Some of the data in Figure 4 can be understood on the basis of the known binding sites on DNA. For example, Hg and Ag are both reported to form complexes only with nucleotide bases [28], which may explain why they are additive. When multiple metals are present at low concentrations their effects are likely to be additive, but at higher concentrations synergism or antagonism may predominate [29].

The present study shows that the toxicity of combinations of metals can be antagonistic or synergistic as well as additive. This demonstrates that knowledge of metal concentrations alone (for example, from ICP-MS or ICP-AES) might not reliably predict sample toxicity. Data from the present study show that Hg(II) ions alter the structure of DNA, inducing changes in the aromatic region of the NMR spectrum. It was also observed that a complementary decamer oligonucleotide containing a T–T mismatch changes its conformation when it binds Hg(II). This is consistent with the release and consequent loss of fluorescence of the DNA-bound reporter dye (data not shown).

The intrinsic fluorescence of at least some our environmental samples implies that they contain organic toxicants, and some samples tested toxic despite the absence of metal ions. Although we did not investigate them here, any molecule that alters the structure of DNA (such as a carcinogen) is likely to be identified by the DNA-dye assay as biotoxic. The toxic effects of organic molecules on the DNA-dye assay are currently being investigated, but it is clear from our data that an analysis of 16 common heavy metal ions can only partially explain the toxicity of nearly half of the samples.

Live organisms clearly have their place when determining biotoxicity of water samples, but there is a growing tide of opinion that opposes the use of animals, particularly vertebrates, for testing products consumed by people, including water. The present study demonstrates that different bioassays respond differently to chemically toxic water samples, which is consistent with the reports of others [10].

The molecular mechanism for the biotoxicity of *C. dubia* and Microtox might be due to the metal ions interacting with different metabolic pathways. In the case of Microtox, toxicants appear to target the light-emitting enzyme complex [29], but little is known of the mechanism by which chemical toxicants affect *C. dubia*. For the DNA-dye assay, the molecular mechanism is related to the different binding sites on DNA. The fact that DNA has a sensitivity comparable to that of other bioassays and that it is portable, easy to use, and produces quantitative results in minutes suggests that it is well suited for use as an initial screening tool for potentially biotoxic chemicals in water samples.

SUPPLEMENTAL DATA

Table S1. Complete elemental analysis for 16 metals (189 KB DOC).

Fig. S1. Effect of pH on DNA-dye fluorescence intensity for HAZ-1 and HAZ-2 (149 KB DOC).

Fig. S2. Titration of wastewater samples (1,047 KB DOC).

Fig. S3. Titration of 16 metal ions (576 KB DOC).

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