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Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative

K. Wah Chu, King L. Chow *

Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

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Abstract

Caenorhabditis elegans, a free-living nematode species, was adopted for a toxicity bioassay of 10 heavy metals. The lethal concentration (LC) of these metals was determined. Based on these data, we conducted pairwise and triple metal combination testing and demonstrated that these heavy metals displayed synergistic killing effects on *C. elegans* larvae. Drastic increases in mortality rate up to 100% could be observed at low metal concentrations. The results illustrate the complexity of toxicity tests in biological systems and show that physical–chemical monitoring of toxicants may underestimate biohazards in environmental samples. We also demonstrate that a transgenic derivative nematode strain, KC136, carrying a heat shock promoter driven *gfp* reporter gene could be used to reduce the duration of an assay so that the synergistic effects among toxicants could be revealed. This derivative strain allows rapid and frequent monitoring of environmental hazards, which usually requires the handling of a large number samples.

Keywords: Transgenic nematode, Heavy metals, Synergistic toxicity

1. Introduction

Bioassays are commonly used in monitoring scheme in toxicological studies (Weber, 1993). Indicator organisms are used to provide early warnings of potential environmental hazards, including the presence of pollutants like toxic heavy metals and organic contaminants. Such assays are often used to monitor the acute toxicity of aquatic effluents according to the guideline set by the regulatory authority. These tests usually entail a 96 h assay using invertebrates and cadmium as a reference toxicant (Weber, 1993).

Physical and chemical methods employing sophisticated equipment such as Inductively Coupled Plasma–Atomic Emission Spectrometry (ICP–AES) and Gas Chromatogaphy–Mass Spectrometry (GC–MS) are also used for such monitoring. These equipments have superior accuracy and sensitivity but require known chemical components as references. Since field samples often contain unidentified components, appropriate

* Corresponding author. Tel.: +852-2358-7342; fax: +852-2358-1559

E-mail address: bokchow@ust.hk (K.L. Chow).

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referencing of all toxicants may not be practical. Thus, whole sample analysis with bioindicator animals was proposed by the U.S. Environment Protection Agency to circumvent this limitation (Weber, 1993). By exposing test organisms directly to field samples, the toxicity of any chemicals including those not on the standard test list can be revealed in a single test. In addition, the bioavailability of toxicants influenced by its affinities to the organisms as well as other unfavorable factors could be uncovered. Hence, bioassays provide a complementary alternative in toxicity monitoring, where physical and chemical measurements reveal less directly the potential hazards that living organisms experience. Nevertheless, bioassays are not frequently used due to practical limitations and the availabilty of the test animals.

Several criteria must be fulfilled for an organism to be adopted as a bioindicator. The organism should be sensitive to the testing toxicants, easy to manage in the laboratory and available throughout the year. A free-living nematode, *Caenorhabditis elegans*, widely used in developmental biology studies satisfies all these criteria. This nematode feeds on bacteria in the soil or water, or *Escherichia coli* in laboratory cultures, and is found in all parts of the world with few geographical restrictions. Its culture conditions, developmental staging, the anatomy and genetic properties are well defined (Sulston and Brenner, 1974; Sulston and Hodgkin, 1988). Certainly, its short life cycle, low cost and high tolerance to pH, salinity, water hardness and temperature make it a robust model. In fact, *C. elegans* has been used in some acute aquatic toxicity tests with some success particularly when its sensitivity to heavy metals was exploited (Williams and Dusenbery, 1988, 1990; Donkin and Williams, 1995). The availability of recombinant technology also makes it possible to improve the sensitivity of assays by transgenesis (Candido and Jones, 1996).

Stress response is a universal phenomenon characterized by the induced synthesis of a unique set of polypeptides called the heat shock proteins (HSPs). Pre-existing heat shock transcription factors (HSTF) are activated by elevated levels of cellular stress. In turn, they bind to heat shock elements (HSEs) in the promoter regions of heat shock responsive genes and thereby activating their transcription (Mutwakil et al., 1997). Hence, *hsp* gene expression represents a rapid read-out sensitive to sub-lethal doses of stress caused by environmental insults. Experiments in *C. elegans* have demonstrated the feasibility of using chimeric transgene consisting of regulatory region from a stress inducible heat shock protein gene attached to the structural region of a reporter gene for detection of toxic compounds (Candido and Jones, 1996; Guven et al., 1994, 1995, 1999). Most of these studies employed the PC72 or CB4027 strains harboring galactosidase reporters driven by *hsp* promoters from nematode and *Drosophila*, respectively. While PC72 strain was found to be more sensitive than the CB4027 strain, the optimized assay protocols developed for these strains often require incubation of animals in heavy metal containing solution at a raised temperature from 25 to 32 °C for a period of 7–24 h. Subsequently, an enzymatic assay was conducted for an extended period from 30 min to 24 h (Candido and Jones, 1996; Guven et al., 1994, 1995; Mutwakil et al., 1997; Jewitt et al., 1999). As an alternative to the *hsp* promoter–reporter system, transgenic strain JF2.1 carrying a metallothionein *mtl-2* promoter driven galactosidase reporter has also been reported (Cioci et al., 2000). This strain is induced selectively by some heavy metals but not all environmental stress. In general, these reporter animals have a single heavy metal detection limit at around 10–100 μM. While most of these studies used only single test toxicant, the impact of different toxicants in combination has not been examined vigorously.

Metals species in the environment almost always exist in mixtures. Yet few studies have focused on their combined effects on living organisms. There have been scattered reports on synergistic or neutralizing effect of specific heavy metals in biological systems, e.g. copepod *Amphiascus tenuiremis* (Hagopian-Schlekat et al., 2001), nematode *C. elegans* (Power and de Pomerai, 1999) and human keratinocytes (Bae et al., 2001). However, no systematic testing of synergism among metals has been conducted. Also, there is no consistent explanation for the effect of metal

interactions due to their complex relationship in biological systems. Each metal may be involved in a spectrum of metabolic pathways to elicit specific toxic effects. Yet, little is known about the intertwining relationship among these pathways. We therefore made the first attempt to investigate the potential interactions of metal toxicants, as found in nature, by this *C. elegans* based bioassay. We observed in both the lethality and stress tests the presence of synergistic and neutralizing effect among different heavy metals. Our results raise the notion that physical/chemical monitoring in most environmental studies may not reflect accurately the toxicity imposed on living system, where the biological impact of a combination of toxicants may require re-examination.

2. Materials and methods

2.1. Preparation of nematode cultures

C. elegans were maintained on nematode growth medium plates seeded with *E. coli* strain OP50 at 20 °C as described (Brenner, 1974). Gravid *C. elegans* hermaphrodites were washed off the plates into centrifuge tubes and were lysed with a bleaching mixture (0.45 N NaOH, 2% HOCl). The eggs were washed with double-distilled water twice, followed by washing with K medium once (50 mM NaCl, 30 mM KCl, 10 mM NaOAc, pH 5.5) and then transferred to a beaker with K medium (10 ml) for 24 h to arrest their growth at L1 stage in the absence of food.

2.2. Lethality test using a single metal

There were in total 10 heavy metals used in the lethality tests. The water-soluble salts for the 10 metals were $ZnSO_4 \cdot 6H_2O$, $HgCl_2$, $NiSO_4 \cdot 6H_2O$, $CdCl_2$, $CuSO_4 \cdot 5H_2O$, $PbCl_2$, $MnCl_2 \cdot 4H_2O$, $AlCl_3$, $CoCl_2 \cdot 6H_2O$, $K_2Cr_2O_7$. Assays were performed in disposable borosilicate tubes with constant shaking at room temperature. Aliquots of 900 μl of an *E. coli* (OP50) suspension in K medium at a concentration of 1 OD550 unit per ml were dispensed into the tubes. Appropriate concentrations of heavy metals were added so that the final volume of an assay was 2 ml. The nematodes were suspended in the K medium at a concentration of 10,000 animals per ml and 100 μl (1000 nematodes) of this suspension was added to each sample. The tubes were tilted on a supported surface with constant shaking for 48 h at room temperature (22 °C). Subsequently, about 500 animals were counted under a dissecting microscope, where the inactive ones were scored.

2.3. Lethality tests with multiple metals

In lethality tests with two or more metals, the procedures were primarily the same as in the single metal lethality test. The concentration of metals used in this test, however, was determined by the results of the single metal lethality tests. Lethal concentrations (LC) causing death of 10, 15, 20 or 30% of the larval culture were used (LC_{10}, LC_{15}, LC_{20}, LC_{30}). Thus, for a particular experiment, the LC_{10} concentration of one metal was mixed with the LC_{10} concentration of another, resulting in a final solution of two metals at different concentrations.

2.4. Construction of transgenic nematodes

Both plasmid constructs *hsp16-2–gfp* and *hsp16-41–gfp* were derived from plasmids pPD99.44 and pPD99.52 (gift from A. Fire). Transgenic nematode lines were generated by microinjecting the transgene, along with the dominant *rol-6* marker plasmid, into wild-type nematodes. The resulting transient transgenic lines were then exposed to ultraviolet-radiation to establish an integrated transgenic line (Metani, personal communication) The stable transgenic strain carrying *hsp16-2–gfp* was designated as KC136 and that with transgene *hsp16-41–gfp* was designated as KC125. These integrated strains were outcrossed twice. Both outcrossed strains did not display any abnormal phenotype. They retained the ability to express GFP upon heat shock or heavy metal induction and displayed similar sensitivity to heavy metals as N2 strain did.

2.5. Stress test of transgenic strains

In the stress tests, the synchronized L3 stage of the transgenic nematodes displaying the most prominent response were used. Synchronized L1 larvae were released and grown on bacteria-seeded plates for 28 h to the L3 stage. They were washed off the plates and resuspended at a concentration of 5000 animals per ml. The appropriate concentrations of the heavy metal (see toxicity tests) were added to the test tube with 100 μl of nematodes to a final assay volume of 1 ml. The incubation condition was identical to that of the lethality test except that *E. coli* was omitted. After treatment for 5 h, the nematodes in the test tube were allowed to settle for 10 min, pipetted onto an agar pad on a glass slide, mounted and observed for the induced fluorescent signal at the pharyngeal bulb. More than 100 nematodes were counted for the statistical analysis.

2.6. Analysis of data

All toxicity and stress tests were replicated six times. SAS was used to calculate the mean and standard deviation of each experimental data point, where the data for the reference toxicant fell within the acceptable control limits described by Freeman et al. (1998). In lethality tests for single metal, Probit analysis was performed to calculate the LC_{50} value of each metal species, while the best-fit dose response curve of each set of experiments was plotted with the SigmaPlot 4.0.

3. Results

3.1. Sensitivity of larval versus adult nematodes

All previous toxicity tests using *C. elegans* were conducted on adult animals or mixed population. The age of adult animals and the composition of a population may impact on the readout of the assay. We reasoned that animals in the early larval stages were more susceptible and sensitive to toxicants. To test this idea, a 48-h toxicity assay using the reference metal, cadmium, was performed with synchronized animal populations at different developmental stages. Under our defined assay conditions, we noted that animals in the late larval stages had similar sensitivities as those of adults (data not shown) while L1 animals were more sensitive. When synchronized L1 and young adult nematodes were subjected to cadmium in the same concentration range, a clear difference in their response was observed (Fig. 1A). While animals in the control medium continued to develop and were active after 48 h (Fig. 1B), L1 larvae could not tolerate cadmium at concentration beyond 1500 μM and were all dead (Fig. 1C). They displayed a LC_{50} value of 595 μM. In addition, the mortality curve on the L1 population had a sharp slope between 0 and 1500 μM, suggesting that they were highly sensitive to changes of metal concentration in this range. On the other hand, a LC_{50} value of 5520 μM was obtained with adult animals, suggesting that adults are 10-fold less sensitive to cadmium. Based on this result, L1 animals were used in all the subsequent lethality assays.

3.2. Lethality tests of metal and establishment of the LC value

A total of 10 metals were assayed in our lethality test with L1 larvae to establish the LC value of each metal. The LC_{50} values determined were tabulated in Table 1. The descending order of toxicity is mercury, copper, lead, chromium, nickel, cadmium, aluminium, cobalt, zinc and manganese. The log scale graph revealing the mortality rate of L1 larvae at different metal concentrations allows us to categorize the metals into two major groups (Fig. 2). The more toxic class, including mercury, copper, lead and chromium, had a killing curve with a steep slope and a LC_{50} ranging from 20 to 300 μM. The range of tolerable concentrations was very narrow and all animals could not survive beyond 300 μM. The less toxic group, however, exhibited a flat shallow killing curve when concentrations of the metals were below 600 μM. The mortality was minimal in this range and increased gradually as the metal concentration increased from 1000 to 3000 μM. This group of metals, including nickel, cadmium,

aluminium, cobalt, zinc and manganese, had LC_{50} values ranging from 400 to 2100 μM.

3.3. Lethality tests of paired metals

Heavy metals in field samples never exist as a pure solution. Therefore, the analysis described above represented only a simplified assay without reflecting the actual conditions in the environment. To examine the potentially complex situation, we performed pairwise metal assays with L1 larvae. Based on the single metal test results, the concentrations of each metal that caused different mortality rates, LC_{10} to LC_{30}, were determined accordingly. We combined two metal ions in the same treatment, where the dosage of each of them could result in only a low level of mortality. For example, the concentration of metal A that caused 10% mortality (LC_{10}[A]) was added to metal B with the same lethal concentration (LC_{10}[B]). Should the metals have a simple additive effect, a mortality rate of around 20% would be observed. If there were interactions between the two metals because they have common cellular targets or uptake mechanisms, we would expect the mortality rate to deviate. In this study, we defined a synergistic result as a combination of two metals having a mortality rate 25% higher than the one

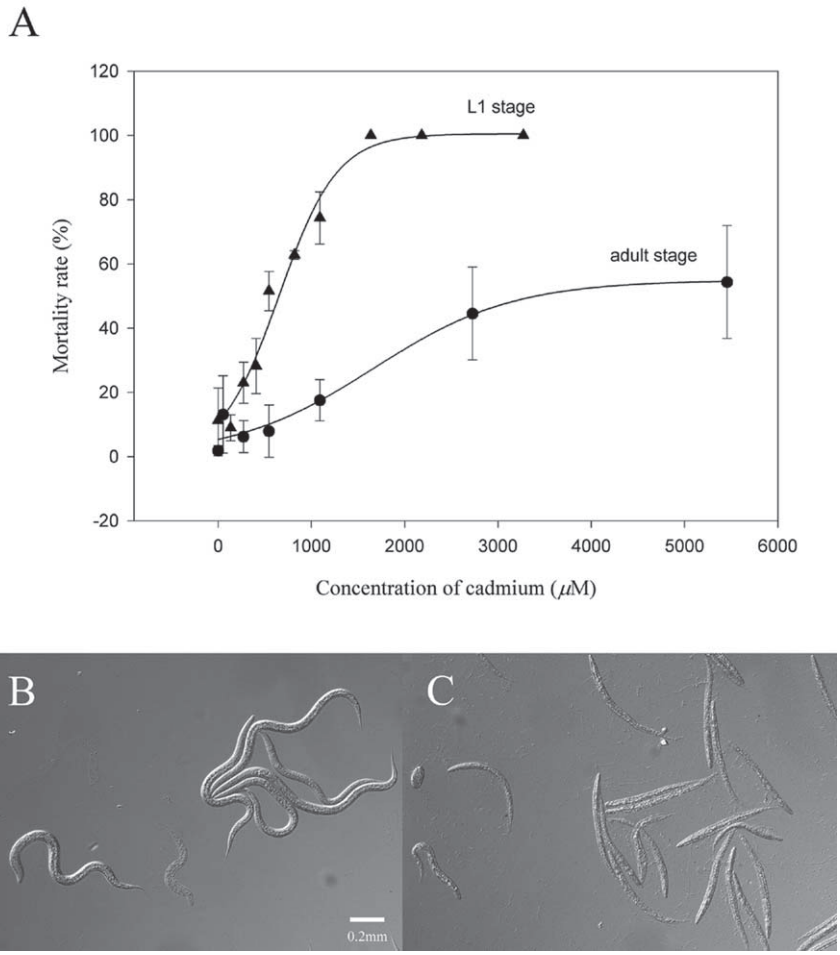

Fig. 1. (A) Mortality rate of L1 larvae versus young adult nematodes. (B) Control population with less than 5% mortality. (C) Population with 100% mortality.

Table 1
The lethal concentration (LC_{50}) value and the corresponding 95% confidence level of different heavy metals

Type of metal	LC_{50} (μM)	95% Confidence level
Hg	18.16	18.34–17.97
Cu	93.36	94.76–92.00
Pb	124.93	126.08–123.77
Cr	237.01	239.49–234.50
Ni	436.02	453.71–418.78
Cd	594.60	604.91–584.39
Al	672.75	684.83–660.53
Co	735.95	745.53–726.28
Zn	1697.58	1714.13–1680.81
Mn	2110.49	2148.79–2072.24

expected. For example, when LC_{10} of each metal was used, the combined effect would be catagorized as synergistic if the mortality rate was significantly above 25% [(10 + 10%) × 1.25] (Fig. 3A, Cd + Cu). Similarly, a neutralizing effect would be suggested if the mortality rate was 25% lower than expected (Fig. 3A, Cd + Zn).

Experiments were first initiated to test six metals, cadmium, copper, lead, mercury, zinc and nickel, in all combinations. The summary is shown in Table 2A. Copper appeared to have a strong synergistic effect in combination with the other five metals, e.g. LC_{20} of cadmium and copper in combination resulted in a 100% mortality of the animals in 48 h (Fig. 3A). Cadmium had variable interactions with different metals. Zinc, on the other hand, often exhibited a neutralizing effect on a number of metal ions tested (Fig. 3A). When zinc was combined with cadmium, no obvious increase in toxicity was observed as if the toxicity had been neutralized. Subsequently, four more metals, cobalt, chromium, aluminium and manganese, were tested against these three representative metals (Table 2B). Consistently, the synergistic effect conferred by copper, the synergistic or additive effect by cadmium and the neutralizing effect by zinc were observed in these assays. The results suggest that though the cellular targets of these toxic effect are unknown, these metals could interact and impair cellular functions and animal survival.

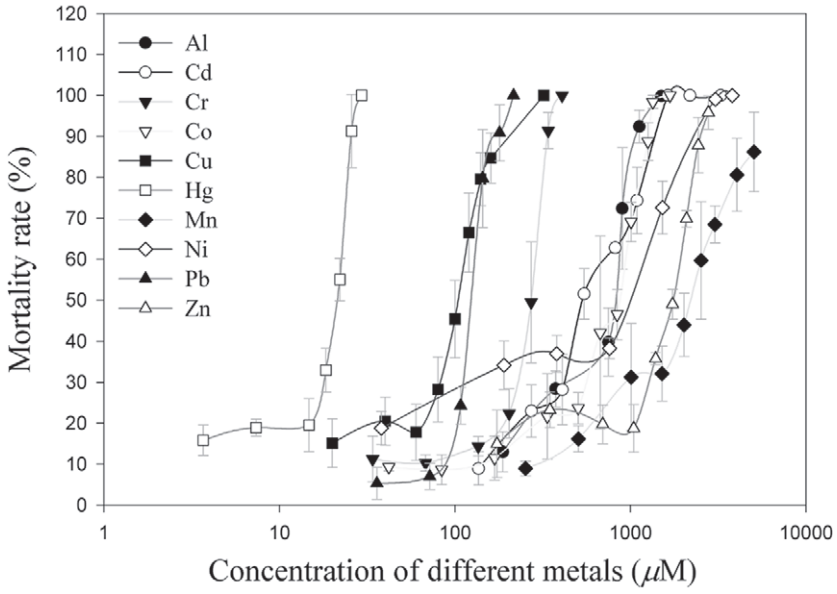

Fig. 2. Dose response curve of different metals. The mortality of L1 nematodes is plotted against the testing concentration on a log scale.

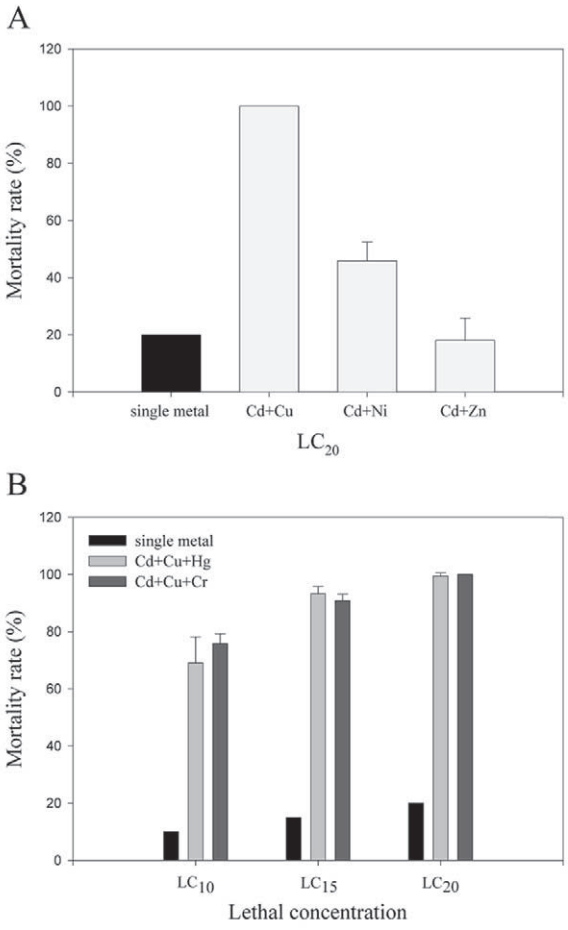

Fig. 3. (A) Dosage combinations of different metal pairs to illustrate synergistic (Cd/Cu), additive (Cd/Ni) and neutralizing (Cd/Zn) effects, respectively. (B) Triple-metal lethality test for cadmium, copper and mercury as well as cadmium, copper and chromium combinations.

3.4. Lethality tests with three metals

While both synergistic and neutralizing effects could be demonstrated with two metals, we further conducted triple-metal lethality tests to see if we could detect a dramatic synergistic impact on animal survival. In case of the combination of chromium, cadmium and copper, which had displayed synergistic effects in the pairwise tests, the mortality rate increased further when they are combined. At the LC_{10} concentration of these three metals, the mortality rate was already at 71.3%, while almost 90–100% mortality was reached at the LC_{15} (Fig. 3B). When cadmium, copper and mercury combination was tested, the LC_{10} concentration produced an even a higher mortality rate at 75.8% (Fig. 3B). With this combination at the LC_{15} concentrations, over 90% mortality was observed. These results argue that potential metal interaction in environmental samples should not be neglected since minute amount of each metal in combination could generate a severe lethal impact.

3.5. Stress test for toxic heavy metals

The lethality test offers a sensitive tool for monitoring the toxicity of an aqueous solution. The duration of the experimental procedure lasting for more than 48 h, however, could have been shortened. Stress test was introduced for this purpose. We reasoned that if heavy metals were toxic, they would impact living organisms through environmental stress and thus induce a stress response. By monitoring such response, which occurs within minutes to hours, we would be able to obtain a rapid perception of the environmental stress. We used the heat shock protein gene promoters, *hsp16-41* and *hsp16-2*, to generate two *gfp* reporter transgene constructs. Both *hsp* promoters are active upon heat induction making them suitable for this test (Stringham and Candido, 1994).

Two stable transgenic lines, KC125 and KC136, carrying *hsp16-41* and *hsp16-2* promoter driven reporter genes, respectively were established. The production of the green fluorescent protein could be induced rapidly and could be conveniently monitored by fluorescent microscopy. When these strains were tested for heavy metal induction, *gfp* expression in KC125 strain was weak after 5 h of incubation in a heavy metal solution. Even though more definite induced expression could be detected when incubation period was prolonged, this strain would not serve the purpose of reducing the assay time. On the other hand, the KC136 strain re-

Table 2
Summary of metal interaction in the pairwise metal lethality assay

Metals	Cadmium	Copper	Mercury	Nickel	Lead
Zinc	N	S	S	N	A
Lead	S	S	A	S	
Nickel	A	S	A		
Mercury	S	S			
Copper	S				

Metals	Cadmium	Copper	Zinc
Cobalt	A	S	N
Chromium	S	S	A
Aluminium	S	S	N
Manganese	A	S	N

A, simple additive effect; N, neutralizing effect; and S, synergistic effect.

sponded more positively within 5 h although a low level of background *gfp* expression in about 40% of the transgenic animals was observed in the control medium (not capturable by photography, Fig. 4C). The induced *gfp* signal at the pharyngeal bulb was strong and allowed a distinct identification of induced expression (Fig. 4A). Hence, this KC136 strain demonstrated a clear-cut contrast between uninduced background and a brighter positive signal.

To distinguish the positive results from the background in this stress test, only metal concentrations that could induce 50% of the transgenic animals to display strong *gfp* signal were taken as having positive effect (Fig. 5, above dotted line). It allows us to define the effective concentration of each metal for *gfp* induction in this KC136 strain and have the value compared with the LC_{50}. When tested with a few representative metals, it was found that copper at 48 h LC_{50}[Cu] concentration, i.e. about 100 μM, was sufficient to induce the reporter gene expression in 5 h. Cadmium had the identical effect at 273 μM, which corresponds to LC_{20}[Cd]. Similarly, mercury induced *gfp* expression at 15 μM, i.e. LC_{20}[Hg], under the same assay conditions. These results show a good improvement on the sensitivity of detection and a major reduction of the assay time. Zinc, on the other hand, did not induce *gfp* expression in KC136 efficiently, and thus the inducible dose could not be determined.

3.6. Combinatorial effect could be revealed by stress tests

If the stress tests gave a result more rapidly than the lethality tests, it would offer a better alternative for toxicity detection provided that the synergistic toxic effect between two metals revealed in the lethality tests could also be monitored. When different combinations of the metals were tested, some combinations at low concentrations, e.g. Cd/Hg at LC_{10} or LC_{20} could generate

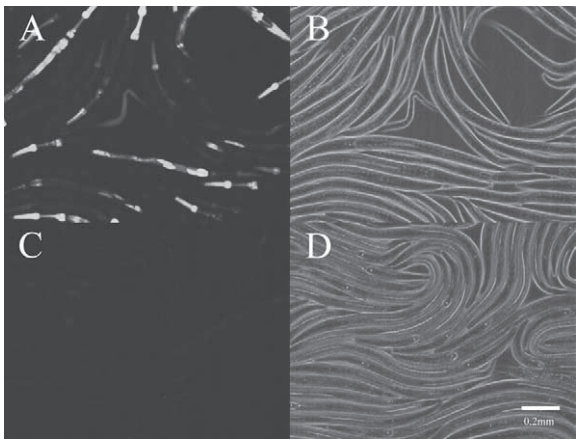

Fig. 4. Transgenic animals after the stress test. (A) KC136 population with *gfp* induction by cadmium and its corresponding bright field image (B). Control KC136 population in the absence of cadmium (C), and its corresponding bright field image (D).

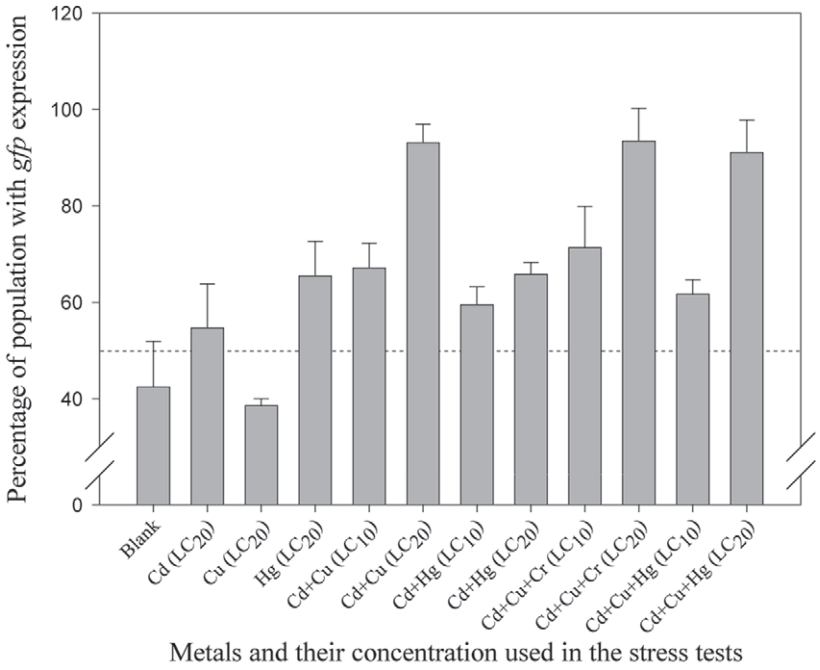

Fig. 5. Synergistic interaction between individual heavy metals can be reproduced in transgenic nematodes in the stress assay. Significant induction of *gfp* expression (50% of a population, above the dotted line) was observed in LC_{10} or LC_{20} doses of single and combined metals, while the blank control gave a signal beyond the detection limit of photography (reference to Fig. 4).

positive fluorescent signal, although no significant increase of fluorescence positive animals was observed with increased metal concentration. However, the Cd/Cu pair did show positive induction of *gfp* at both LC_{10} and LC_{20} concentrations, where stronger response was noted at LC_{20} (Fig. 5). Similar observation was found in the triple-metal stress tests with Cd/Cu/Hg and Cd/Cu/Cr. Stronger *gfp* induction was detected at the LC_{20} concentration than LC_{10} level (Fig. 5). Thus, our results suggest that the use of transgenic animals may indeed allow us to monitor the stress impacts on animals of low metal concentration at around LC_{10} to LC_{20} level and efficiently uncover potential toxicity of samples within 5–6 h.

4. Discussion

Based on 96-h acute toxicity test results, the sensitivity of *C. elegans* to heavy metals has been compared with that of other invertebrates, Daphnia, and vertebrate, salmonid fish (Williams and Dusenbery, 1990; Sprague, 1987). The range of 96-h LC_{50} of *C. elegans* was from 0.05 to 1.64 μM and that of Daphnia was from 0.05 to 0.76 μM (Williams and Dusenbery, 1990). For salmonid fish, the LC_{50} range was from 5.46 to 13637 μM (Sprague, 1987). *C. elegans* has a sensitivity comparable to that of Daphnia in the μM range and is at least one order of magnitude more sensitive than the fish bioindicator. The most important of all, the various requirements for illumination, feeding, medium changing can be eliminated by using *C. elegans*, making it a more user-friendly indicator animal for toxicity monitoring.

At present, there are a few research groups using *C. elegans* as a model for bioassays (Guven et al., 1994; Jones and Candido, 1999; Mutwakil et al., 1997; Power and de Pomerai, 1999; Williams and Dusenbery, 1990). With the use of adult animals or mixed population, the order of metal toxicity determined in their studies varied. This inconsistency could be due to difference in

methodology and the nature of the chemicals used (Williams and Dusenbery, 1990). The difference in ionic concentration of the incubation media has been suggested to result in different stress response to a variety of metals (Donkin and Williams, 1995). The number and density of animals used in each study could also account for the discrepancy. For example, more than 1000 nematodes were used by Jones and Candido (1999) per test instead of less than 10 by Williams and Dusenbery (1990). Since the body cuticle of these animals may act as a sink for metals, the population size might have impact on the bioavailability of the metals in the sample solution (Freedman et al., 1993). In addition, the incubation period in the heavy metal solution also differ from one study to another. Therefore, it is difficult to have an accurate comparison of the sensitivity of these different assay protocols.

In this study, we demonstrate that the developmental stages of the animals is important to the sensitivity of an assay. When young adults were used, the detectable limit was an order of magnitude higher than that defined with L1 animals (Williams and Dusenbery, 1988, 1990; Jones and Candido, 1999). L1 *C. elegans* larvae have a superior sensitivity range in our 48-h assay regime (Fig. 1). In fact, extrapolating from the reported data of 96-h toxicity test with adult animals, we believe that L1 larvae probably represents one of the most sensitive invertebrates so far adopted for metal toxicity assay. Moreover, the toxicities detected for different heavy metals as revealed by our bioassays match quite well with the predictions based on the metals' physical and chemical properties with just some minor difference (Tatara et al., 1997, 1998; Nieboer and Fletcher, 1996).

With respect to the use of transgenic nematodes in toxicological study, the protocols again differ among studies. There are two commonly used strains, the PC72 (Stringham and Candido, 1994) and the CB4027 (Guven et al., 1994), which use a β-galactosidase reporter driven by the *hsp16-1* promoter and fly *hsp70*, promoter respectively. Both strains gave good induction effect with heat shock, and the exposure time required for heavy metal induction is from 7 to 24 h. However, the detection of induced gene expression requires permeabilization of the animals followed by the enzymatic assay. It uses additional reagents and can run for 30 min to 24 h (Candido and Jones, 1996; Guven et al., 1994; Mutwakil et al., 1997; Jewitt et al., 1999). No such procedure is needed in our assay. Immediate result can be observed under a microscope. This is particularly important when timely measurement is necessary, although the non-quantitative measurement of fluorescent intensity may compromise the sensitivity to a small extent. However, it should be noted that though biochemical assays using 4-methylumbelliferyl-β-D-galactopyranoside (MUG) or *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrates for β-galactosidase may take a long time, they do have the flexibility to adjust the reaction time for signal amplification and increase of detection sensitivity (Guven et al., 1994). In addition, the adoption of different promoter reporter genes as well as the addition of other chemical additives, e.g. oxygenated perfluorocarbon or non-ionic surfactant could improve the uptake of heavy metal by the animals and thus the assay sensitivity (Dennis et al., 1997; Freeman et al., 1998; Jewitt et al., 1999). Such modifications should be equally applicable for further improvement of our assay.

Little was known about the cellular cause of toxicity in organism and the impact of multiple metals. One hypothesis explaining the interactions focuses on the stability between metal ions and their binding sites (Nieboer and Fletcher, 1996). The stability of the metal ions binding to the cellular targets are governed by two stability indexes, the covalent index and ionic index. For a fixed value of the covalent index, the stability of metal ions–cellular target complex increases with the magnitude of ionic index. For a fixed value of ionic index, the complex stability increases with the value of covalent index. Furthermore, metals with high covalent index tends to displace the metals with high ionic index for the binding sites. Except for Al, all the metal tested have similar ionic index, where they have a descending order of covalent index as Hg, Pb, Cu, Cd, Co, Ni, Zn, Mn and Al. This order is similar to that of toxicity determined in this study, except copper, nickel and aluminum, which appeared to be more toxic. The covalent and ionic indexes of

chromium were not determined previously (Nieboer and Fletcher, 1996), and thus would not allow verification with our experimental results. Moreover, our results show that the more toxic metals with higher covalent index, Hg, Pb and Cu, tend to have synergistic effect. Those metals with low covalent index, e.g. Cd, Co, Ni, Zn, Mn and Al, are more likely to have a variable impact in our pairwise analyses. It also appears that metal with low covalent index, e.g. Zn, tends to neutralize the toxic effect of other metals with lower covalent index, e.g. Cd, Ni, Co, Al and Mn.

In addition to the notion of complex stability and the competition of metal ions for common target sites, interaction of metal species could act through multiple biochemical and physiological processes subjected to the influence of external and biological factors (Nieboer and Fletcher, 1996). Bioaccumulation of a metal can be modulated by the differential uptake, transport and sequestration within an animal (Dallinger and Rainbow, 1993). Thus, in transgenic animal assay, different induced expression patterns of the *hsp* promoter β-galactosidase reporter have been reported to be influenced by different metals, e.g. induced expression in the gut by mercury, in the pharynx by cadmium, in the neurons by copper and in the hypodermis by zinc (Stringham and Candido, 1994). We are far from understanding the biological processes leading to lethality in animals and the synergistic killing effect of metals. Other than having multiple metals acting on a common cellular target, it is possible that lethality was the result of a combination of tissue function failure. The observation made in this study reveals the complexity of the organism–environmental interaction, arguing that the biological impact and toxicity of a low dose of heavy metals in environmental samples could have been mistakenly neglected due to insufficient emphasis on biological monitoring.

The definition of different endpoints provides the venue for developing rapid and cost-effective tests to be used in routine environmental assessment, where protocols with improved sensitivity and short manipulation procedures are often desired. While end points used in most studies were based on different behavioral traits, e.g. rate of movement, feeding and fecundity, they are far from being clear-cut (Donkin and Williams, 1995). The induced expression of stress protein genes turns out to be a convenient endpoint, particularly when it can be quantified. In our single metal stress test, the tested metals gave a reproducible induction of *hsp–gfp* expression (Fig. 5). In terms of sensitivity of detection, 10–100 μM of the more toxic metals could induce the *gfp* expression in $>50\%$ of the animals where 20–300 μM of these metals was needed to kill half of the tested L1 animals. Thus, a stress test offers at least a two- to three-fold improvement of sensitivity over lethality test. In terms of processing time, a stress test takes only 5 h instead of 48 h as in the lethality test, i.e. almost a 10-fold improvement of efficiency. The combination of these two factors in using KC136 transgenic strain in a stress test can raise the detection sensitivity by more than 20-fold. In addition, positive detection of a stress effect could be observed at even lower metal concentrations when they exist in combination. Therefore, with a simple set up and minimal processing time, this KC136 transgenic line allows hundreds of samples to be handled with indicative results within 5–6 h without compromising the sensitivity. If we can couple both the stress test with the lethality test in a single assay, the toxicity of a sample can be revealed in 5 h and be confirmed in 48 h. Such a combined approach would provide superior sensitivity, efficiency and accuracy. With further scaling up of such operations and better quantification of the stress signal, such as by reading from a fluorometer instead of by counting animals, we have no doubt that this rapid bioassay could be applicable to routine monitoring of a large number of environmental samples.

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