Toxicity ranking of heavy metals with screening method using adult Caenorhabditis elegans and propidium iodide replicates toxicity ranking in rat

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The utility of any model system for toxicity screening depends on the level of correlation between test responses and toxic reactions in humans. Assays in Caenorhabditis elegans can be fast and inexpensive, however few studies have been done comparing toxic responses in this easily cultured nematode with data on mammalian toxicity. Here we report that a screening assay for acute toxicity, using adult C. elegans grown in axenic liquid culture, replicated LD50 toxicity ranking in rat for five metals. This assay utilized the COPAS Biosort and propidium iodide (PI) as a fluorescent indicator of morbidity and mortality after 30-h exposures. We found that chronic toxicity assays of 2-week treatment duration, followed by analysis of PI induced red fluorescence levels, produced less consistent results than the acute assays. However, other chronic toxicity endpoints were compound and concentration specific, including changes in vulval and gonadal morphology, intestinal thickness and integrity, and the presence of retained internal eggs in post-reproductive animals. Some of these endpoints reflect similar findings in mammals, indicating that measurements of morbidity and mortality in conjunction with morphology analyses in C. elegans may have the potential to predict mammalian toxic responses.

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1. Introduction

Toxicology studies are a vital step in ensuring the public safety. Safety studies for evaluating adverse effects of substances often utilize large numbers of rodents, and are extremely expensive and time consuming. This has resulted in a backlog of over 10,000 compounds that are in need of prioritization for further testing (Dix et al., 2007). Through various laws, agreements, and memorandum of understanding (MOU), several government agencies have collaborated to outline the need for expedited development of fast, reliable alternative methods for screening potential toxins (Congress, 2000; National Research Council, 2000). A recent MOU between the National Institutes of Health, the U.S. Environmental Protection Agency, and the U.S. FDA, stressed exploration of high-throughput assays and tests that utilize phylogenetically lower organism with the speed and lower expense ratios of in vitro testing (Cole et al., 2004; Boyd et al., 2007; Leung et al., 2008). As with any model system, C. elegans will only prove useful in toxicity screening if results are predictive of toxic responses in other organisms, yet very few studies have been done to evaluate correlations between responses in C. elegans and toxicity ranking in mammals (Boyd et al., 2010). In one such study, ranking of median lethal concentration (LC50) values in C. elegans for eight metal salts paralleled ranking for rat median lethal dose (LD50) values, and the LC50 values from C. elegans were found to be equally predictive of relative toxicity in rats as LD50 values were from mice to rats (Williams and Dusenbery, 1988). Another study analyzing motility in adult C. elegans found that for fifteen organophosphate compounds, toxicity ranking order was significantly correlated to rat LD50s (Cole et al., 2004). Both of these studies utilized approximately 100 animals or less per condition, and did not use methods that would lend themselves to scaling for high-throughput screening.

The COPAS™ Biosort (Union Biometrica) is a microfluidic device that automates the analysis, sorting, and dispensing of hundreds of
C. elegans nematodes per minute. As each worm passes the sensor, axial length, extinction (a measure of optical density), and fluorescence excitation in three wavelength ranges can be evaluated (Pulak, 2006). Axial length and extinction increase as C. elegans hatch from eggs and develop through the four larval stages (L1–L4) into an adult (Sprando et al., 2009), and we have shown that COPAS measurements of growth can be used to significantly correlate toxicity ranking in C. elegans to rat LD50 ranking (Ferguson et al., 2010). In previously published reports of COPAS analyses of growth, various sophisticated methods of statistical analysis were utilized to evaluate the differences among treatment group data sets, each comprised of thousands of measurements (Boyd et al., 2009, 2010; Ferguson et al. 2010). One of the goals of this study was to evaluate the reliability of simpler methods of analyzing COPAS outputs. Additionally, in an effort to compare C. elegans data to rat LD50 ranking in a similar assay, we sought a means to measure C. elegans death that would lend itself to use in high-throughput techniques. Studies have been done using a fluorescent marker of death in C. elegans (Gill et al., 2003), but we found that the fluorescent dye used in these studies only identifies C. elegans that have died from heat shock. Thus, the objectives for this work were threefold: (1) to develop a COPAS method to quantitatively assess toxicity by measuring death in adult C. elegans using a dye that will fluoresce in worms that died from any cause, (2) to identify a simple, fast, and reliable method to analyze the resultant COPAS data output from thousands of worms per condition, and (3) to assess the usefulness of this method in ranking compounds based on their known toxicity.

2. Materials and methods

2.1. C. elegans strains and culture

The C. elegans N2 Bristol strain used in these experiments was obtained from the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. Worm cultures were maintained in vented tissue culture flasks in C. elegans habitation media (CehM), an axenic liquid culture media containing C. elegans habitation reagent (CeHR) (Rao et al., 2005; Sprando et al., 2009) and non-fat cows milk, and maintained in incubators at 22 °C on shakers set to 80 rpm. Synchronized cultures were obtained by bleaching gravid adult worms, collecting the eggs, and hatching the eggs overnight in M9 buffer (adapted from Nass and Hamza (2007)). At 22 °C, cohorts reached mid-fourth larval stage (L4) between 54 and 56 h after switching the hatchlings from M9 to CehM. In all assays of synchronized cohorts, 25 µL 5-fluoro-2′-deoxyuridine (FUDR) was added to all tested cultures at 56 h post hatching feeding to prevent progeny production, and this time point was counted as day 0 of adulthood.

Dosing was accomplished by preparing metal solutions in distilled water at twice the desired final concentration; (CeHR) was also prepared in concentrated form and then mixed with dosing solution or water as previously described (Sprando et al., 2009). All CehM-dosing mixtures tested neutral with phthdram™ paper (Micro Essential Laboratory, Brooklyn, New York). For one-day trials of acute toxicity, 30-h treatments began at day 0 of adulthood in CehM or CeHR, as indicated. For two-week trials of chronic toxicity, treatment began at the 56 h post-hatching feeding time point, and CehM containing 25 µM FUDR plus indicated concentrations of metals was changed every 3–4 days. All experimental animals were maintained at approximately 5 µL dosing mixture per worm. In 25 cm flasks, this provided a view of 10–30 worms per 4–objective field. Rough estimates of live vs. dead fractions in treatment flasks were obtained by observing all worms in three fields. Unanaesthetized adult C. elegans that appeared healthy were not observed to be immobile at any time, therefore; worms that were immobile for 30 s were assumed dead.

2.2. Dye testing

SYTOX green nucleic acid stain (Molecular Probes), was used according to the manufacturer's instructions. For propidium iodide (PI) staining, 750 µL of 1 mg/mL PI was added to C. elegans in 10 mL CehM without treatment metals added 17–24 h before analysis. All cultures were incubated in CehM without additives for at least 2 h prior to analysis to flush excess PI from the intestinal tract of live worms.

2.3. Reagents

Cadmium chloride, copper chloride, mercury chloride, potassium chloride, sodium arsenite, PI, and FUDR were purchased from Sigma–Aldrich (St. Louis, MO). Stock solutions were prepared in filtered distilled water and further diluted for use.

2.4. Microscopy image analysis

Images of C. elegans were obtained using a Nikon Eclipse 80i microscope, Intensilight C- HGFI illuminator (Nikon Instruments, Melville, NY), and SPOT camera and software (SPOT Imaging Solutions, Sterling Heights, MI). Images were analyzed using SPOT and Image (National Institutes of Health, USA) software. For presentations, phase contrast (oblique illumination) images were balanced for brightness and contrast and, when necessary, rotated for a consistent anterior–posterior, dorsal–ventral presentation format in Photoshop CS5. All fluorescence images within a single figure received identical brightness/contrast manipulations. All morphology analysis was done using images taken with a 10× objective of sodium azide anaesthetized C. elegans held between a coverslip and a 2% agarose pad. Live vs. dead microscopy analyses were done without anaesthetic. Death was assumed when no movement was detected over a 30 s observation period.

2.5. COPAS

COPAS sample pressure was calibrated using CP Control Particles (Union Biometrica, Holliston, MA) according to the manufacturer’s instructions. Briefly, with sample pressure at 4.95 instrument specific units, 15–20 CP Control Particles passed the sensors per second. This sample pressure was then maintained throughout the study. Live, anaesthetized C. elegans were washed twice and then diluted in M9 buffer to obtain a maximum of 15 readings per second. A reading refers to the data obtained when an object passes the COPAS sensors. A minimum of 3000 readings were obtained from each treatment sample. Each reading included red fluorescence, extinction (∫E), and the time of flight (TOF) information. Red fluorescence indicates the level of PI uptake. ∫E measures the decrease in laser light when a particle or organism passed through the laser beam, giving an indication of optical density. TOF measures the amount of time the instrument microprocessor was detained in the analysis of a signal, giving a relative indication of the length of an object.

3. Results

3.1. Dye testing

Standard methods for determining death in C. elegans involve assessment of individual worms for response to light pressure from a thin platinum wire or eyelash probe, or assessment of small populations for movement using images in conjunction with tracking software. In order to utilize the COPAS to screen for the live/dead fraction in a population, we first needed to identify a universal fluorescent marker of death. SYTOX™ (Molecular Probes) has been successfully used with the COPAS to measure death in heat shocked populations of C. elegans (Gill et al., 2003). However, we found that SYTOX did not increase fluorescence in worms that had died from causes other than heat stress (data not shown), limiting its usefulness. PI is a red fluorescent DNA intercalating dye that is excluded from live cells in culture. We found that incubation with PI caused worms that had died of heat shock (Fig. 1A and B), sodium arsenite (NaAs) toxicity (Fig. 1C and D), and old age (Fig. 1E and F) to fluoresce brightly. Additionally, only very low levels of staining were observed in live, healthy worms. Together, these data indicate that PI can be used as a marker of death from a variety of causes.

Levels of PI fluorescence in C. elegans determined to be dead by observed immobility varied greatly (Fig. 1B). In order to determine a PI red fluorescence level cut off for toxicity, we utilized the COPAS sorting feature on 2 week old adults. We found that when sorting for PI treated C. elegans with red fluorescence values of up to 50 instrument specific units, the COPAS only selected live, healthy worms (Fig. 2A and B). COPAS PI red fluorescence values above 50 (CR > 50) were associated with dead worms, or live worms with structural damage. A small fraction of control animals were observed to have gonadal lesions at 2 weeks of adulthood, but only those live animals that also had vulval abnormalities were observed to have PI fluorescence in gonadal tissues (Fig. 2C and D). Sorting for COPAS red fluorescence values over 100 selected live animals with a great deal of structural damage or degradation (Fig. 2E and F) and dead animals (Fig. 2G and H). In further analyses, we therefore utilized the fraction of COPAS readings with red
fluorescence values greater than 50 as a measure of morbidity and mortality in each sample.

3.2. Refining COPAS data analysis

The strength of the COPAS is that it can analyze hundreds of C. elegans nematodes per minute. Technical difficulties with the system include: (1) inclusion of background noise as a low diagonal line of readings in recorded data (Fig. 3A, lower right quadrant), (2) eggs and other debris with low EXT and TOF values are not distinguished from worms (Fig. 3A, lower left corner), and (3) overlapping worms can pass the sensors resulting in a single reading with artificially high TOF and EXT values (Fig. 3A, upper right quadrant). Small changes in conditions such as temperature, handling and age of salt solutions, efficiency of aspiration during wash steps, and worm concentration in analyzed samples can have large effects on the number of these artifactual readings. Therefore, defined limits are required to exclude these readings from analyses.

Using COPAS data from PI treated control C. elegans in scatter charts to plot TOF vs. EXT, we noted that all of the data points in the low “line” of background noise had red fluorescence values of zero. We then utilized the COPAS sorting feature on adult day-14 C. elegans treated with PI to select for units with red values of zero to one, and found that only media was selected (data not shown). We therefore excluded readings with red values of zero from data analyses (Fig. 3B).

While average TOF values are excellent indications of differences in axial length between groups, individual TOF values do not correlate precisely to length. This may be due to some worms passing the sensors sideways or folded, or to small changes in pressure and flow which can alter the time of flight for two worms of the same length. However, we found that sorting for units with TOF values below 200 did not select whole adult worms. Additionally, sorting for five units with TOF values above 1500 resulted in the selection of ten worms, indicating that each reading with a TOF value above 1500 was for two worms together. Therefore, by utilizing only TOF values between 200 and 1500, COPAS data can be limited to the size of single adult worms.

3.3. Acute sodium arsenite toxicity is detected by PI fluorescence

The CERCLA Priority List of Hazardous Substances, maintained jointly by the Agency for Toxic Substances and Disease Registry and the Environmental Protection Agency, ranks arsenic first among substances that pose significant threats to human health (ATSDR and DHHS, 2007). Sodium arsenite (NaAs) is toxic to C. elegans, with an approximate survival rate of 70% after exposure to 195 ppm for 24 h on young adult worms (Liao et al., 2010), and as little as 7 ppm altering larval growth rates (Liao et al., 2010; Sperando et al., 2009). Using NaAs as a model toxicant with 195 ppm as an intermediate dose, we tested whether COPAS analysis would correlate red fluorescence values from PI with NaAs concentration.
Fig. 2. COPAS PI red fluorescence values above 50 (instrument specific units) corresponded to damaged and dead C. elegans. Using the sorting feature of the COPAS on adult day-14 C. elegans, worms were selected for the red fluorescence values indicated at left. (A and B) COPAS sorting for units with red fluorescence readings of 50 and below selected apparently healthy, mobile worms. The majority had dark anterior intestines which nearly fill the body cavity, and normal age-associated gonadal atrophy. (C and D) PI staining of dead tissue within gonadal lesions (arrows) is apparent in live worms with vulval abnormalities (asterisk). (E and F) Pictured worms are alive, but have severe internal degradation and no longer move normally. (G and H) Dead worms do not necessarily fluoresce more brightly than very sick worms. Fluorescence images are all 100 ms exposures with identical brightness and contrast manipulations for presentation.

Fig. 3. COPAS analysis: distinguishing data from artifacts. (A) All readings from age synchronized, PI treated adult day-5 C. elegans, plotted for EXT vs. TOF. (B) The same data after readings with red fluorescence values of zero were removed from the data set.
After a 30-h treatment in CeHM using age synchronized adult day-4 *C. elegans*, we found that increasing concentrations of NaAs from zero to 585 ppm did not induce changes in observed size or optical density (Fig. 4A and C), which was reflected in similar graphs of COPAS EXT vs. TOF values for control and 585 ppm NaAs treated populations (Fig. 4E and F). When looking at red fluorescence levels however, the differences between the four treatment groups of 0, 65, 195 and 585 ppm NaAs were marked. For the control group, only two out of every hundred worms had a CRF > 50 (Fig. 4B and G). The fraction of the 65 and 195 ppm NaAs treatment groups with CRF > 50 increased to 0.07 and 0.22, respectively (Fig. 4I). At 585 ppm NaAs, the fraction of the treated population with CRF > 50 increased to 0.96 (Fig. 4D, H, and I). These data demonstrate that PI fluorescence can be used to assess acute toxicity and dose response in *C. elegans*.

3.4. Acute toxicity ranking in adult *C. elegans* using PI and COPAS analysis mimics rat LD50 ranking

To determine if PI fluorescence as measured by the COPAS can be used with adult *C. elegans* to rank toxicity, we selected five metal salts, cadmium chloride (CdCl2), copper chloride (CuCl2), mercury chloride (HgCl2), potassium chloride (KCl), and sodium arsenite (NaAs), for further testing using a 30-h treatment on day-4 adults. These compounds have rat LD50s ranging from 1 to 26000 mg/kg (Table 1). M9, a buffered salt solution commonly used in *C. elegans* manipulations, when combined with high concentrations of CdCl2 or CuCl2, induced the formation of apparently crystalline precipitates on the order of 10–100 μm in diameter. High concentrations of CdCl2 in CeHM induced formation of precipitated matter in clumps of 1 mm or more. Therefore, this 30-h assay was performed in CeHR. In three separate trials, at all concentrations tested, nearly all of HgCl2 treated *C. elegans* had CRF > 50, making HgCl2 the most toxic compound tested in this assay (Fig. 5A). At 1000 and 500 ppm, NaAs also killed most of the worms, with both concentrations producing CRF > 50 in a minimum 0.98 of the treated populations. A dose response was seen at the lower NaAs concentrations, with 250 and 125 ppm giving CRF > 50 fractions of 0.89 ± 0.01 (mean ± S.D.) and 0.22 ± 0.05 respectively. CdCl2 treatment induced a clear dose response at all tested concentrations, with 1000, 500, 250 and 125 ppm inducing CRF > 50 fractions of 0.62 ± 0.08, 0.40 ± 0.04, 0.23 ± 0.06, and 0.15 ± 0.03 respectively. At 1000 ppm CuCl2, the CRF > 50 fraction was 0.36 (±16). Lower CuCl2 concentrations had CRF > 50 fractions of 0.89 ± 0.01 (mean ± S.D.) and 0.22 ± 0.05 respectively. CdCl2 at concentrations above 62.5 ppm caused elements in CeHM to agglomerate, preventing the wash steps that are required prior to COPAS analysis. As dietary deprivation in post-larval *C. elegans* extends rather than shortens lifespan (Lee et al., 2006; Szewczyk et al., 2006), death in samples with agglomerated matter was likely caused by cadmium toxicosis rather than by reduced nutrient availability. CuCl2 at 500 ppm also killed all of the animals by day 11; however, at 125 ppm CuCl2 approximately 80 to 90% of animals were observed to still be moving on day 11. By visual inspection, KCl did not have an apparent effect at any tested concentration.

Using the four controls in this experiment to determine a baseline of variability from sample to sample within the same experiment, we found the fractional population with CRF > 50 varied from 0.06 to 0.07, average EXT from 809 to 848, and average TOF from 914 to 969 (Table 2). COPAS values for 500 ppm KCl fell within the range or very close to those for controls, indicating that it is not toxic to *C. elegans* at tested concentrations. As CeHR contains over 5000 ppm potassium in the form of potassium citrate and potassium phosphate, this finding is not surprising.

At 7.8 ppm, the most striking difference in COPAS values among tested compounds was for CdCl2 with a reduced average EXT, which corresponded to observed intestinal thinning in this treatment group (Fig. 6A and B). Intestinal abnormalities, including disrupted cytosomes and shortened microvilli, have been reported in *C. elegans* treated chronically with as little as 1 ppm CdCl2 (Popham and Webster, 1979). In several other trials not reported here, we have found that low EXT values correspond with thinning or other abnormalities of the intestine, indicating that for adult *C. elegans* grown in liquid culture, COPAS EXT values have the potential to be used as an indication of intestinal health.

At 31.25 ppm, HgCl2 had the greatest effect on fractional CRF > 50 values (Table 2) and by microscopy observation, most HgCl2 treated worms were dead at this concentration. All CdCl2 treated worms observed by microscopy appeared small and pale at this concentration (Fig. 6E), and this was reflected in low TOF and EXT values. The fractional CRF > 50 for HgCl2, CdCl2, and CuCl2 was 0.81, 0.66, and 0.19 respectively. The fractional CRF > 50 for 500 ppm KCl was 0.06, therefore the toxicity ranking in this initial 2-week trial was HgCl2 > CdCl2 > CuCl2 > KCl.

3.5. Evaluation of chronic toxicity testing with metal chlorides

The symptoms of acute and chronic poisoning with the same compound differ, as do recommended tests and treatments (Lussier et al., 1985; Ratnaile, 2003). Additionally, relative toxicity ranking differs among species used as environmental indicators of contamination (Lussier et al., 1985). Having demonstrated the utility of screening *C. elegans* using the COPAS with PI to measure acute toxicity, we next wanted to test this model’s effectiveness in measuring chronic toxicity. Depending on environmental conditions such as temperature and nutrient availability, wild type *C. elegans* have a life span of approximately 3–5 weeks. We therefore conducted a preliminary two-week chronic toxicity trial using heavy metal chlorides HgCl2, CdCl2, CuCl2 and KCl at four concentrations in 4-fold steps from 7.8 to 500 ppm in CeHM to determine concentrations that would maximally affect adult *C. elegans* without killing all of them before the end of the test.

One limitation of COPAS analysis of PI fluorescence levels at a single two-week time point was that *C. elegans* that had been dead for extended periods did not fluoresce as brightly as those that had died recently (data not shown). Additionally, differences in the efficiency of centrifugation between live and dead animals favors recovery of live animals in wash steps that are required for changing media and COPAS analysis. Therefore, samples in which all animals were dead in 11 days or less were not analyzed.

HgCl2 at 500 and 125 ppm killed all adult *C. elegans* within 2 days. About half of the 500 ppm CdCl2 treated animals were dead by day 8 and all were dead by day 11. At least half of the animals at 125 ppm CdCl2 were dead by day 11 (data not shown). Unfortunately, the sample with 125 ppm CdCl2 could not be analyzed, as CdCl2 at concentrations above 62.5 ppm caused elements in CeHM to agglomerate, preventing the wash steps that are required prior to COPAS analysis. As dietary deprivation in post-larval *C. elegans* extends rather than shortens lifespan (Lee et al., 2006; Szewczyk et al., 2006), death in samples with agglomerated matter was likely caused by cadmium toxicosis rather than by reduced nutrient availability. CuCl2 at 500 ppm also killed all of the animals by day 11; however, at 125 ppm CuCl2 approximately 80 to 90% of animals were observed to still be moving on day 11. By visual inspection, KCl did not have an apparent effect at any tested concentration.

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3.6. Morphological observations subsequent to chronic heavy metal exposure

Among the control and 31.25 ppm CuCl2 treatment groups after 2 weeks of treatment, encapsulated gonadal lesions were observed
Fig. 4. Acute sodium arsenite toxicity was detected by COPAS PI red fluorescence values. (A) Control adult day-5 C. elegans. (B) 100 ms Exposure of the same field. (C and D) After 30 h in 585 ppm NaAs followed by PI staining, most worms were dead and fluoresced brightly. (E) The same data as shown in Fig. 3A, with readings removed that had zero for red fluorescence and/or TOF values of less than 200 or greater than 1500. (F) In the 585 ppm NaAs treated group, readings for EXT and TOF were similar to controls. (G) COPAS values of red fluorescence plotted against TOF show that PI staining was generally low in controls, and (H) increased dramatically after treatment with 585 ppm NaAs. (I) Data plotted as the fraction of each population with CRF > 50.
in 3 of 20 and 2 of 11 respectively, with the remainder showing normal gonadal atrophy (Fig. 6C and F). In contrast, among the 31.25 ppm HgCl2 and CdCl2 treatment groups, 9 of 20 and 17 of 20 were observed to have encapsulated gonadal lesions, respectively (Fig. 6D and E). Thin but intact intestines were observed in 3 of 20 controls, and 4 of 11 from the 31.25 ppm CuCl2 treatment group with the remainder having smoothly outlined anterior intestines which nearly filled the body cavity, while thin or apparently degraded intestines were observed in 11 of 20 and 18 of 20 from the 31.25 ppm HgCl2 and CdCl2 treatment groups, respectively. These intestinal differences were reflected in COPAS EXT values for each group (Table 2). The 2-week treatment with 125 ppm CuCl2 also induced a very low average EXT, and correspondingly, the highest CRF > 50, but TOF and EXT values were highly variable (Fig. 7). In contrast, while TOF and EXT values for CuCl2 were consistently close to control values, CRF > 50 fractions varied from control to CdCl2 levels. COPAS values for CdCl2 fell between HgCl2 and CuCl2 values for CRF > 50, TOF and EXT in most, but not all trials. Every effort was made to maintain consistent conditions and handling, however, small changes in temperature can have large effects on COPAS analysis of PI fluorescence in C. elegans. Media changes and wash steps may be selective. We conclude that COPAS analysis of PI fluorescence in C. elegans is a less reliable method to rank toxicity than 30-h treatments on adults.

### Table 1
Toxicity ranking as measured by rat oral LD50s (ScienceLab, 2010).

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Rat Oral LD50 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercuric chloride</td>
<td>1</td>
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<tr>
<td>Sodium arsenite</td>
<td>41</td>
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<tr>
<td>Cadmium chloride</td>
<td>88</td>
</tr>
<tr>
<td>Copper chloride</td>
<td>584</td>
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<tr>
<td>Potassium chloride</td>
<td>2600</td>
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### Table 2
COPAS measurements on wild type C. elegans after a 2-week treatment with metal chlorides and subsequent PI staining.

<table>
<thead>
<tr>
<th>Compound ppm</th>
<th>n</th>
<th>CRF &gt; 50</th>
<th>TOF</th>
<th>S.D. EXT</th>
<th>S.D.</th>
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</thead>
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<tr>
<td>HgCl2</td>
<td>0</td>
<td>2887</td>
<td>0.06</td>
<td>969</td>
<td>189</td>
</tr>
<tr>
<td>HgCl2</td>
<td>7.8</td>
<td>2912</td>
<td>0.16</td>
<td>981</td>
<td>186</td>
</tr>
<tr>
<td>HgCl2</td>
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<td>2934</td>
<td>0.81</td>
<td>815</td>
<td>195</td>
</tr>
<tr>
<td>HgCl2</td>
<td>125</td>
<td>500</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CdCl2</td>
<td>0</td>
<td>2960</td>
<td>0.06</td>
<td>914</td>
<td>205</td>
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<tr>
<td>CdCl2</td>
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<td>0.13</td>
<td>940</td>
<td>157</td>
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<td>CdCl2</td>
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<td>0.66</td>
<td>681</td>
<td>167</td>
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<tr>
<td>CdCl2</td>
<td>125</td>
<td>500</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuCl2</td>
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<td>2856</td>
<td>0.07</td>
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<td>194</td>
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<tr>
<td>CuCl2</td>
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<td>0.11</td>
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<td>180</td>
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<tr>
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<tr>
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<td>500</td>
<td>2939</td>
<td>0.06</td>
<td>899</td>
<td>196</td>
</tr>
</tbody>
</table>

a n Represents the number of readings with red values greater than zero and TOF values between 199 and 1501 for each condition analyzed by COPAS in a single experiment.
b The fraction of worms in each sample with a COPAS red fluorescence value from PI greater than 50 (instrument specific units).
c The average and standard deviation of EXT and TOF values for each sample condition.
d All animals were dead before the experiment was completed and were not analyzed by COPAS.

CdCl2, and in only 1 of 20 from the 31.25 ppm HgCl2 treatment groups, while no internal eggs were observed in the control or 31.25 ppm CuCl2 groups.

### 3.7 In C. elegans, evaluations of chronic toxicity using COPAS data from a single concentration at a single two-week time point produce inconsistent results

For further chronic toxicity testing, we selected a single concentration of 31.25 ppm. In four trials, HgCl2 consistently produced the highest CRF > 50, but TOF and EXT values were highly variable (Fig. 7). In contrast, while TOF and EXT values for CuCl2 were consistently close to control values, CRF > 50 fractions varied from control to CdCl2 levels. COPAS values for CdCl2 fell between HgCl2 and CuCl2 values for CRF > 50, TOF and EXT in most, but not all trials. Every effort was made to maintain consistent conditions and handling, however, small changes in temperature can have large effects on C. elegans lifespan. Additionally, C. elegans that have been dead for several days do not settle during centrifugation steps with the same efficiency as live and recently dead animals, therefore media changes and wash steps may be selective. We conclude that COPAS analysis of PI fluorescence in C. elegans is a less reliable method to rank toxicity than 30-h treatments on adults.

### 4. Discussion

The three Rs strategy of alternatives to animal testing involves reduction in the use of higher animals, refinement of current techniques, and ultimately replacement with alternative methods (Russell and Burch, 1959). To further these goals, a collaborative effort to develop new methods of toxicity analysis was begun in 2008 by the National Institutes of Environmental Health Sciences, the National Human Genome Research Institute, and the U.S. Environmental Protection Agency, and joined by the U.S. Food and Drug
Administration in 2010. One of the central tenets of this collaboration, informally called Tox21, is the exploration of novel high throughput screening assays and tests using phylogenetically lower animal species such as *C. elegans*.

In developing replacements for testing in higher animals, it is essential to define the limits within which alternative animal models have the potential to provide human relevant results. Advantages of utilizing *C. elegans* as a model organism include low...

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**Fig. 6.** Two-week treatments with metal chlorides induce morphologic changes in adult *C. elegans*. (A) In most adult day-14 *C. elegans* from control groups, the anterior intestine (arrowhead) is dark, relatively smooth, and nearly fills the body. Scale bar in (A) is for images A and B. (B) Representative image demonstrates that anterior intestines were thinner in the group treated with 7.8 ppm CdCl₂ for 2 weeks. (C) Image of control FUdR-treated *C. elegans* shows intact vulva (asterisk), normal age-associated atrophy of the uterus (arrows), and oocytic material visible as circles at the sides of the image. (D) Vulval abnormalities and gonadal lesions (arrowheads) were frequently observed in the 31.25 ppm HgCl₂ treatment group. (E) 31.25 ppm CdCl₂ also induced Vulval and gonadal changes. Thin, pale intestines correspond to low COPAS EXT values for this group. (F) The majority of *C. elegans* treated with 31.25 ppm CuCl₂ for 2 weeks appeared similar to controls. (E) Vulval abnormalities (asterisk) and internal eggs were frequent in the 125 ppm CuCl₂ treatment group. Scale bar in (G) is for images C and G. All images presented are of live adult day-14 *C. elegans*.
C. elegans is the first multicellular organism to have its complete genome sequenced, and genetic analysis indicates a high degree of conservation with mammals (Bargmann, 1998; Consortium, 1998). Disadvantages of using C. elegans for toxicity screening include a lack of many structures common to higher organisms, such as respiratory and cardiovascular systems. Adult somatic cells are post-mitotic, therefore C. elegans is not likely to be a good model for the assessment of carcinogens. Additionally, C. elegans has a tough collagenous cuticle which protects it from the environment. Therefore, as a model organism for toxicity screens, C. elegans is likely to be useful in the study of oral routes of exposure, rather than toxicity following dermal absorption or inhalation.

Wild type C. elegans hermaphrodites lay approximately 275 eggs by self-fertilization (Byerly et al., 1976). There are several methods for maintaining a C. elegans experimental cohort without progeny, and each has disadvantages. One method is to individually transfer adult worms away from eggs and hatchlings to a fresh environment daily during the reproductive period (Johnson and Wood, 1982). This process is time consuming, and manual manipulations carry the potential for structural damage to the worms. Another method utilizes temperature sensitive mutant strains that are fertile at permissive temperatures and infertile at non-permissive temperatures, but apparently wild type in all other respects (Nelson et al., 1978). Strain stocks are maintained at permissive temperatures while experimental cohorts are transferred to non-permissive temperatures during development so that testing is carried out on sterile adults. However, the low yet significant rate of approximately 0.1 progeny per adult raised at the non-permissive temperature (Mitchell et al., 1979) is unacceptable in liquid culture experiments with thousands of worms per flask. The thymidylate synthetase inhibitor FUdR (Cohen et al., 1958) completely blocks embryogenesis in C. elegans grown in axenic liquid culture, and has been reported to have no significant effects on the rates of growth, aging, or pharyngeal pumping when used at 25 μM on wild type worms (Gandhi et al., 1980). Recently however, the widespread use of FUdR in lifespan analyses of C. elegans has been called into question due to studies which have shown that FUdR at concentrations of 50 μM and above in agar based media can significantly increase the lifespan of specific genetic mutant strains (Aitkhadj and Sturzenbaum, 2010; Van Raamsdonk and Hekimi, 2011), and that FUdR is protective in some models of stress (Suzanne Angeli and colleagues, personal communication). We selected FUdR for synchrony maintenance due to its widespread use and ease of application in large scale experiments, but acknowledge that it may have metabolic effects with the potential to interact with specific toxic responses.

We found that PI can be used as an indicator of morbidity and mortality resulting from a variety of causes, in both FUdR synchronized and mixed age populations. Given that the intensity of red fluorescence varied considerably among dead and dying worms, with structurally damaged live worms and those dead for intermediate periods fluorescing more brightly than those recently and long dead, we found that measuring the fraction of C. elegans with red fluorescence from PI of greater than 50 COPAS specific units provided a measure of morbidity and mortality within a treatment population. Our results with increasing concentrations of NaAs indicate that PI used in conjunction with the COPAS can provide a sensitive method to measure acute toxicity in C. elegans. We also demonstrated that population fractions with CRF > 50 after a 30-h treatment using four different concentrations of five metals with a wide range of rat LD50s on adult C. elegans could rank these metals the same order as they are ranked in mammals, with very similar results from three separate trials. Further testing is required to determine if finer distinctions in toxicity ranking can be made with this method.

Results from analyses of PI staining levels by COPAS from chronic treatments were less consistent than those from the 30-h assays. While the average fractional populations with CRF > 50 ranked the three heavy metal chlorides we studied by this method.
in the order HgCl₂ > CdCl₂ > CuCl₂, there was a good deal of variability from one trial to the next, especially for copper. Part of this variability may be due to the loss of dead animals during centrifugation steps required for the 2–3 times weekly changes in media. Since small changes in temperature result in significant changes in C. elegans lifespan, it may be that differences in ambient temperature during the short time that worms were removed from the incubator for manipulations and examination resulted in these differences from one experiment to the next. It may be that chronic toxicity assays in C. elegans that measure CRF > 50 could provide consistent results if certain experimental parameters were altered from the ones we used here, such as multiple concentrations, multiple time-points for COPAS analysis, and/or a later initiation of treatment, as we used in the acute toxicity assays.

Our morphology analyses from chronic toxicity trials did provide potentially interesting mammalian-correlative data. For the more toxic heavy metal chlorides in our two-week study, HgCl₂ and CdCl₂ at 31.25 ppm, abnormal intestinal, gonadal, and Vulval morphology were indicators of chronic toxicity, while visible oocytic material was associated with health in adult day-14 control and CuCl₂ treated C. elegans. Cadmium toxicity is associated with histopathology of the intestine across phyla, including cellular necrosis and abnormalities of the villi in mice (Valberg et al., 1977). Paneth cell vacuolation and abnormalities of the villi in rats (Phillpotts, 1986), cellular necrosis and vacuolation of mucosa and enterocytes in fish (Kruatrachue et al., 2003), and abnormalities of cytosomes and microvilli in C. elegans (Popham and Webster, 1979). After a two-week treatment begun at the fourth larval stage, we observed intestinal thinning in C. elegans treated with 7.8 and 31.25 ppm CdCl₂, and these findings were corroborated by low COPAS EXT values. Copper is known to inhibit reproduction in water fleas (Dave, 1984) and sheep (Murawski et al., 2006), and to inhibit egg laying in C. elegans (Calafato et al., 2008). We observed high levels of retained eggs in 125 ppm CuCl₂ chronically treated C. elegans. Taken together, these data indicate that C. elegans may be a useful predictor of some toxic effects in mammals.

Measuring growth rates, frequency of protruding vulva, brood size, and levels of internal hatching are established methods of determining toxicity in C. elegans (Anderson et al., 2001; Boyd et al., 2010; Calafato et al., 2008; Sprando et al., 2009). To this list, we add analysis of intestinal width and integrity, gonadal lesions, internal eggs, and lack of visible oocytic material in post-reproductive FUDR treated C. elegans as measures of chronic toxicity. Some morphological differences were reflected in COPAS values, as in the observed correlation between intestinal thinning or disruption and low EXT, while others such as gonadal lesions in the absence of Vulval disruption were not, stressing the importance of visual analysis, especially when analyzing potential toxins with unknown effects. Thus, toxicity screens using C. elegans should include morphology analysis in addition to methods such as the one detailed here, metabolic testing, and/or gene expression analyses.

In summary, we have developed a method to quantitatively assess toxicity by measuring morbidity and mortality in C. elegans using axenic liquid culture, a fluorescent dye, and the COPAS. We assessed the usefulness of this method in ranking a small number of compounds of varied but known toxicity. We found that 30-h assays replicated rat LD₅₀ toxicity ranking for five metals and were highly reproducible. While chronic toxicity assays with this method were less consistent, interesting morphology observations provided potentially mammalian-relevant data. We are presently evaluating the usefulness of this method in assessing the toxicity of a number of different chemical compounds including other heavy metals, mycotoxins and sea food toxins. It is our hope that, using C. elegans as an experimental model, we will be able to develop a rapid and low-cost screen to identify potentially toxic compounds. The identification of these potentially hazardous compounds would facilitate a more rational choice for further in vivo testing which would ultimately have the potential to result in reduced toxicity testing in mammals.

**Conflict of Interest**

The authors declare that there are no conflicts of interest.

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