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MEDIUM- AND HIGH-THROUGHPUT SCREENING OF NEUROTOXICANTS USING *C. ELEGANS*

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Abstract

The U.S. National Toxicology Program, the U.S. Environmental Protection Agency, and other national and international agencies are committing significant resources towards the development of alternative species to be used as replacements for mammalian models in toxicological studies. *Caenorhabditis elegans* is a well-characterized soil nematode that is becoming a useful model in the assessment of neurotoxicants. To determine the effects of potential neurotoxicants on *C. elegans*, four medium-throughput (feeding, growth, reproduction and locomotion) and two high-throughput (growth and reproduction) assays have been developed. Three of these assays use the COPAS Biosort, a flow cytometer capable of rapidly measuring thousands of nematodes in minutes. Medium-throughput feeding, growth, and reproduction assays were used to assess the toxicity of eight suspected neurotoxicants. For several of the neurotoxicants examined, significant effects were observed at similar concentrations between assays. High-throughput reproduction and growth assays were used to estimate the toxicity of thousands of chemicals in two libraries. These assays will prove useful in evaluating the role of alternative toxicological models in tiered toxicity testing of thousands of chemicals.

Introduction

Caenorhabditis elegans is a small (~1.5 mm) free living soil nematode. It was originally chosen in 1963 by Sydney Brenner as a model organism to study the genetics of development and the nervous system of a simple metazoan. Today, it is likely one of the most thoroughly studied metazoans in terms of its cell biology, genetics, development, and behavior. *C. elegans* occur in two sexes: self-fertilizing hermaphrodites and males. Hermaphrodites contain 959 somatic cells, while the males have 1031 cells [36]. The difference in cell number is attributed to the specialized structures associated with mating and the male tail including muscles, neurons and neuronal support cells, proctodeal, and hypodermal cells [24]. Although there are a limited number of cells, *C. elegans* has highly differentiated digestive, reproductive, muscular, and nervous systems.

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C. elegans develop from fertilized egg to gravid adult in about 3 days at 20°C [32,69]. A single hermaphrodite has the ability to produce approximately 300 offspring. Offspring mature through four larval stages, L1 - L4, growing in spurts between stages after molting old cuticles [14,41]. At two points in the lifecycle, if food is not available, nematode growth will arrest. Animals will survive for several days as starved hatchlings that arrest as L1's, or for several months when starved L2's develop into dauer larvae [42,52].

There is a wealth of knowledge available on *C. elegans* including its complete genomic sequence [59], the technology to quickly produce transgenic nematodes [44], and the ability to observe all of the cells in the living nematode by microscopy [32,65,66]. The available microscopy techniques in *C. elegans* have led to the development of an exceptionally detailed database on *C. elegans* anatomy, as well as on cell and developmental biology [2,32]. This database maps the development of every cell from the fertilized egg to the reproductive adult [57] and all of the neuronal pathways and connections [66].

A detailed map of the complete *C. elegans* nervous system, consisting of the physical location of all of the hermaphrodite neurons and all neuronal connections (neuron-neuron and neuromuscular), was originally described in 1986 by White, *et al* in “The Mind of the Worm” [66]. The adult *C. elegans* hermaphrodite contains 302 neurons in two independent nervous systems: 282 in the somatic nervous system and 20 in the pharyngeal nervous system [56,57,66]. There are approximately 6500 chemical synapses, 900 gap junctions, and 1500 neuromuscular junctions [32]. The pharyngeal nervous system is completely contained within the *C. elegans* pharynx (Figure 1, *inset*), with only four synapses between the pharyngeal and somatic nervous systems [1,8]. These nervous systems control locomotion, feeding, defecation, reproduction, and environmental sensing. *C. elegans* has the ability to sense differences in temperature, chemicals, odorants, and food. The male nervous system contains 473 neurons with the additional neurons involved in mating behavior [22,23,55]. Detailed descriptions of the *C. elegans* neuroanatomy can be found in [32], [66] and [2].

One of the strengths of *C. elegans* as a model organism is the high degree of evolutionary conservation in its biological processes. In addition to evolutionary conservation in basic biological functions, such as intermediate metabolism, DNA, RNA and protein synthesis; orthologs for many of the proteins and regulatory pathways induced as part of the “stress response” in vertebrates have been identified in *C. elegans* [27,29,34,53,64,68].

In terms of neuropharmacology, *C. elegans* express many of the neurotransmitters and associated receptors that are found in higher eukaryotes. These include dopamine, acetylcholine, γ -aminobutyric acid (GABA), glutamate, and serotonin. In addition, several neuropeptides encoding genes have been identified including those encoding insulin-like, FMRFamide (Phe-Met-Arg-Phe-NH₂)-like neuropeptides. Many of the neurons that express specific neurotransmitters have been identified [50].

Tools in robotics, image acquisition and analysis, gene knockout, and gene and protein expression measurements make it possible to study complex biological processes in *C. elegans* in medium- and high-throughput fashions. The defined nervous system, evolutionary conservation in neuronal structure and function, and the ability to utilize medium- and high-throughput technologies make *C. elegans* an excellent organism for the rapid detection of potential neurotoxicants in a non-mammalian organism.

Medium-Throughput Screening Assays

We have developed several semi-automated, medium-throughput assays that can assess the toxicity of approximately five compounds per week. These assays allow for the quantification of the effects of chemicals on growth and development, reproduction, feeding, and locomotion

at different developmental stages. Feeding and locomotion, measured using adult nematodes, are two endpoints that directly assess neurotoxicity. Growth and development from *C. elegans* L1 larvae through 48 h post-hatch provides an indirect measurement of neurotoxicity. Reproduction, measured using young adult animals, provides an indication of the effects of a potential toxicant on development and indirectly, the neuromuscular activity of egg-laying.

Feeding, reproduction, and growth assays utilize the COPAS Biosort, a flow cytometer capable of sorting, dispensing, and measuring size and fluorescence parameters of individual nematodes [47]. In the Biosort, nematodes pass through a flow cell where axial length, extinction, and fluorescence are simultaneously measured using a pair of lasers. Axial length, or time of flight (TOF), is the length of time that a nematode interrupts the light beam. Extinction (EXT), an estimate of the nematode's optical density, is the amount of light that is blocked as a nematode passes in front of the laser. Both TOF and EXT are indicators of the size of an individual nematode, which increase as nematodes develop through larval stages into adults [47].

In these assays, between five and several hundred *C. elegans* are exposed to toxicants in liquid media [3] in 96-well plates. In addition to buffer and toxicant, each well contains *E. coli* OP50 as a food source [54]. After incubating nematodes at 20°C for 4 - 48 h, depending on the assay, they are aspirated from the wells and their behavior (feeding, locomotion) or size characteristics (growth, reproduction) are measured either by the Biosort or by microscopy. The following sections provide details and examples for each assay.

Feeding

Feeding in *C. elegans* is accomplished by the coordinated pumping of three sections of the pharynx (Figure 1, *inset*), which simultaneously push bacteria into the intestine and expel excess liquid out of the mouth [7]. The neuromuscular activity of feeding is regulated by a self-contained nervous system [1]. Feeding activity is affected by internal and external factors, including nutritional status and exogenous application of chemicals [6,37]. Feeding phenotypes have been described by directly observing and manually counting pharyngeal contractions of individual nematodes by microscopy [5], or by recording action potentials of pharyngeal muscles [48]. These methods provide useful information but, for practical reasons, only on few animals per test substance can be evaluated. In addition, these methods require specialized technical experience. As such they are not applicable towards the examination of the differential effects of chemical exposures on pharyngeal activity, in which many more measurements are needed to obtain sufficient statistical power for proper analyses.

We developed a feeding assay capable of measuring hundreds of individual nematodes in minutes using the Biosort [13]. In this assay, 25 synchronized adult nematodes (3d post-hatch) [54] are dispensed into each well of a 96-well plate containing varying concentrations of test toxicant. Following an incubation period of 24 h, red fluorescent microspheres (0.5 µm) are added to each well and nematodes are allowed to feed for 15 min. The amount of accumulated microspheres is determined from the level of fluorescence in each nematode. In addition to fluorescence, nematode sizes (TOF and EXT) are recorded. The feeding activity can then be expressed as size-corrected red fluorescence for cohorts of nematodes [13]. To model

decreasing feeding activity with increasing dose, the Hill expression [35] $Hill = \frac{Vm * dose^x}{km^x + dose^x}$, is subtracted from $Vm+a0$. EC₅₀s are calculated by fitting the medians of the log(red fluorescence)/log(TOF) values for each cohort to the decreasing Hill equation as a function of dose (Figure 1):

$$\text{red fluorescence} = \frac{V_m}{1 + \left(\frac{\text{dose}}{k_m}\right)^x} + a_0 \quad (\text{eq. 1})$$

Here, $V_m + a_0$ denotes the red fluorescence for the non-treated nematodes and is the maximum value the expression can have. The value of the expression decreases to the lower asymptote, a_0 , as toxicant concentration increases. The lower asymptote represents a maximum possible decrease in red fluorescence. For the neurotoxicants and concentrations presented in this review, a_0 ranged from 18% (chlorpyrifos) to 36% (cocaine base) of $V_m + a_0$. The parameter, k_m , is the estimated concentration that reduces the red fluorescence to halfway between $V_m + a_0$ and a_0 , which is, the EC_{50} . Finally x is the shape parameter that governs the steepness of the decrease.

The effect of varying concentrations of chlorpyrifos oxon on feeding is presented in Figure 1. Here, the EC_{50} is estimated as 0.31 μM . A similar approach was used to test the effects of seven other known developmental neurotoxicants on *C. elegans* feeding (Table A). The EC_{50} s for these neurotoxicants range from $\sim 0.14 \mu\text{M}$ (chlorpyrifos) to $\sim 122 \mu\text{M}$ (cadmium chloride).

Reproduction

A common phenotype observed after exposure to chemicals or as a result of genetic mutations is a change in the number of offspring produced by a single nematode. The production of offspring may be affected at many stages including oocyte and sperm formation, fertilization, embryonic development, or egg-laying. Egg-laying behavior is regulated internally by signals to the vulva muscle (Figure 2, *inset*), which can be disrupted by environmental conditions such as exposure to chemicals [10,61,63]. *C. elegans* reproduction is routinely measured by manually counting the number of offspring as a function of time [58]. An alternative assay was developed that measures the amount of chitinase, a molecule released after egg hatching. This is an indirect measure of reproduction, but provides information on oocyte fertilization and ability to hatch [40].

We developed a reproduction assay that uses the Biosort to directly count the number of offspring produced. In this assay, five age-synchronized L4 larvae are placed into each well of a 96-well plate containing varying concentrations of neurotoxicants. After incubating for 48 h, the size and stage of development of the adults and offspring are measured. The number of larvae produced during this time period is an indication of the effect of the toxicant on nematode fecundity. The numbers of nematodes are directly fit to the Hill expression used to analyze the feeding assay (eq. (1)). Over all assays, the maximum number of control offspring ranged from 268 to 318 per replicate.

The effect of varying concentrations of hexachlorophene on reproduction is presented in Figure 2. The EC_{50} is estimated as 6.3 μM . The effect of eight known developmental neurotoxicants on *C. elegans* reproduction was determined and EC_{50} values estimated using equation 1 (Table A). The rank order of EC_{50} s in the reproduction assay was similar to those observed in the feeding assay with chlorpyrifos, chlorpyrifos oxon, and methyl mercury being the most toxic and cadmium chloride, chlordiazepoxide, and tebuconazol being the least toxic.

Growth

We developed a 48-h growth assay to measure nematode growth rates at several stages of development. In this assay, 50 synchronized L1 larvae are dispensed into each well of a 96-well plate containing varying concentrations of toxicant. Following incubation for 48 h at 20°

C, the time necessary for untreated nematodes to develop to the final larval stage (L4), the size distribution of nematodes in each well is determined by measuring TOF and EXT.

Using the log(EXT) measurements, growth is defined as the change in the distribution of sizes of nematodes measured when first loaded into the plates, compared to the distribution of sizes when measured after 48 h. Because the initial measurement on a specific nematode can not be matched with its final measurement, our method models the growth distribution of the initial cohort of nematodes. Briefly, the range of log(EXT) is divided into a number of bins. Each nematode is assumed to advance from its initial bin to larger log(EXT) measurement according to a uniform probability model. A weighted mixture of the lognormal distribution and the modeled nematode distribution is fit to the observed measurements. Using the estimated number of bins advanced, values are derived for the mean size (log(EXT)) and growth rates in terms of log(EXT)/h at each toxicant concentration. To estimate EC₅₀ values, the decreasing Hill expression (eq. (1)) is fit as a function of toxicant concentration to both the estimated means and estimated growth rates. In cases where estimated means and growth rates are both observed at the lower asymptote, the estimated EC₅₀s are very similar (Table B). Figure 3 illustrates the effects of 48 h exposures on the mean log(EXT) values at six concentrations of chlorpyrifos on *C. elegans* development. Based on the analysis of estimated log(EXT) means, an EC₅₀ of 22.9 μ M was calculated. For the growth assay, chlorpyrifos and chlorpyrifos oxon were the most toxic, while cadmium chloride and tebuconazole were the least (Table B).

Motion tracking

C. elegans locomotion is regulated by the same neurotransmitters as in mammals, with acetylcholine being the primary excitatory neurotransmitter [51]. *C. elegans* locomotive behavior has been studied on solid, agar surfaces and in liquid medium [17]. On solid surfaces, nematodes move forward or backward by bending the body, making sinusoidal waves [11].

Abnormal locomotive behavior, referred to as uncoordinated movement, is a commonly observed phenotype in *C. elegans* behavioral assays. Characterization of *C. elegans* movement has historically been made qualitatively by visual examination. Recently, different locomotive behaviors have been categorized using computer-aided visualization techniques. In these studies, single nematodes are observed using microscopes equipped with automated tracking systems [9,18,26,28,33,38]. Once identified, the single nematode can be tracked for several minutes to hours. Analysis of the nematodes can be quantified by over 60 movement parameters.

Another approach is to track tens to hundreds of nematodes simultaneously at low magnification [67]. Centroids of multiple nematodes are tracked using dark-field microscopy, where *C. elegans* appear as bright objects. The computer then tracks nematode movement in real time. Using this system, the effects of metals, ethanol, solvents, and organophosphate and carbamate pesticide exposures on movement have been quantified [3,4,12,15,20,30,45].

In our assay, we use a *myo-2::GFP* strain of *C. elegans* (CB5584), which expresses high levels of green fluorescent protein in pharyngeal muscles. Motion tracking is performed on an inverted fluorescence microscope equipped with a CCD camera, motorized stage and an incubator; which maintains constant temperature and humidity throughout the image capture process. In this system, computer tracking monitors head movement rather than the centroid of the nematode. After exposing L4 *C. elegans* to neurotoxicants for 4 h, nematodes are transferred to agar pads. Each agar pad holds approximately 40 animals, which are tracked at low magnification for ~35s (3.5 frames/sec). Motion tracking software then generates Computer Assisted Sperm Analysis-like statistics on each nematode [19,62], which can then be compared between treated and untreated nematodes. Figure 4 illustrates the effects of methyl mercury on *C. elegans* behavior after a 4 h exposure. Fitting the Hill equation (eq. (1)) to the

average velocity yields an estimated EC₅₀ of 10.0 µm/s. A similar analysis was performed on *C. elegans* exposed to chlorpyrifos with an estimated EC₅₀ of 1.1 µm/s. Similar to feeding and reproduction, chlorpyrifos was more toxic than methyl mercury.

High-Throughput Screening Assays

We have tested both reproduction and growth assays as high-throughput screening tools to estimate the potential toxicities using two chemical libraries: the National Toxicology Program's (NTP's) 1408 [70] and the EPA's ToxCast 320 pesticides actives [21]. Working stock solutions of the NTP or EPA chemical libraries were prepared in 96-well plates and dissolved in DMSO. Multiple 96-well plates with varying concentrations were prepared for each set of chemicals. For each plate, eight chemicals were diluted to the desired concentration in 1% DMSO for exposure plates and were replicated in three treatment wells per chemical at a single concentration. Each plate also contained four vehicle control wells and four positive control wells (1 and 4 % DMSO, respectively). Remaining wells were either rinse wells, to avoid carryover of nematodes between wells, or were empty.

Within the NTP library of 1408 chemicals, approximately 20% caused significant decreases in 48-h reproduction, compared to controls. However, the variability in the number of L4's loaded and the inherent biological variability of egg production and egg-laying led to wide variability in control groups.

In contrast, the 48-h growth assay showed much less variable from plate-to-plate and therefore the data were more consistent. The growth of control populations does vary from day-to-day, however, the change in log(EXT) is predictable over the 48-h exposure period. The 48-h growth assay was used to screen the EPA's ToxCast 320 at a single concentration of 200 µM. For approximately half of the chemicals, nematode growth was clearly reduced, while another quarter of the chemicals did not affect growth relative to control groups. Analytical techniques are being developed to determine the relative toxicity of the remaining chemicals that were not clearly positive or negative.

Discussion

Four medium- and two high-throughput assays are described that measure the toxicity of potential neurotoxicants using *C. elegans* as an alternative to mammalian model organisms. The sensitivity of the four endpoints (growth, feeding, reproduction, and locomotion) varied between chemicals. Similar EC₅₀s were estimated for effects on feeding, reproduction, and growth for chlordiazepoxide and hexachlorophene (Tables A, B). Cocaine, cadmium chloride, and tebuconazol affected reproduction more than feeding and growth, while chlorpyrifos and chlorpyrifos oxon caused greater effects on feeding and reproduction than growth at similar concentrations. Locomotion after methyl mercury exposure was also affected at similar concentrations as reproduction and growth (Tables A, B and Fig. 4). Differences in EC₅₀ values between endpoints may be a consequence of the mechanism of action of the neurotoxicant. For example, a neurotoxicant that specifically targets neuromuscular activity may have similar EC₅₀s for feeding, motion and reproduction, but not growth. In contrast, a toxicant that affects cell differentiation or development may significantly inhibit growth and reproduction without affecting feeding or locomotion.

In the development of any medium- or high-throughput *in vivo* assay, classification of chemical toxicity is usually based on comparing treated individuals with animals under non-exposed or control conditions. Therefore, the assay with lower control variability is considered to be more reliable. Feeding, reproduction, and growth were examined as potential endpoints in high-throughput screening protocols. Feeding behavior varies greatly between isogenic individuals and is strongly influenced by environmental factors such as food availability and temperature

[49]. Although environmental conditions are carefully controlled in all experiments, control feeding activity varies across experimental days and a large range of feeding activities are observed in control populations within the same day [13].

Variability between wells within a plate, plates within a day, and day-to-day was observed in the reproduction assay. Some of this variability is due to technical limitations of the COPAS Biosort. The Biosort dispenses nematodes into wells with ~90% accuracy (Union Biometrica, personal communications). However, slight inaccuracies in the number of nematodes dispensed into wells can result in a large variability in the numbers of offspring within a well.

Day-to-day variability is also routinely observed in data from the 48-h growth assay. Some of this variability can be attributed to differences in individual growth rates. However, overall, the 48-h growth assay has the lowest variability among controls in the three assays. For this reason, the 48-h growth assay was selected for future high-throughput screening of chemical libraries.

Comparability to mammalian neurotoxicants

Many technical and statistical challenges in using *C. elegans* as an alternative test organism for *in vivo* medium- and high-throughput screening have been met. The next challenge is to use the data obtained using *C. elegans* to help in the prediction of toxicity in humans. To meet this challenge, the results obtained using *C. elegans* will have to be linked to data obtained using laboratory rodents and, where possible, from humans. Several studies have already demonstrated that changes in *C. elegans* following chemical exposures appear to be predictive of developmental shifts and/or neurological damage seen in laboratory studies using rodents [3,20,25,31,³⁹,43,46,60,67]. Linking cell-based high-throughput screening data to *in vivo* screening results (*C. elegans*, *Drosophila*, zebrafish) to inform on the toxicity of different agents in humans is the ultimate goal of a collaborative research program established by the NTP, the EPA, and the National Institutes of Health Chemical Genomics Center (NCGC) [16].

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Figure 1. Effects of chlorpyrifos oxon on *C. elegans* feeding

For each of two replicate experiments (shown as blue and red), groups of 25 adult *C. elegans* were exposed to one of five concentrations of chlorpyrifos oxon (plus controls) for 24 h. Medians of log(RFP)/log(TOF) values are plotted and the data fit to the Hill equation. *inset*, adult *C. elegans* pharynx.

Figure 2. Effects of hexachlorophene on *C. elegans* reproduction

For each of three replicate experiments (shown as blue, red, and green), groups of five L4 nematodes were exposed to one of five concentrations of hexachlorophene for 48 h. The numbers of observations (larvae and embryos) were fit to the Hill equation. *inset*, region around an L4 *C. elegans* vulva: *I*, intestine; *UL*, uterine lumen; *Hyp*, hypodermis; *Vul*, vulva.

Figure 3. Effects of chlorpyrifos on *C. elegans* growth

For each of three replicate experiments (shown as blue, red, and green), groups of 50 L1 nematodes were exposed to one of five concentrations of chlorpyrifos for 48 h. *Left panel*, observations measured and estimated means fit to Hill equation. *Right panel*, modeled frequency histograms showing the effects of different concentrations of chlorpyrifos (0, 0.1, 0.3, 1.0, 3.0, and 10 μM) on nematode size distribution (log(EXT)) for one of the replicates.

Figure 4. Effects of methyl mercury on *C. elegans* movement

Groups of 40 L4 nematodes were tracked after exposure to one of five concentrations of methyl mercury for 4 h. *Left panel*, average velocity of *C. elegans* motion, mean $\pm$ s.e. *Right panel*, computer generated tracks for control nematodes (*top*) and those exposed to 10 μ M methyl mercury (*bottom*).

Table A
Toxicity of Eight Developmental Neurotoxicants on *C. elegans* Feeding and Reproduction

Toxicant	EC ₅₀ (μM)	
	Feeding	Reproduction
chlorpyrifos	0.141 (0.051, 0.23) ^a	0.09 (0, 0.23)
chlorpyrifos oxon	0.31 (0, 0.81)	0.28 (0, 1700)
hexachlorophene	16.6 (8.1, 25)	6.3 (4.0, 8.5)
chlordiazepoxide	68.6 (0, 290)	49.9 (23, 77)
cocaine base	94.5 (0, 5800)	4.2 (2.5, 6.0)
methyl mercury	112 (0, 270)	1.42 (0.75, 2.1)
cadmium chloride	122 (0, 406)	18.0 (14, 22)
tebuconazol	180 (76, 280)	46.7 (11, 82)

EC₅₀, effective concentration that reduces feeding or reproduction activity to half the maximum activity

^a95% confidence interval

Table B
Effects of Eight Developmental Neurotoxicants on *C. elegans* Growth

Toxicant	EC ₅₀ (μM)	
	Means	Growth rates
chlorpyrifos	22.9 (13, 33) ^a	3.9 (2.4, 5.4)
chlorpyrifos oxon	8.8 (0, 65)	5.0 (3.3, 6.7)
hexachlorophene	18.2 (12, 24)	19.3 (16, 23)
chlordiazepoxide	64.8 (39, 91)	66.3 (28, 105)
cocaine base	34.6 (0, 86)	72 (0, 180)
methyl mercury	11.4 (10, 13)	11.2 (9.5, 13)
cadmium chloride	1131 (1097, 1166)	502 (447, 557)
tebuconazol	481 (284, 677)	241 (179, 302)

EC₅₀, effective concentration that reduces mean growth or growth rate to half the maximum

^a95% confidence interval