TOXICOLOGICAL ASSESSMENT OF CHEMICALS USING CAENORHABDITIS ELEGANS AND OPTICAL OXYGEN RESPIROMETRY

KATHERINE SCHOUEST,† ALICE ZITOVA,‡ CHARLES SPILLANE,† and DMITRI B. PAPKOVSKY*‡
†Genetics and Biotechnology Lab, Department of Biochemistry, BioSciences Institute, ‡Biophysics and Bioanalysis Lab, Department of Biochemistry, Cavanagh Pharmacy Building, University College Cork, College Road, Cork, Ireland

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Abstract—Oxygen consumption is indicative of an organism’s metabolic state, whereby alterations in respiration rate can result from the presence of different stimuli. Here, we develop a novel approach based on quenched fluorescence oxygen sensing and respirometry method for toxicity screening assays using the nematode Caenorhabditis elegans. Previously, C. elegans was established as a useful model in soil and aquatic toxicology studies. For existing toxicology screening approaches with C. elegans, however, the endpoint is lethality. In addition, the assay time frame for the existing approaches is considerably longer than that for the approach described here. We present a sensitive, robust, high-throughput platform using standard laboratory equipment for toxicological studies by measuring respiration rate in C. elegans animals using a phosphorescent probe.

Keywords—Toxicity screening assay Oxygen consumption Quenched fluorescence oxygen sensing Optical oxygen respirometry

INTRODUCTION

The widespread use and release of both natural and synthetic chemicals in the environment (singly or as complex domestic and industrial effluents) has acted as a driver for the development of rapid and cost-effective toxicity testing methods to protect humans and other biota. Also, the pharmaceutical and drug discovery industry are now dealing with an increasing number of new chemical entities and compound libraries for which rigorous toxicity and safety assessment is the key component of the preclinical drug development process. Toxicity assessment with conventional animal models (typically rodents and fish) entails some major drawbacks, including high cost, need for ethical approval, and long assay times.

In recent years, a range of alternative animal models for toxicity screening have received considerable attention because of their small size, high fecundity, and lower costs for toxicological analysis of chemical and environmental samples. For instance, a number of toxicity assays have been developed using bacteria (Escherichia coli) [1], mammalian cell lines [1], invertebrates (Artemia salina) [1], and vertebrates (zebrafish) [1]. Toxicity testing using animal models has been based primarily on assessment of mortality, which is a relatively long and subjective assay with a low level of automation (throughput) and moderate sensitivity. Because of these bottlenecks, new biomarkers and analytical systems, which allow rapid, cheap, and high-throughput assessment for toxicity screening, are being introduced.

Molecular oxygen is an important substrate of aerobic or facultative aerobic microorganisms that can provide valuable information regarding their metabolic activity, health, and responses to various stimuli. Respirometric toxicity assays that are based on monitoring the rate of oxygen utilization by organisms have high potential for toxicity screening. Traditional manometric and polarographic techniques for oxygen measurement produce high-quality data but are expensive, have limited throughput, and are difficult to apply for toxicity screening. Hence, a variety of oxygen sensing and respirometric systems have been developed that are based on fluorescence quenching of dedicated oxygen-sensitive materials [3]. Such systems have been used successfully for toxicological assessment of chemical and environmental samples with a range of biological samples, including prokaryotic and eukaryotic cell cultures [4], and also in whole-model animals, such as Danio rerio (zebrafish) and A. salina (brine shrimp) [1].

Caenorhabditis elegans is a free-living, nonparasitic, multicellular metazoan with a short life cycle of approximately 3 days that has been used as a model organism in developmental biology, behavior, anatomy, and genetics laboratories for more than 30 years [5]. Caenorhabditis elegans is also a highly attractive model for toxicology studies. It is easy to maintain under laboratory conditions, can be grown en masse, and is available throughout the year [6]. A soil-dwelling organism with a wide geographical distribution, it has a high tolerance to pH, salinity, and water hardness [6].

From a toxicological screening viewpoint, C. elegans is sensitive to a wide range of toxicants, including heavy metals, organic phosphates, and pesticides. Such studies have established C. elegans as a powerful model for rapid testing of the toxicity of soil and water samples as well as pharmaceutical compounds. Caenorhabditis elegans offers a wide variety of ecologically and toxicologically relevant endpoints for toxicology screening, such as mortality, life span, behavior/movement, feeding, growth, and reproduction [7]. For mortality or behavior/movement endpoint assays, a short incubation time (24 h [8] or 48 h [6]) commonly is used. Whereas these endpoints generally are used and are easily scored under a microscope, they fail to reveal the more subtle effects of toxic compounds and can be prone to identification of false positives.

* To whom correspondence may be addressed (d.papkovsky@ucc.ie).

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Previous studies on oxygen consumption of C. elegans employed optical fibers and silicone grease as a sealant [9]. Such approaches have limitations, including specialized equipment and the necessity to measure partial pressure of dissolved and gaseous oxygen in the headspace. One assay [10] indirectly measured oxygen consumption by using a TR-2 carbon dioxide gas respirometry system (Sable Systems International, Henderson, NV, USA) to measure the amount of CO$_2$ produced by C. elegans. They used approximately 50 animals per measurement and spent 5 to 6 h per measurement to detect the effective signal. Another assay [11] that measures the oxygen consumption of C. elegans in liquid suspension using Clark-type electrodes also has limitations, such as the consumption of oxygen, relatively long response time, and chemical instability [12].

In the present study, we have merged C. elegans biology and optical oxygen respirometry to develop simple, automated systems for toxicological assessment of chemical samples that can be deployed on a much wider scale and are suitable for the analysis of individual animals. These assays are based on the phosphorescent oxygen-sensing probe MitoXpress$^\text{TM}$ (Luxcel Biosciences, Cork, Ireland) and monitoring of oxygen uptake on commercial fluorescent readers [13]. Monitoring of probe fluorescence indirectly monitors the levels of sample oxygenation. From the relation between probe fluorescence intensity and oxygen concentration, relative respiration rates and changes in respiration rates can be easily determined. Such respiration profiles also are indicative of the metabolic state of the organism and the effect of exposure to a toxicant, particularly at sublethal doses.

**MATERIALS AND METHODS**

**Materials**

A phosphorescent oxygen probe, type A65N-1, which is excitable in the regions of 340 to 400 nm or 520 to 540 nm and emits at 630 to 690 nm, was obtained from Luxcel Biosciences. The ZnSO$_4$ (purity, ≥99%), CdCl$_2$ (purity, >99.99%), dimethyl sulfoxide (DMSO; solvent), rotenone (mitochondrial inhibitor), phenol (organic compound), microcystin-LR (MCLR; cyanotoxin), and paraoxon (organophosphate insecticide) were obtained from Sigma-Aldrich (Dublin, Ireland). Organic chemicals and MCLR were prepared in 100% DMSO and then diluted to the required concentration in K-medium [14]; the final DMSO concentration was 1% (v/v). Stock solutions of heavy metal ions were prepared in Milli-Q water (Millipore) and diluted to the required concentration in K-medium (32 mM KCl and 51 mM NaCl) [14]. The K-medium was used to prevent metals precipitating from the medium, as recommended [14].

**Nematode strains and culture methods**

Wild-type C. elegans (N2 strain), obtained from the Caenorhabditis Genomics Center (Minneapolis, MN, USA), were cultured on nematode growth (NG) media with E. coli OP50, at 20°C according to standard techniques [5]. The NG media were made as described previously (18 g/L of agar, 2.5 g/L of peptone, 3 g/L of NaCl, autoclaved; 5 mg/L of cholesterol, 1 mM MgSO$_4$, 1 mM CaCl$_2$, and 25 mM KH$_2$PO$_4$, pH 6) [5]. The E. coli OP50, a uracil-deficient bacteria, was maintained on luria broth (LB)—streptomycin agar plates at 4°C and cultured overnight at 37°C in LB broth.

All experiments were carried out on synchronized C. elegans adults (24 h post L4 stage) derived from dauer preparations [14]. Dauer larvae are an alternate larval state of arrested growth that occurs in the absence of food [5]. Embryos were isolated from adult nematodes by hypochlorite treatment as described previously [15], and dauer larvae stocks were maintained on NG plates until use, when they were transferred to NG plates spread with OP50 bacteria (NG-OP50) [16].

**Measurement of C. elegans respiration**

Measurements were carried out in standard, 96-well microtiter plates made of clear polystyrene (Sarstedt, Dublin, Ireland) in a sample volume of 150 µl; in black, 384-well microplates (Greiner, Frickenhausen, Germany) in a sample volume of 75 µl; in low-volume, 96-well plates (type MPU96-U1; Luxcel Biosciences), in a sample volume of 10 µl; and in LightCycler$^\text{®}$ glass capillary cuvettes (Roche, West Sussex, UK) in a sample volume of 20 µl. The phosphorescent oxygen probe was used according to the manufacturer’s instructions at final concentrations of 5 µM for the capillaries, 0.5 µM for the Luxcel plates, and 0.1 µM (fivefold lower) for the 96- and 384-well plates because of the larger optical path in these microplates compared to that in the Luxcel plates. For the microplate platforms, sample wells were read on a GENios Pro$^\text{TM}$ plate reader (Tecan, Mänedorf, Switzerland) in time-resolved fluorescence mode using 380-nm excitation and 650-nm emission filters, delay time of 40 µs, and gate time of 100 µs. The LightCycler capillaries were read on a LightCycler fluorescent reader (Roche) originally developed for quantitative polymerase chain reaction. To initiate the assay, test samples in 96- and 384-well plates were sealed with 0.1 or 0.05 ml, respectively, of mineral oil (Sigma), whereas Luxcel plates were sealed with adhesive tape. Measurements on the LightCyclers were carried out after spinning the capillaries at 800 rpm for 10 s in a centrifuge to bring the individual C. elegans and medium to the bottom; they were capped without any additional seal. Data collection was performed for 0.5 to 2 h at room temperature (22°C). Measured profiles of probe fluorescence for each sample were processed as described previously [1] to determine the initial rates of increase of fluorescent signal, which reflect the rate of depletion of dissolved oxygen and the rate of organism respiration.

**Assessment of effects of toxicants on nematodes**

The use of a food source, E. coli OP50, was described previously for the 24-h exposure to greatly reduce control mortality (0 µM toxicant) [16]. Saturated OP50 was pelleted by centrifugation at 15,000 g for 15 min, resuspended in an equal volume of K-media, and washed three times in K-media. After the third wash, the bacteria were resuspended in K-media, and the desired concentration of the toxicant solution was tested. The concentration of E. coli was consistent between toxicant concentrations. Caenorhabditis elegans were removed from NG-OP50 plates with K-media, gently centrifuged, and rinsed three times in K-media to remove excess OP50 bacteria. Caenorhabditis elegans were resuspended in K-media, and equal aliquots were added to the 2-ml microfuge tubes containing E. coli OP50 and toxicant (assay volume, 1 ml), after which the tubes were placed on a rocker for 24 h at room temperature 22°C [6]. Mock-treated C. elegans were incubated for 24 h without toxicant (0 µM) as a positive respiratory control in each experiment. Steady-state body residue of toxicants over the time were not tested.

Following 24 h of incubation, C. elegans were washed in
K-media and transferred to NG-OP50 plates. Individual *C. elegans* were then transferred to capillaries or Luxcel plates at the required numbers (see Results) in the presence of the appropriate toxin concentration being tested. For the elimination of unwanted bacterial respiration, *C. elegans* were washed before plating to remove bacteria. To compensate for some bacteria being transferred from the plate to the Luxcel plate platform, a negative respiratory control with bacteria but no *C. elegans* or toxicants was included in each respiration assay. If anything, the amount of bacteria in this negative respiratory control overcompensated for the bacteria present with the *C. elegans*. Data were corrected to the negative respiratory control.

Each concentration of the toxicant was analyzed in eight replicates on the Luxcel, 96-well, and 384-well plate assays or in five replicates in the capillary assays. Measured time profiles of probe fluorescence from each sample were normalized for the initial intensity (signal at time zero) and then used to determine their respiration rates relative to controls (mock [0 μM]-treated *C. elegans*). These slopes were related to untreated *C. elegans* (positive respiratory control shows 100% respiration) and corrected for those without *C. elegans* (negative respiratory control shows 0% respiration). A one-way analysis of variance with a Dunnett’s comparison to the control test was used to determine if the difference in respiration for each treatment group from the 0 μM control was statistically significant. Sigmoidal fits in OriginPro 7.5G software (OriginLab, Northampton, MA, USA) were used to determine the half-maximal effective concentration (EC50) for the toxicants (i.e., the median concentration that causes a 50% reduction in respiration compared to untreated *C. elegans* after 24 h of incubation). Sigmoidal fits in OriginPro 7.5G software also were used to determine the median lethal concentration (LC50) for the toxicants (i.e., the concentration that kills 50% of *C. elegans* to compared with mock-treated [no toxicant] *C. elegans* after 24 h of incubation).

A preliminary toxicity test was conducted for each toxicant. This test enabled a range of concentrations to be tested for use in the final toxicity test and gave an approximate 24-h EC50. A definitive toxicity test was conducted when the approximate value given by preliminary test was not sufficient; this permits calculation of the 24-h EC50 and determination of concentrations corresponding to 0 and 100% immobilization. Zinc and cadmium were examined in a preliminary test at final concentrations of 0, 1, 10, and 1,000 μM and in a definitive test at final concentrations of 0, 0.27, 2.7, and 81.0 μM. Phenol was tested at final concentrations of 0, 0.1, 1, 10, 100, 1,000, and 10,000 μM. The effect of DMSO on *C. elegans* respiration was tested at final concentrations of 0, 0.1, 1, 2.5, and 10% (v/v). Rotenone, microcystin, and paraoxon were prepared in 100% DMSO and tested in 1% (v/v) DMSO. Rotenone was tested in a preliminary test at final concentrations of 0, 0.27, 0.81, 2.43, and 7.29 μM. Microcystin was tested at final concentrations of 0, 1, 2.5, and 5 μM. Parafoxon was tested in a preliminary test at final concentrations of 0, 0.1, 1, 10, and 100 μM and in a definitive test at final concentrations of 0, 0.1, 1, 10, and 100 μM and in a definitive test at final concentrations of 0, 0.1, 1, 10, and 100 μM.

The lethal test following a 24-h exposure and using immobilization of *C. elegans* as the endpoint was performed for all toxicants like parallel assay. For calculation of the LC50, *C. elegans* were prepared as described above. After a 24-h incubation, washing, and transfer to NG-OP50 plates, dead (not responsive to touch) and live (responsive to touch) animals were counted. Zinc and cadmium were tested at 0, 1, 10, 100, 1,000, 1,250, 2,500, and 5,000 μM. Phenol was tested at 0, 1, 10, 100, 1,000, 1,250, 2,500, 5,000, and 10,000 μM. The DMSO was tested at 0, 1, 5, 10, and 20%. Rotenone was tested at 0, 0.1, 1, 10, 12.5, 25, 50, and 100 μM. Microcystin was tested at 0, 0.5, 1, 2.5, and 5 μM. Parafoxon was tested at 0, 0.1, 1, and 10 μM.

**RESULTS**

Evaluation and selection of optimal platforms and parameters

To establish a toxicity assay using *C. elegans* as a model organism, and to use respiration rate as an endpoint readout, the following criteria had to be optimized: The measurement platform, the number of individual nematodes, and the assay conditions (Fig. 1). Three different respirometric platforms (standard, 96-, and 384-well plates with mineral oil seal; sealable, low-volume, 96-well Luxcel plates; and glass capillaries) [13] were tested to determine their suitability, sensitivity, and analytical performance with *C. elegans*.

To evaluate and allow cross-comparison of different respirometric platforms with different numbers of *C. elegans*, the relative respiration rates were determined. The raw profiles of probe fluorescence and processed data (initial slopes/relative respiration rates) are presented for the respiration of different numbers of *C. elegans* on both the Luxcel plate platform (Fig. 1A) and the 96-well plate platform (Fig. 1B). A flat fluorescence profile (line with a slope of zero) indicates no changes in sample dissolved oxygen concentration; oxygen consumption because of animal respiration (if any) is balanced by back-diffusion of ambient oxygen. The ambient oxygen is able to diffuse into the sample through the seal and plastic body of the plate. When the respiration rate/animal number exceeds a certain threshold, it results in the development of oxygen gradients within samples; thus, respiration becomes measurable. The steeper the slope of the probe’s fluorescent signal, the higher the respiration rate. The slope of each line was calculated and corrected for the corresponding negative respiratory control (no *C. elegans*) to compensate for possible non-specific optical effects during the measurement (temperature fluctuations and probe photobleaching), and the average respiration rate for each number of *C. elegans* was determined and graphed (Fig. 1C).

Using the Luxcel plate platform (Fig. 1A and C), the respiration of one or five *C. elegans* adults was not significantly different from the negative respiratory control (*p* > 0.05), and fluorescence intensity was low with respect to error bars. The respiration of 7, 10, or 15 *C. elegans* adults is significantly different from negative the respiratory control at a 99% confidence interval (*p* < 0.0001). Therefore, for toxicity assays on Luxcel plates, 10 *C. elegans* per well were used; this gave reliable signal change measurements because of variations in animal respiration. The even distribution of individual nematodes in the shallow wells of the Luxcel plate provided for consistent and reproducible results (relative standard deviation [RSD], 4.5%). In addition, the relatively low numbers of *C. elegans* required per well indicated that this platform is well suited for toxicity assays using *C. elegans*.

Using the 96-well plate platform (Fig. 1B and C), the respiration of 10 or fewer animals was not detectable at a level significantly different (10 animals, *p* = 0.070) from the negative respiratory control. A minimum of 25 *C. elegans* adults
Fig. 1. Caenorhabditis elegans respiration profiles measured using different platforms. Four platforms were tested for sensitivity with different numbers of C. elegans adults (A and B). Shown are the average dose–response curves (normalized intensity vs time [h]) obtained for different numbers of C. elegans using (A) the low-volume, 96-well, Luxcel plate platform and (B) the 96-well standard plate were typical of other platforms (not shown). The respiration rate of different numbers of C. elegans were calculated from these dose–response curves for 96-well Luxcel plates and 96-well plates (C). Statistical significance was calculated using a one-way analysis of variance with Dunnett’s comparison with control: Negative respiratory control (no C. elegans) versus each number of C. elegans. Using the capillary platform, five biological replicates of single C. elegans adults reproducibly consume more oxygen (and yield consistent dose response curves) than the negative respiratory control (D). NS = not statistically significant ($p > 0.05$). *$p < 0.05$, **$p < 0.0001$. 
was required to detect a significant difference from the negative respiratory control \((p = 0.0202)\). For the reliable measurement of inhibitory effects of toxicant on respiration, however, even higher numbers \((25 \leq n \leq 50)\) of \(C.\ elegans\) are required \((p < 0.0001)\). On the 384-well plate, a minimum of 10 \(C.\ elegans\) adults was required to detect a significant difference from the negative respiratory control \((p = 0.0107)\), with 25 \(C.\ elegans\) giving a stronger signal \((p < 0.0001)\;\text{data not shown}\). A large RSD \((15\%-43\%)\) was observed between the replicate wells, however, which we attribute to the relatively large volume \((75\, \mu\text{l})\) and/or uneven distribution of \(C.\ elegans\) in the wells. Because of the large numbers of \(C.\ elegans\) required, the large standard deviation, and poor analytical performance, the 96-and 384-well platforms were not suitable for toxicity assays.

The capillary platform (Roche) was the most sensitive (Fig. 1D) platform analyzed. In this platform, the respiration rate of a single \(C.\ elegans\) adult could be reliably detected \((p = 0.0002)\) (Fig. 1D). Increasing the number of \(C.\ elegans\) produced an increase in measured respiration rates, but the relationship between the number of animals and the respiration rate was nonlinear. An increased respiration rate was detected when two rather than one \(C.\ elegans\) were measured \((p = 0.0044)\), but no significant difference was seen in comparisons between two, three, four, or five adults \((0.5098 \leq p < 0.9994)\;\text{data not shown}\). This could be attributable to the geometry of the capillary cuvette and detector optics and to the high sensitivity of the capillary platform. The \(C.\ elegans\) likely aggregate at the bottom of the cuvette and interfere with the optical signal because of their significant size (this can be observed as increased signal noise at higher numbers). From the toxicological screening perspective, the opportunity to analyze individual organisms provided by this platform is very attractive. Variability in measured respiration profiles of individual \(C.\ elegans\) was 15\%, which was calculated as the RSD of respiration rate profile \((n = 10)\).

Because of superior performance, the low-volume, scalable, 96-well Luxcel plates with 10 \(C.\ elegans\) animals per well and glass capillaries with one \(C.\ elegans\) animal per capillary were selected for further validation experiments with known toxicants. Good temperature control \((\pm 0.1^\circ\text{C})\) and optical alignment of the capillary platform are important for oxygen respirometry and result in better-quality data (in terms of sensitivity and reproducibility) \([17]\). The Luxcel plate/fluorescent reader platform provides increased sample throughput, lower cost, and consumption of the oxygen probe.

To determine the most appropriate age of \(C.\ elegans\) for use in the study, different developmental stages were tested: L2/L3, L4, and young adults (Fig. 2). For this assay, 15 animals per well were used on a 96-well Luxcel plate platform. The observed result of older \(C.\ elegans\) consuming more oxygen (Fig. 2) correlates with the increasing size of \(C.\ elegans\) with age: L2/L3 are 360 to 510 \(\mu\text{m}\) in length, L4 are 620 to 650 \(\mu\text{m}\) in length, and young adults are 900 to 940 \(\mu\text{m}\) in length (http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm). The radius of young adult \(C.\ elegans\) is approximately 30 \(\mu\text{m}\), with a corresponding volume of 2.82 \(\times 10^6\) \(\mu\text{m}^3\), and the volume proportion of 15 \(C.\ elegans\) versus a Luxcel plate well of approximately 4\%. In the respiration test, the synchronized young adult \(C.\ elegans\) (72 h after feeding dauers) displayed the most acceptable response (changes in oxygen concentration [slopes] were seen in 0.2 h) and thus were used in all subsequent studies (Fig. 2).

**Effects of different toxicants on \(C.\ elegans\) respiration**

The chemicals chosen for testing of the respirometric assay with \(C.\ elegans\) were selected as representatives of different classes of known toxic compounds: Heavy metal ions (\(\text{Zn}^{2+}\) and \(\text{Cd}^{2+}\)), organophosphate insecticide (paraoxon), mitochondrial inhibitor (rotenone), organic compounds (phenol), and a cyanobacterial toxicant (MCLR).

To determine the effects of these toxicants on respiration, \(C.\ elegans\) were incubated with different concentrations of each compound as described in **Materials and Methods**. In a previous study \([18]\), Namalwa cells were treated with DMSO concentrations ranging from 0.01 to 20\% (\(\text{v/v}\)); respirometry results showed a detectable drop in oxygen consumption at a DMSO concentration of 1\%. In the present study, DMSO at 1\% (\(\text{v/v}\)) did not affect \(C.\ elegans\) respiration \((p = 0.9999)\) (Fig. 3) and was used to dissolve MCLR and the pesticides (paraoxon and rotenone). The toxicity data were processed as dose–response curves; the initial slopes were calculated for
Fig. 3. The effect of dimethyl sulfoxide (DMSO) on *Caenorhabditis elegans* respiration. Respiration rates of 10 *C. elegans* adults were measured at room temperature (−22°C) using the 96-well Luxcel plate platform after exposure to 0, 0.1, 1, 5, or 10% (v/v) DMSO for 24 h. All data were normalized and are shown as a percentage of the 0% positive respiratory control (100%). No *C. elegans* were included in the negative respiratory controls. Error bars represent the standard deviation among eight replicates. Statistical significance was calculated using a one-way analysis of variance with Dunnett’s comparison with control: 0% DMSO versus each concentration of DMSO. NS = not statistically significant (p > 0.05).

Fig. 4. The effect of cadmium and zinc on *Caenorhabditis elegans* respiration. Respiration rates of 10 *C. elegans* adults were measured at 22°C using the 96-well Luxcel plate platform after exposure to 0, 30, 90, 270, or 810 μM cadmium (■) or zinc (□) at room temperature (−22°C) for 24 h. All data were normalized and are shown as a percentage of the 0 μM positive respiratory control (100%). No *C. elegans* were included in the negative respiratory controls. Error bars represent the standard deviation among eight replicates. Statistical significance was calculated using a one-way analysis of variance with Dunnett’s comparison with control: 0 μM control versus each concentration of toxin. NS = not statistically significant. *p < 0.001, **p < 0.0001.

Table 1. Half-maximal effective concentration (EC50) and median lethal concentration (LC50) of tested chemical classes for model organism *Caenorhabditis elegans* measured by oxygen respirometry.

<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Toxicant</th>
<th>24-h EC50 (μM)</th>
<th>24-h LC50 (μM)</th>
<th>Reference data (LC50 [μM])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>96-Well Luxcel plate</td>
<td>Capillary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heavy metals</td>
<td>Cadmium</td>
<td>60.85 ± 16.9</td>
<td>94.77 ± 34.07</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td></td>
<td>Zinc</td>
<td>135.72 ± 38.6</td>
<td>NA</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Aromatic compound</td>
<td>Phenol</td>
<td>909.81 ± 281.47</td>
<td>NA</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Polar aprotic solvent</td>
<td>Dimethyl sulfoxide</td>
<td>7.67% ± 2.64%</td>
<td>NA</td>
<td>10.02 ± 7.14</td>
</tr>
<tr>
<td>Pesticides</td>
<td>Rotenone</td>
<td>0.87 ± 0.33</td>
<td>NA</td>
<td>11.74 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>Paraoxon</td>
<td>22.8 ± 5.8</td>
<td>NA</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Cyanotoxicant</td>
<td>Microcystin</td>
<td>&gt;5</td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>

Experiments were performed in triplets; the data for a representative experiment are given. The LC50 refers to the concentration at which 50% of *C. elegans* were dead (not responsive in touch assay). NA = not available.

a EC50 for paraoxon-ethyl.
Fig. 5. The effect of rotenone on Caenorhabditis elegans respiration. Respiration rates of 10 C. elegans adult were measured at 22°C using the 96-well Luxcel plate platform after exposure to 0, 0.27, 0.81, 2.43, or 7.29 μM rotenone at room temperature (−22°C) for 1 h (□), 4 h (□), or 24 h (■). All data were normalized and are shown here as a percentage of the 0 μM positive respiratory control (100%). No C. elegans were included in the negative respiratory controls. Error bars represent the standard deviation among eight replicates. Statistical significance was calculated using a one-way analysis of variance with Dunnett’s comparison with control: 0 μM control versus each concentration of toxin. NS = not statistically significant. *p < 0.05, **p < 0.0001.

**Discussion**

The aim of the present work was to establish a robust toxicity screening assay by which different toxic compounds affecting the respiration of the model nematode C. elegans could be monitored via noninvasive, quenched fluorescence oxygen sensing. We have established the optimal conditions for respirometric monitoring of C. elegans and have tested the assay with six different model compounds that have known toxicological effects on biological systems, albeit via different biochemical mechanisms.

The type of platform on which a bioassay is developed is critical to ensuring that the assay is robust, reliable, accurate, sensitive, and economical. For our analysis of C. elegans, we investigated a number of possible respirometric platforms, including 96- and 384-well plates sealed with mineral oil; low-volume, 96-well, Luxcel plates sealed with a dedicated lid/adhesive film [30]; and unsealed glass capillaries [31]. For some experimental purposes, it may be desirable that the respiration of a single animal (nematode) be analyzed; for other purposes, analysis of nematode populations may be preferable. Hence, experimental parameters, including platform and number of C. elegans, were taken into consideration to measure oxygen consumption. Our investigations regarding the number of C. elegans required for each platform demonstrated that relatively smooth respirometric profiles can be obtained (Fig. 1A). An increase in organism number showed a higher change in fluorescent signal over time (initial rate). This increase eventually levelled off, indicating depletion of oxygen.

Luxcel plates and capillaries were chosen for further toxicity assays, because they provided a good distribution of animals and allowed a low number of C. elegans per well. The glass capillaries are impermeable to oxygen and are more sensitive than Luxcel plates [32]. Our results indicate that only one C. elegans adult is required to quantify oxygen consumption in the capillary platform, which allows monitoring of a single individual, and with a 32-sample capacity (32 capillaries), this platform is suitable for medium-throughput analysis. To our knowledge, no other bioassay platforms currently allow monitoring of oxygen consumption in a single nematode. On the other hand, the cost of LightCycler capillaries (used on a disposable basis) is significant, and probe consumption is high (because of the nonoptimal instrument optics).

The Luxcel plate also was a sensitive platform and could detect a single C. elegans adult, but to achieve a greater robustness of detection, 10 C. elegans adults were used per well. The Luxcel plate platform provides a higher throughput, offering a 96-sample capacity, which may be better suited to some assays. It is compatible with a broad range of standard fluorescent readers and is more robust and economical. Respiration profiles of C. elegans were reproducible and conclu-
sive for both the capillaries and the Luxcel plate platforms. For further testing of the pesticides, we chose to use the Luxcel plate platform.

The primary limitation identified for this system is the relatively high variation of the measured parameters (see Table 1), respiration rates, and 24-h EC50s. This concurs with the results of Braeckman [11], who found that even though C. elegans have a set cell lineage and cell number, the biomass can vary between individual C. elegans. Braeckman suggested that such variation in biomass accounts for variation in respiration. This could account for the variable respiration observed between individuals as well, and we propose that it is in line with the deviation seen between other individually tested, multicellular metazoans [1]. This variation can be compensated for by running an appropriate number of replicates for each concentration point (4 < n < 8 for our systems). On the other hand, the use of metabolic markers, such as oxygen consumption, with C. elegans instead of endpoint mortality (mobility) assessment is highly advantageous, because it eliminates false-negative results. Screening of compound libraries for significant toxicological effects on C. elegans can be done at a single dose with a small number or replicates. The assay is applicable to the toxicants for which the mechanism of action affects organism respiration and bioenergetics.

Caenorhabditis elegans is a multicellular animal that is widely used as a biomedical and genetic model organism. Hence, it is an attractive organism for developing toxicity assay procedures. Chronic or delayed effects of environmental agents often are difficult to evaluate in mammalian models because of their long life cycles. Caenorhabditis elegans, however, is a good model organism for rapid preliminary toxicity studies because to its short life cycle, and this method of oxygen sensing can be easily performed in any laboratory. In addition, approximately 40% of C. elegans genes are directly orthologous with human genes [33], and C. elegans shares many common biochemical pathways with mammals (mitogen-activated protein kinase [34]). Caenorhabditis elegans exhibits a repertoire of behaviors that are analogous or homologous to those in humans (nicotine dependence [35] and aging [36]) and that can be studied by genetic, anatomical, and pharmacological approaches. The widespread use of C. elegans as a basic model for studying human diseases (poly-cystic kidney disease [37] and Alzheimer’s disease [38]) can facilitate more subtle approaches to toxicity screening targeted toward specific human diseases or basal behaviors.

Our results demonstrate that fluorescence-based oxygen respirometry is a useful tool for toxicological assessment and screening of chemical and environmental samples. Compared to the conventional toxicity testing system Microtox® (Strategic Diagnostics, Hampshire, UK), which uses Vibrio fischeri, the C. elegans respirometric assay described in the present work was less sensitive for some toxicants but more sensitive for others. Different organisms will have different toxicity responses, and multispecies approaches that include C. elegans oxygen respirometry approaches will be most informative. Direct comparison of oxygen respirometry and the conventional Microtox assay is not possible because of the different incubation times. For Zn²⁺, the Microtox assay gave a 15-min EC50 of 38.2 μM [1], whereas the respirometry assay gave a 24-h EC50 of 135.72 ± 38.6 μM. The lethality assay, a visual assessment of mortality, gave a 48-h LC50 of 1,697.58 μM [6]. In comparison with the respirometry assay, the mortality test LC50 is approximately 10-fold higher than that in the present work, and the incubation time is longer. For most of the toxicants, the respirometric assay was more sensitive than the lethality assay; DMSO was the only toxicant tested that gave similar 24-h LC50s and 24-h EC50s.

Respirometry assays using Jurkat cells, E. coli, A. salina, and zebrafish have been conducted [1], so comparisons with the C. elegans system are possible. The effect of zinc on C. elegans after a 24-h incubation corresponded to that observed for Jurkat cells and E. coli, with 24-h EC50s of 147.2 ± 28.3 μM and 121.2 ± 16.6 μM, respectively. For other animal models, such as zebrafish and A. salina, the 24-h EC50s were slightly different, with values of 389.7 ± 203.6 μM and 16.1 ± 11.8 μM, respectively.

Microcystin-LR has been found to be toxic to liver tissue [26]. Caenorhabditis elegans seems to be relatively immune to MCLR at concentrations that are toxic to mice [26]. Whereas C. elegans is a good model to study the toxic effects of chemicals on mitochondrial respiration, it is of very low complexity and may not be such a good model for organ toxicity (no liver). This needs to be considered when using the assay for development of pharmacological compounds, especially those that may have liver toxicity.

Toxicological effects apply not only to toxicant bioassays but also to drug screening. The C. elegans-based approach presented here, using quenched fluorescence oxygen sensing, can be extended to initial prescreening of pharmaceutical compounds that affect cellular respiration. However, follow-on screening considerations, including organ-specific toxicity in mammals, need to be considered for such pharma-toxicant screening.

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