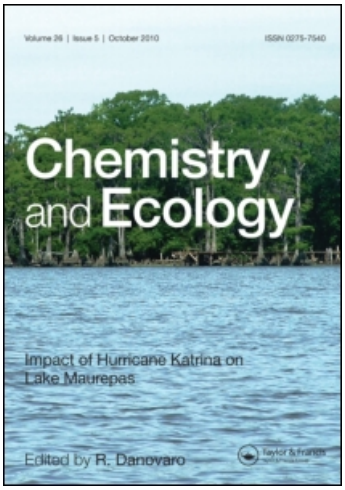

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Respirometric acute toxicity screening assay using *Daphnia magna*

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Oxygen consumption rate is a universal and sensitive biomarker of viability and metabolic status of aerobic organisms. We applied the optical oxygen sensing method to monitor the respiration of individual *Daphnia magna* (*D. magna*) and to develop a simple, automated screening assay for the assessment of acute toxicity of large numbers of chemical and environmental samples. *D. magna* were exposed to the toxicants and effluent samples for 24 h or 48 h and then analysed for changes in respiration relative to untreated controls, using standard microtiter plates and a fluorescence reader. Our assay showed the ability to detect sub-lethal effects of reference toxicants, including potassium dichromate, sodium lauryl sulfate, zinc and cadmium, ease of generation of dose-response curves and EC_{50} values. The assay showed comparable sensitivity and robustness to the conventional assay, and higher sensitivity when testing industrial effluents. It is therefore well suited for environmental monitoring.

Keywords: optical oxygen respirometry; oxygen sensing; *D. magna* toxicity testing

1. Introduction

Daphnids, particularly *D. magna* have been used for many years in toxicity testing of chemicals [1,2] and effluents [3–5]. Due to their widespread occurrence and sensitivity to a broad range of chemicals and environmental pollutants, they have been recognised as a general representative of freshwater zooplankton species [6]. Due to their ecological significance (broad distribution and important link in pelagic food chains), parthenogenetic reproduction, short life cycle, ease of laboratory culture, discrete growth, small size, ease of handling, high fecundity, the low cost and minimal equipment required for bioassays, daphnids are now accepted as the invertebrates of choice for aquatic toxicologists. They also facilitate the use of a large number of test specimens for good statistical design and analysis.

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Several standard assays use daphnids for measuring toxicity of chemical substances in aquatic samples, including one developed by the US Environmental Protection Agency (EPA) to assess relative toxicity of effluents and surface water [7]. The EPA test employs neonates (<24 h old) of *Ceriodaphnia dubia* during a three-brood, 7-day static renewal test, with test results measured in terms of survival rate and reproductive parameters. Another bioassay assessing the effects of chemicals on the reproductive output of *D. magna* runs for 21 days [2]. Toxicity tests that include exposure through the entire life cycle (with survival rate and the number of viable offsprings as endpoints) are useful but time consuming. Several short-term tests have been developed for acute toxicity, showing compromised speed, sensitivity and cost, which are based on the assessment of immobilisation (or mortality) of *D. magna* after 24 [1,8], 48 [9,10] and 72 h [11].

Monitoring the rate of oxygen consumption – a sensitive metabolic biomarker of test organisms – has high potential for toxicity screening. Previous studies with daphnids have employed either a Strathkelvin respirometer [12], a calibrated oxygen electrode employed in BOD bottles [13,14] or in a through-flow system [15], and the chemical Winkler method [16,17], where the amount of dissolved oxygen reflects the biological activity of water masses [18]. However, all of these respirometric techniques require high numbers of *D. magna*, are relatively labour-intensive and slow, and have limited sample throughput. In contrast, optical oxygen sensing and respirometry employs a fluorescence-based probe which is added to the sample. Probe fluorescence is quenched by dissolved oxygen, thus allowing real-time quantification of the latter. Measurement of probe fluorescence in multiple samples in standard 96-well plates on a fluorescence plate reader allows monitoring of oxygen concentration. From these data, respiration profiles can be obtained for each sample, which are indicative of their metabolic response to the toxicant. This approach has been shown to provide simple, high throughput analysis of toxicological effects of various stimuli on test organisms. It was demonstrated with several model animals including *Artemia salina* (brine shrimp) [19,20], *Danio rerio* (zebrafish) [19], *C. elegans* [21] and with prokaryotic and eukaryotic cell cultures [22–25].

In this study, we applied optical oxygen respirometry to the measurement of *D. magna* oxygen consumption rate, with the aim of developing a simple, automated system for toxicological assessment of chemical and environmental samples. The approach included the initial method development for *D. magna* by evaluating several microplates-based respirometric platforms, optimisation of key assay parameters, and assay validation with a set of reference toxicants in comparison with standard immobilisation-based *D. magna* tests. Finally, a panel of effluents retrieved from various industries with activities such as surface coatings, metals, food and drink, chemicals, wood, and paper, were examined for their dose- and time-dependent patterns of toxicity using *D. magna* and mammalian cells.

2. Materials and methods

2.1. *Materials*

A phosphorescent oxygen sensing probe, MitoXpress, comprising a macromolecular conjugate of Pt-Porphyrin dye, excitable at 340–400 nm and emitting at 630–690 nm [26] and sealing oil were obtained from Luxcel Biosciences (Cork, Ireland). Analytical grade $ZnSO_4$* $7H_2O$, $CdCl_2$, $K_2Cr_2O_7$, sodium lauryl sulfate (SLS), were supplied by Sigma-Aldrich (Ireland). Effluent samples collected from different sites (EPA license classification) were obtained from the Shannon Aquatic Toxicology Lab (Shannon, Co. Clare, Ireland). Solutions of chemicals and effluents were prepared using Millipore grade water.

2.2. *Culturing of* D. magna

D. magna, collected from continuous culture at the Shannon Aquatic Toxicity Laboratory, was maintained in continuous culture under semi-static conditions at 20 °C ± 2 °C in 1 litre beakers in de-chlorinated water, using 16 h:18 h of light:dark photoperiod and a density of 20 adults per litre. Water having characteristics similar to dilution water [8] (total hardness 250 ± 25 mg/l ($CaCO_3$), pH 7.8 ± 0.2, Ca/Mg molar ratio of about 4:1 and dissolved oxygen concentration of above 7 mg/l) was used as both culture and test medium. Medium was renewed three times a week and beakers were washed with a mixture of mild bleach and warm water. Stock cultures and experimental animals were fed daily with *Chlorella* sp. algae (0.322 mg carbon/day). This algal culture was also cultivated continuously using freshwater algal culture medium [27]. Three-week-old offspring of *D. magna* were separated from cultures at regular intervals and used for the production of juveniles (≤24 h), which were then used in the toxicity tests.

2.3. *Measurement of* D. magna *respiration*

Measurements with *D. magna* were carried out using: (i) standard 96-well microtiter plates (96WP) made of clear polystyrene (Sarstedt, Ireland) and sample volume 150 μl; (ii) black 384-well microplates (Greiner, Germany) (384WP) and sample volume 75 μl; and (iii) low-volume sealable 96-well plates, type MPU96-U1 (Luxcel Biosciences, Ireland) (Luxcel plate) and sample volume 10 μl. The MitoXpress probe (supplied dry in a vial) was reconstituted in MilliQ water and gave 1 μM stock. The probe was used at the following final concentrations in medium: 0.1 μM for the 96 WP and 384 WP and 0.5 μM for the Luxcel plates. For all the platforms, plates were read on a fluorescence plate reader Genios Pro (Tecan, Switzerland) in time-resolved fluorescence mode, using a 380 nm excitation and a 650 nm emission filters, delay time of 40 μs and gate time 100 μs. The required number of *D. magna* (see Results) was transferred with a Pasteur pipette into each assay well, which contained medium with probe. To initiate the respirometric assay, samples in standard 96- or 384-well plates were sealed with 100 μl or 50 μl of oil, respectively, while samples in Luxcel plates were sealed with adhesive tape. The plate was then placed in the fluorescent reader set at 25 °C. Kinetic fluorescence measurements were performed over 0.5–2 h, with readings in each assay well being taken every 2 min. Measured time profiles of probe fluorescence from each treated sample and also untreated *D. magna* (used as positive control – 100% respiration) were normalised for the initial intensity (i.e. signal at time zero) and then processed to determine the initial slopes (typically for 10–20 min time intervals). These slopes were corrected for those without *D. magna* (negative control – 0% respiration) and related to positive control. A one-way ANOVA with a Dunnetts comparison was used to determine if the difference in respiration for each treatment group was statistically significant compared with the positive control. Each assay point was usually run in 6–8 repeats and each experiment was repeated 2–3 times to ensure consistent results.

Fluorescence of an oxygen-sensitive probe is related to oxygen concentration as follows [28]:

$$[O_2] = (I_0 - I)/IK_{s-v},$$

where I_0 and I are emission intensities of the oxygen probe in absence and in presence of oxygen concentration $[O_2]$, and K_{s-v} = Stern–Volmer constant (quenching characteristic).

2.4. *Exposure of* D. magna *to toxicants and acute toxicity testing*

For acute toxicity testing, 20 *D. magna* juveniles (≤24 h) were randomly placed in 50 ml glass beakers, containing 40 ml of de-chlorinated (fresh) water with different concentrations

of toxicants/effluents and without (untreated controls). As in the standard test [8], *D. magna* were not fed during the incubation. Following 24 h or 48 h incubation, individual *D. magna* were transferred by Pasteur pipette into microplate wells containing medium and the toxicant. SLS and $K_2Cr_2O_7$ were tested from 20–80 mg/l and from 0.2–1 mg/l, respectively. Cadmium was tested in the concentration range 0.5–1.5 mg/l and 0.1–1 mg/l for 24 h and 48 h incubation time, respectively. Zinc was tested in the concentration range 2–15 mg/l and 0.2–2 mg/l for 24 h and 48 h incubation time, respectively. Each concentration of the toxicant was normally analysed in 8 replicates and data processed as above. Sigmoidal fits produced with OriginPro 7.5G software were used to determine EC_{50} values [29], i.e. the mean concentration that causes a 50% reduction in respiration compared with untreated *D. magna*.

2.5. *Toxicity testing of effluents*

Effluent samples were tested for their toxicity on *D. magna* and also Jurkat cells, a human leukaemia T-cell line (the American Type Culture Collection). Samples were initially analysed undiluted, using *D. magna* and 24 h exposure and Jurkat cells and 1 h exposure, and a procedure similar to that used for the pure chemicals (see above). Subsequently, toxic samples were analysed at different dilutions. In parallel with respirometric measurements, standard definitive toxicity tests [8] were also conducted to determine the percentage of *D. magna*, which become immobilised after the exposure to different effluent concentrations. Corresponding EC_{50}-24 h values were calculated and compared with the respirometric values.

With the eukaryotic cell model (Jurkat cells), effluent toxicity was examined using 1:10 initial dilution (for retaining essential medium composition) and 1 h and 24 h exposure times, using the procedure described in detail elsewhere [19]. Briefly, cells were cultured in 75 cm^2 flasks (Sarstedt) in a humidified atmosphere of 5% CO_2 in air, at 37 °C, in RPMI-1640 medium (Roswell Park Memorial Institute) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin to a concentration of approximately 1×10^6 per ml. The cells were then centrifuged and resuspended in fresh medium at the required concentration. Toxicity assays were conducted at 5×10^6 cells/ml. For 24 h exposure, the cells were dispensed in 4.5 ml aliquots into 20 ml culture flasks (Sarstedt), mixed with 0.5 ml of effluent (undiluted or diluted with medium), cultured for 24 h under standard conditions, and then dispensed into the wells of standard 96 WP, 150 μl/well. For 1 h exposure the cells were dispensed directly into the wells of 96 WP. 1.5 μl of oxygen probe stock (concentration in stock 10 μM) was then added to each assay well followed by the addition of 100 μl of oil. The plate was then read on the Genios Pro reader at 37 °C, as described above, using the same instrument settings. Parameters of toxicity (dilutions causing significant and 50% effect) were calculated as described earlier.

3. Results

3.1. *Selection of optimal platform for the measurement of* D. magna *respiration*

Different model organisms differ in their size and respiration rate. To establish *D. magna* as a model organism for respirometric toxicity testing, an optimal measurement platform had to be determined and the main assay parameters optimised. Three different respirometric platforms previously developed by the team [30], namely, standard 96WP and 384WP with mineral oil seal, and Luxcel plate, were tested for their suitability and analytical performance with respect to the measurement of *D. magna* respiration. For all of these platforms, samples are only partly sealed (with oil or lid), and ambient oxygen can still diffuse slightly into the sample through the

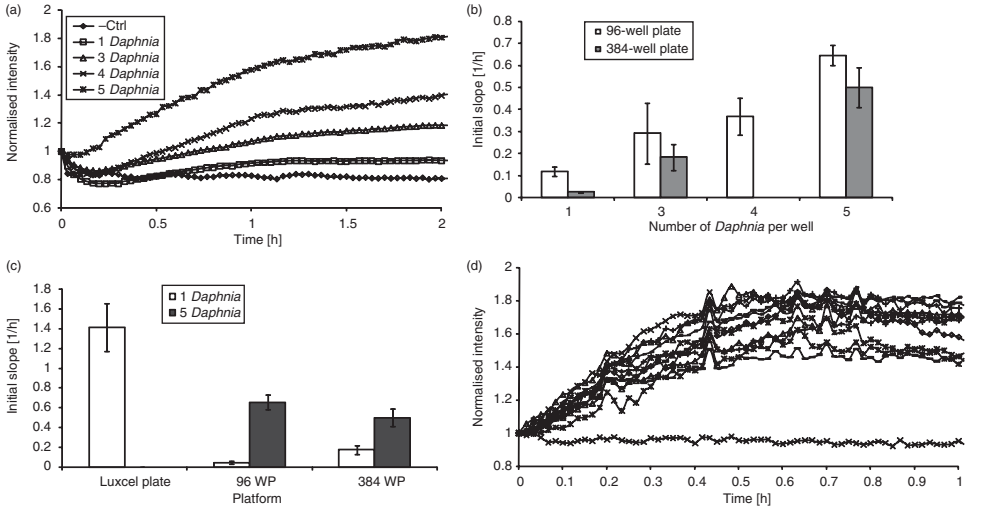

Figure 1. *Daphnia magna* respiration measured with different platforms. (A) Raw fluorescent data for standard 96-well plate: profiles for different numbers of *D. magna*. Signal profiles for each sample with test organisms were normalised for the initial intensity signal. (B) Processed data for standard 96- (open bars) and 384- (solid bars) well plates: initial slope (dI/dt, Fluorescence Intensity Units per min) as a function of animal number of *D. magna*. (C) Comparison between the three platforms as Luxcel plate, 96 WP and 384 WP. (D) Reproducibility of profiles with one animal per well for the Luxcel plate. Measured at $22 \pm 2\,^{\circ}\mathrm{C}$, for error bars (SD) $N = 8$.

plastic body of the plate and the seal. When respiration rates of organisms are too low, oxygen consumption by the sample is balanced by back-diffusion of oxygen from air, so that dissolved oxygen concentration and probe signal remain unchanged (flat line). When sample respiration rate exceeds a certain threshold, oxygen gradients develop within samples and respiration becomes measurable, seen as an increase in probe fluorescence. The steeper the slope of the probe signal, the higher the respiration rate. When the dissolved oxygen is depleted the signal levels off.

Figure 1 shows the effect of the number of *D. magna* neonates on respirometric profiles for each of the three platforms. Although respiration of single organisms was measurable both in 96 WP and 384 WP (Figure 1(A)–(C)), signal changes were too small for the monitoring of inhibitory effects on respiration (common for most of the toxicants). Cross-comparison of different respirometric platforms with different numbers of *D. magna* is given in Figure 1(B) & (C), showing the initial slope for each profile corrected for corresponding negative control (no animal) to compensate for possible optical effects during the measurement (temperature fluctuations or probe photobleaching), and in the case of real samples for bacteria contamination. Processed data (initial slopes) for the respiration of 1 or 5 *D. magna* on the Luxcel plate, the 96 WP and 384 WP are shown in Figure 1(C).

For the 96 WP platform (Figure 1(A) & (B)), respiration of a single animal was not significantly higher than for negative controls; with 3 and 5 *D. magna* significant difference was achieved ($p < 0.5$ and $p = 3.9 \times 10^{-5}$, respectively). For the 384 WP, a minimum of 5 animals were required ($p = 1.7 \times 10^{-3}$). Large standard deviations between replicate wells (RSD 33–25%) were observed for these platforms, which we attributed to the uneven distribution of organisms within the well and their frequent entrapment in the oil seal. Due to their lower sensitivity and higher variability, the 96 WP and 384 WP are less suitable for toxicity assays with *D. magna*. They require a minimum of five organisms per well, which is not very convenient for manual dispensing, especially for a large number of samples.

In the Luxcel plate (sealed with tape), respiration of individual *D. magna* is easily detectable (Figure 1(C)) and enough space for individual neonates is available in wide and shallow

micro wells. Respiration of single *D. magna* was significantly higher ($p < 0.0001$) than negative controls (no *D. magna*), giving a sufficient window of signal change for reliable measurement of animal respiration effected by a toxicant (i.e. between positive and negative controls). Repeatability between different wells/animals was good (Figure 1(D)) (15% RSD-relative standard deviation), considering the significant variability in respiration rates between different organisms. Due to the small volume of sample wells in Luxcel plates, it was not possible to place more than one *D. magna* in a well.

Due to superior performance and greater sensitivity, the Luxcel plate with a single *D. magna* per well were selected for all subsequent toxicity testing experiments with reference to chemicals and effluents. This platform, coupled with a standard fluorescent reader, provides high sample throughput, low cost and low probe consumption. Other parameters such as temperature (20 ± 2 °C), and the age of *D. magna* (≤24-h-old juveniles) were the same as in the standard method [8].

3.2. *Analysis of reference toxicants using* D. magna *and optical respirometry*

The chemicals chosen for testing of the respirometric assay with *D. magna* were classical reference toxicants. The effect of the probe at 0.5 μM on toxicants was tested, and no interaction with the toxicants was observed (data not shown). The tested concentrations of all toxicants are entered in section 2.4.

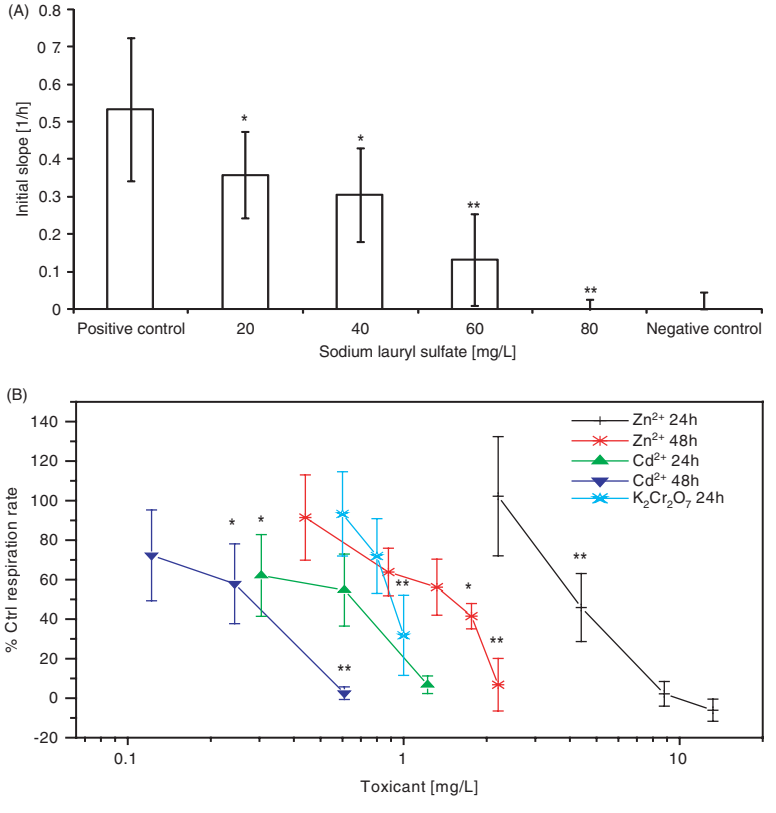

Figure 2. Dose and time dependence of toxic effects on *D. magna* respiration. (A) SLS at 24 h exposure; (B) heavy metals Zn^{2+} and Cd^{2+} at 24 and 48 h exposure and $K_2Cr_2O_7$ at 24 h. The platform used was a 96 Luxcel plate. Statistical difference, between eight identical concentrations of toxicant, was calculated using one-way ANOVA with a Dunnetts comparison; NS = not significant; $^{*}p < 0.05$; $^{**}p < 0.001$. T = 22 °C, $N = 8$.

Table 1. Mean effective concentrations (EC_{50}-24 h/48 h) for different chemicals measured with *D. magna*.

Toxicant	Standard assay EC_{50}-24 h [mg/L]	Respirometric assay EC_{50}-24 h, (C_{min}) [mg/L]	Standard assay EC_{50}-48 h [mg/L]	Respirometric assay EC_{50}-48 h, (C_{min}) [mg/L]
$K_2Cr_2O_7$	1.12 [8], 3.9 [32]	0.899 ± 0.11, (0.8)	—	—
Sodium lauryl sulfate	50 [32]	64.9 ± 8.28, (60)	—	—
Zn^{2+}	—	4.52 ± 0.58, (4)	1.83 ± 0.07 [33]	1.49 ± 0.14, (0.9)
Cd^{2+}	4.66 [34]	0.63 ± 0.23, (0.3)	1.88 [34], (0.615 ± 0.03) [35]	0.16 ± 0.06, (0.08)

Note: C_{min}, the smallest toxicant concentration giving a significant effect ($p = 0.01$) on *D. magna*. Calculated using Student's *t*-test with confidence limits of 99%.

SLS, a surfactant found in many personal care products (soaps, shampoos etc.), reduced *D. magna* respiration following a 24 h exposure at concentrations of 60 mg/l ($p = 1.1 \times 10^{-5}$) and above (see Figure 2(A)). Calculated EC_{50}-24 h value was 33.37 ± 8.72 mg/l (Table 1).

The inorganic reagent $K_2Cr_2O_7$ is widely used as an oxidising agent in various laboratory and industrial applications, for cleaning glassware and etching materials. It is harmful and must be handled and disposed of appropriately, hence its use in aquatic toxicity assays [8,31]. After 24 h exposure, the concentration of 1 mg/l of $K_2Cr_2O_7$ reduced *D. magna* respiration significantly ($p = 4 \times 10^{-4}$) compared with positive controls (see Figure 2(B)). Calculated EC_{50}-24 h value was 0.90 ± 0.11 mg/l, which correlates well with literature data, although being slightly lower (Table 1). Our test met the criteria of EC_{50}-24 h 0.6–2.1 mg/l [8] required for the validation.

Exposure to heavy metal ion Zn^{2+} for 24 h had no significant effect on *D. magna* respiration at concentrations 2.2 mg/l ($p = 0.9$) and lower (Figure 2(B)). However, at 4.4 mg/l and higher it was reduced ($p = 7 \times 10^{-4}$) in a dose-dependent manner. A period of 48-h exposure enhanced the toxic effect, which became significant at 0.88 mg/l ($p = 1 \times 10^{-3}$), giving almost complete inhibition at 2 mg/ml.

Cd^{2+} binds to free sulfhydryl residues, displaces zinc co-factors, and generates reactive oxygen species, thus exposure to Cd^{2+} results in cellular damage [32,33]. *D. magna* exposed to different Cd^{2+} concentrations after 24 h incubation showed a significant reduction in respiration at 0.3 mg/l ($p = 4 \times 10^{-3}$) and 0.6 mg/l ($p < 0.001$) (Figure 2(B)). For 48 h incubation time, significant reduction in respiration was seen at 0.24 mg/l ($p = 0.003$). EC_{50}-24 h and EC_{50}-48 h values for Cd^{2+} and Zn^{2+} were determined as 0.63 ± 0.23 mg/l, 0.16 ± 0.06 mg/l and 4.52 ± 0.58 mg/l, 1.49 ± 0.14 mg/l, respectively.

The inter-assay variation for three independent experiments was in the region of 15–30% for the *D. magna* assay and 5–15% for Jurkat cells. Such variability is quite common for most of the biological assays. It can be compensated for by running appropriate numbers of replicates for each concentration point ($N = 8$ for our systems).

3.3. *Analysis of effluents*

To confirm the efficiency and robustness of the new respirometric *D. magna* test and compare it with the standard method, it was applied to the analysis of 10 industrial wastewater samples that were initially examined for their residual toxicity, undiluted and in a blind manner, i.e. without knowing their source and composition. Subsequent analysis of toxic samples at different dilutions enabled determination of EC_{50} values. The results were then traced to the origin and possible contamination of each sample and compared with toxicity data produced by the standard test. Analysis of the same samples by the standard *D. magna* test showed toxicity in samples 2–9, which were mainly effluents with elements of metals, pesticides, and pharmaceuticals. Samples 2 and 6 gave similar value of EC_{50} for the standard *D. magna* test and respirometric assay: 6.5%

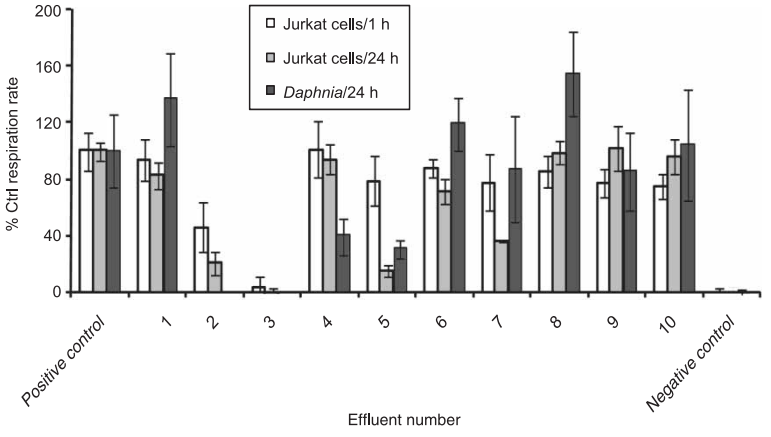

Figure 3. Comparison of toxic effects of effluent samples (diluted 1:10) on Jurkat cells after 1 h (open bars) and 24 h exposure (grey bars), and (diluted 1:1) on *D. magna* (black bars) after 24 h exposure. The assay was conducted at $37 \pm 2\,^{\circ}C$ with $N = 4$ for cell assay and ($22 \pm 2\,^{\circ}C$) with $N = 8$ for *D. magna*.

(2) and 14.3% (6), $14.03 \pm 4.97\%$ (2) and $14.54 \pm 0.74\%$ (6), respectively. Samples 3 and 4 showed a higher sensitivity in standard assay than in respirometric assay with EC_{50} 27.7% (3) and 7.5% (4), and $85.6 \pm 37.39\%$ (3) and $19.85 \pm 3.82\%$ (4), respectively. Conversely, for samples 5 and 7 the respirometric assay demonstrated higher sensitivity than the standard assay with EC_{50} values $4.01 \pm 0.47\%$ (5) and $14.19 \pm 6.05\%$ (7), and 7.4% (5) and 41.4% (7), respectively. These results suggest that the new respirometric toxicity assay with *D. magna* provides very comparable sensitivity with respect to wastewater samples.

For more detailed assessment of effluent toxicity, a eukaryotic model (Jurkat cells) was also applied using 1 h and 24 h incubation times. For 1 h exposure time, significant toxicity was seen only for samples 2, 3. Increasing the exposure to 24 h toxicity was detected for samples 2, 3, 5 and 7. A summary of effects of all 10 effluents on both *D. magna* and Jurkat cell respiration at different incubation times is shown in Figure 3. Effluent number 3 showed the highest toxicity to Jurkat cells during exposure 24 h with an EC_{50}-24 h of 0.63 ± 0.23 (see Table 2).

4. Discussion

The aim of this work was to establish a robust assay, using *D. magna* as an aquatic animal model, with which the effects of different toxicants on respiration can be monitored by the non-invasive optical oxygen sensing method. Due to its short life cycle and robust culturing conditions, *D. magna* is a good model organism for rapid preliminary toxicity studies, and this assay based on oxygen sensing can easily be set up in any laboratory.

The type of measurement platform is critical for ensuring that the assay is robust and reliable. For the analysis of *D. magna* we used standard 96 WP and 384 WP, and the low-volume sealable Luxcel 96-well plate specially developed for respirometry. For practical purposes it would be ideal if the respiration of a small number of animals could be analysed. Animal respiration in the assay chamber (sealed microplate well) causes an increase in fluorescence signal of the probe over time that eventually levels off due to the depletion of dissolved oxygen and cessation of quenching by oxygen. As a result of optimisation, the low-volume, sealable Luxcel plate was chosen for toxicity assays, due to its good sensitivity and performance with one animal per well. This is a high-throughput platform offering 96 assay point capacity which is well suited for screening assays. Respiration profiles were reproducible and conclusive for this platform. The variation

Table 2. Toxic effects of industrial effluents on *D. magna* and Jurkat cells and characterisation of effluent samples.

Effluent no.	Standard assay *D. magna*	Respirometric *D. magna* EC_{50}-24 h [% vol/vol]	Jurkat EC_{50}-1 h [% vol/vol]	Jurkat EC_{50}-24h [% vol/vol]	EPA class	Industrial activity description
1	ND	ER	ND	ND	5	The use of a chemical or biological process for the production of basic pharmaceutical products
2	6.5	14.03 ± 4.97	9.16 ± 0.64	10.16 ± 0.74	8	The manufacture of paper pulp, paper or board
3	27.7	85.60 ± 37.39	2.09 ± 0.52	0.63 ± 0.23	3	The production, recovery, processing or use of ferrous metals in foundries having melting installations
4	7.5	19.85 ± 3.82	ND	ND	12	The surface treatment of metals and plastic materials using an electrolytic or chemical process
5	7.4	4.01 ± 0.47	ND	8.22 ± 4.27	5	The manufacture by way of chemical reaction processes of organic or organo-metallic chemical products
6	14.3	14.54 ± 0.74	ND	ND	5	The manufacture of pesticides, pharmaceuticals or veterinary products and their intermediates
7	41.1	14.19 ± 6.05	ND	1.39 ± 0.15	5	The use of a chemical or biological process for the production of basic pharmaceutical products
8	20	ER	ND	ND	7	Commercial brewing, distilling, and malting installations
9	76.9	ND	ND	ND	12	The manufacture or use of coating materials in processes
10	ND	ND	ND	ND	5	The use of a chemical or biological process for the production of basic pharmaceutical products

Notes: ND, no detectable effect at the highest concentration tested; ER, enhanced respiration of organism.

of measured parameters, i.e. respiration rates and EC_{50}-24 h values (see Table 1) can be largely attributed to the variation in respiration between individual animals (in line with the deviation seen between other individually tested multicellular metazoans [19]).

The results of respirometric testing are comparable with the conventional acute toxicity testing system using *D. magna*.

5. Conclusion

We have optimised conditions for the monitoring of *D. magna* and the effects of toxicants on its respiration. The assay was validated with four reference compounds, which have known toxicological effects on biological systems, and then applied the new test to analyse a panel of different wastewater samples.

The use of metabolic markers such as oxygen consumption with *D. magna*, instead of endpoint mortality (mobility) assessment, is advantageous as it eliminates false-negative results. The assay is particularly useful and sensitive to the toxicants whose action affects the respiration and bioenergetics of test organism. Screening of chemicals and environmental samples for their toxicological effects on *D. magna* can be done at one or different doses, and with a small number or replicates for higher confidence.

The use of a microtiter plate platform (96 wells), on which the test is conducted, significantly increases the number of samples available for testing, miniaturises and automates the whole

system. These factors, combined with the simple, rapid and sensitive nature of the assay, highlight its considerable potential for environmental monitoring and biochemical toxicology.

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